COMPARATIVE CHARACTERIZATION OF DENGUE VIRUS SEROTYPE 2 ISOLATES FROM A SOUTH PACIFIC EPIDEMIC SWEEP

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ABSTRACT

The four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4) cause a wide spectrum of clinical disease, ranging from febrile illnesses to life-threatening hemorrhagic fever. Worldwide incidence has increased alarmingly to where DENV is now the most common mosquito-borne viral disease in the world. However, understanding the mechanisms by which DENV causes disease remains elusive, due both to the complexity of vector-host transmission and the lack of effective *in vitro* and animal models with which to test mechanisms of pathogenesis. Our previous work, in which we carried out phylogenetic analysis of an epidemic sweep of DENV-2 among South Pacific islands that occurred in the early 1970s, suggests that strain variation might have a significant impact on epidemic severity. Specifically, DENV-2 isolates from an outbreak on the island of Tonga – whose epidemiology was notable for being distinctly attenuated compared with outbreaks on all other affected islands – were found to have three unique amino acid substitutions in the prM, NS2A and NS4A gene regions that distinguished them from all other DENV-2 isolates. Therefore, based on the hypothesis that changes in the DENV genome can lead to variations in disease severity and epidemic potential between different DENV strains, this study explores the relationship between genetic changes in DENV-2 epidemic strains and their phenotypic effects using *in vitro* models. Phenotypic differences between DENV-2 isolates from the entire South Pacific sweep were measured on the basis of viral productivity (i.e., viral titer and replication rate or rate of virus production per cell) in both human and mosquito cells. In addition, the specific mutations characterizing the Tonga synapomorphies suggested additional measurements to examine the proportion of infectious virions and possibly the viral effects on host-immune responses. .

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LIST OF ABBREVIATIONS

ADE	antibody-dependent enhancement
bp	base pair
C	capsid
CryoEM	cryo-electron microscopy
DENV	dengue virus
DENV-2	dengue virus, serotype-2
DF	dengue fever
DHF	dengue hemorrhagic fever
dN	nonsynonymous substitution
dpi	days post infection
dS	synonymous substitution
DSS	dengue shock syndrome
E	envelope
ER	endoplasmic reticulum
FcγR	Fcγ receptor bearing cells
HI	hemagglutination inhibition
kb	kilobase
kDa	kilodalton
M	molar
mM	millimolar
MCMC	Markov chain Monte Carlo
min	minute
MID	minimal infective dose
μL	microliter
mL	milliliter
ML	maximum likelihood
MOI	multiplicity of infection
NC	nucleocapsid
NJ	neighbor joining
nm	nanometer
NS	nonstructural
NTR	nontranslated region
ORF	open reading frame
PCR	polymerase chain reaction
prM	pre-membrane
qPCR	quantitative polymerase chain reaction
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second
UTR	untranslated region

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CHAPTER 1: INTRODUCTION

Dengue virus (DENV) is the single-most significant disease-causing, mosquito-borne virus in the tropical and subtropical regions of the world today,[22] producing outcomes as varied as mild fever to hemorrhage and shock leading to death[98]. In spite of considerable research, no antiviral drugs are currently available[145] and vaccine development has been problematic with the only currently licensed tetravalent vaccine (Sanofi-Pasteur's Dengvaxia) showing less than 60% efficacy.[29] This is against a background in which rates of DENV transmission continue to increase[164] such that DENV infections now range from 50–100 million[183] to 390 million[22] a year. Transmitted by several species of the *Aedes* genus of mosquitoes but primarily by *Aedes aegypti*[186], DENV is a member of the Flavivirus genus, which includes viruses responsible for diseases such as yellow fever, Japanese encephalitis, Congenital Zika syndrome, and West Nile neuroinvasive disease.[129,148] DENV itself is an approximately 11-kb positive-strand RNA virus and has four closely related but antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, DENV-4), which do not provide complete cross-protective immunity. Infection with any of the four serotypes can result in asymptomatic infection or a spectrum of clinical symptoms and signs, including non-specific febrile illness, dengue fever (DF), and severe dengue (until recently classified as dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS][248]). In spite of considerable research, the mechanism by which DENV causes disease and the question of why clinical disease shows such considerable variation remains incompletely understood.

A variety of known risk factors for serious dengue disease have been identified, such as viral strain variation,[44,135] vector competency[31] and secondary infection by a heterologous DENV serotype[99]. Research into the mechanisms of DENV pathogenesis has been hampered by the lack

of an animal model and by the multi-factorial nature of its risk factors[153].

Nonetheless, important insights into the question of dengue pathogenesis and DENV epidemic potential may be gained from careful study of past outbreaks. The series of outbreaks upon which this study is based – an epidemic sweep of DENV-2 in the South Pacific that occurred in the early 1970s – represent a unique opportunity to examine the possible contribution of evolution and transmission ecology in the epidemiology of DENV outbreaks. These South Pacific outbreaks represent a kind of natural experiment in which many, if not most, of the confounding factors in dengue disease dynamics were controlled for, thus allowing for clear examination of such factors as evolution, strain variation, and the effects of genomic change on DENV function. In previous research, we carried out phylogenetic analysis of DENV isolated from this series of outbreaks and demonstrated a possible link between strain variation and epidemic severity on these South Pacific islands. That work revealed that DENV-2 isolated from an outbreak on a specific island revealed unique amino acid substitutions that distinguished them from all other isolates, and only occurring on the one island with an outbreak that was distinctly attenuated compared with DENV-2 outbreaks on all other affected islands.[213]

Thus, we believe that strain variation resulting from viral evolution may play a significant role in dengue pathogenesis and DENV epidemic potential. The challenge is to delineate the phenotypic differences arising from strain variation and to identify the role these phenotypes might play in terms of pathogenesis and transmission.

Therefore, this introduction begins with a review of DENV structure and our current understanding of DENV evolution, and then proceeds to offer a brief overview of our historical understanding of dengue as a disease as well as its recent expansion. The introduction continues

with a discussion on diagnosis and clinical effects, dengue transmission and epidemiology and a review of past and current research on dengue pathogenesis; all in the service of providing the context necessary for understanding the research carried out and described herein.

Dengue Virus Structure

Dengue virus (DENV) is a small enveloped virus with a virion approximately 50 nm in size, containing an electron dense core of genomic RNA complexed with capsid (C) protein and surrounded by a membrane composed of lipid bilayers and the **envelope** (E) and **membrane** (M) proteins. The **nucleocapsid** (NC) is the single genome RNA packaged within multiple copies of the small C protein. Formation of the NC is one of the earliest events in the flavivirus assembly.[54] The E glycoproteins are the major antigenic determinant on flavivirus particles and contain both cellular receptor-binding sites and a fusion peptide that mediate membrane fusion during virus entry.[258] Individual M proteins are small proteolytic fragments resulting from the cleavage of the precursor **pre-membrane (prM)** protein during maturation of nascent virus particles within the secretory pathway. Thus, prM probably acts as a chaperone for the folding of the E protein, serving as a lock to prevent the E glycoproteins from being prematurely activated by low pH during their transport through the trans-Golgi network prior to virion secretion at the plasma membrane.[144] However, prM cleavage does not appear to be an "all-or-nothing" phenomenon but instead progressive and relatively inefficient with one study showing approximately 30 to 40% of prM remaining in the extracellular particles of a DENV-2 isolate.[118] Given its function as a "lock" prM cleavage is a prerequisite for DENV maturation and thus it was assumed infectivity. However, recent research suggests that immature DENV may itself play a pathogenic role, in as much as anti-prM antibodies may enhance its uptake by Fc-receptor bearing cells.[184] From the

standpoint of the research described in this dissertation, understanding the role of prM has potential importance because a non-synonymous substitution in the prM gene region defines the attenuated Tonga clade among those isolates collected from the South Pacific DENV-2 outbreaks from 1971 – 1974 (see the section **Phylogenetics of the South Pacific Sweep** for further detail).

The DENV **genome** is an approximately 11-kilobase (kb) single-stranded RNA of positive polarity containing a single open reading frame (ORF) that encodes a polyprotein. Genomic RNA is infective and acts as the viral mRNA for replication, as well as the template for transcription of the complementary negative-sense RNA. The negative-sense RNA serves in turn as the template for genomic RNA synthesis. In common with other flavivirus, the DENV genome has a gene order of 5'–C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5–3' with the three structural genes — C, prM and E — followed by the seven non-structural genes.[161] DENV ORFs range in size from 3387–3396 amino acids, making them the shortest among the mosquito-borne flaviviruses.[83] The ORF is flanked by 5' and 3' untranslated regions (**UTR**) of ~100 and ~400 nucleotides, respectively. These noncoding regions assume a secondary structure that is believed to regulate viral protein synthesis, RNA replication, and packaging through spatial arrangements for interactions with viral or host cell proteins.[28,152] There is evidence that the 5' and 3' ends stack together (in what is sometimes referred to as a 'panhandle structure') to cause cyclization of the genome. This in theory would permit the viral replicase to bind to both ends of the template RNA at the same time, thus producing cyclized positive strands, perhaps during the initial stages of RNA replication.[124,152]

The function of many of the DENV non-structural proteins is currently either unknown or lacking significant details. Because of their relevance to the research discussed in this dissertation,

NS2A and NS4A will be reviewed briefly. **NS2A** is a relatively small hydrophobic protein of around 22 kDa in size, which is cleaved from NS1 by an unknown ER resident host enzyme. Mutations in yellow fever virus NS2A that block virus production can be blocked in turn by a second mutation on the surface of the NS3 helicase domain suggesting that virion assembly involves an interaction between NS2A and NS3. [131] This and other interactions, such as those with NS5 and the 3' UTR, make it seem likely that NS2A is involved in coordinating the shift between RNA packaging and RNA replication. [123] In addition, NS2A also has been shown to act as an interferon (IFN) antagonist by inhibiting IFN antiviral immune response both alone and in coordination with NS4A and NS4B.[142,162] Finally, NS2A genes may also play a role, along with NS4B, in anchoring the viral replicase complex to cellular lipid membranes.[156]

Very little is known about the functions of **NS4A**. The protein is small (16 kDa), hydrophobic, localized to the ER lumen, and membrane associated. NS4A appears to play a key role in the replication function of NS1 based upon mutation studies[139] and its abundant co-localization within replication complexes.[147] The carboxyl-terminal region of NS4A, designated the 2K fragment, serves as signal sequence for the translocation of the adjacent NS4B into the ER.[156] As mentioned above for the NS2A protein, DENV NS4A, along with NS4B can also block type 1 IFN signaling, albeit with NS4B having the strongest neutralizing effect.[162]

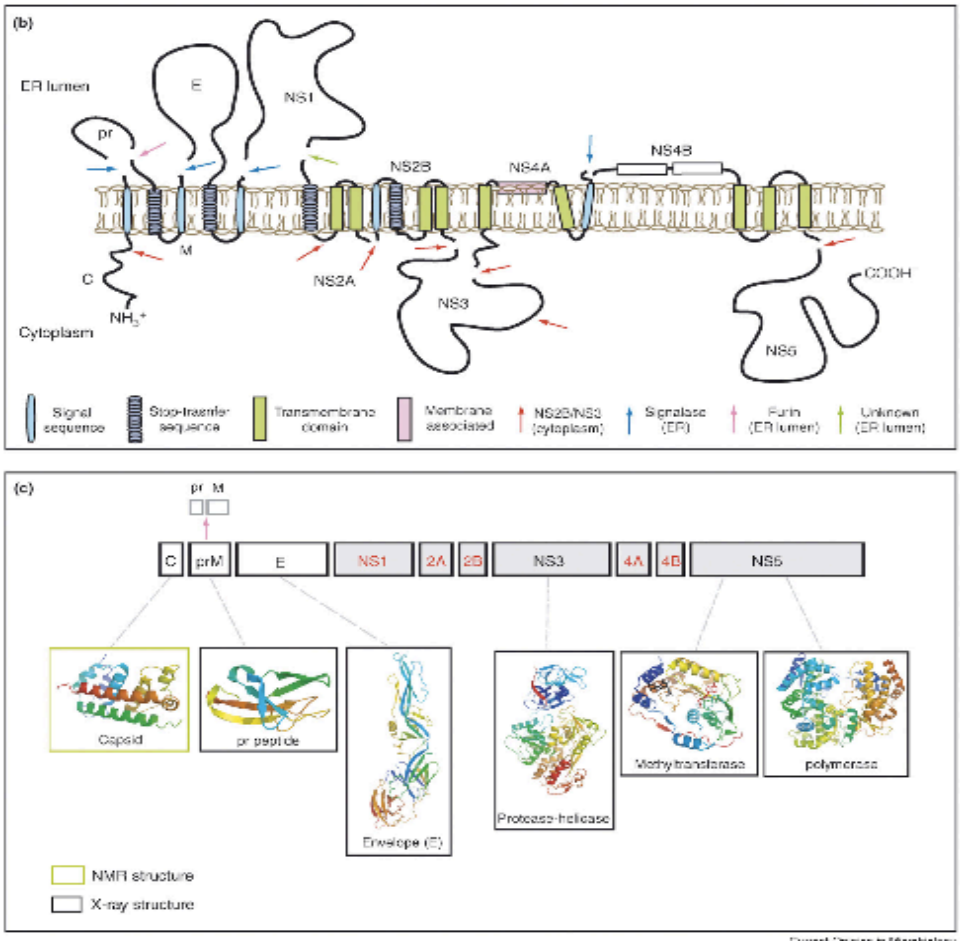


Figure 1. Predicted topology of the dengue virus polyprotein across the endoplasmic reticulum (ER) membrane. Arrows indicate sites of processing by host cell and viral proteases. Enzymatic cleavage sites are indicated by colors as described in the legend. From Perera and Kuhn 2008

Dengue Virus Phylogenetics and Evolution

DENV is a member of the genus *Flavivirus*, family *Flaviviridae*. Among the family *Flaviviridae*, which also includes the genera *Hepaciviruses* and *Pestiviruses*, only the flaviviruses include vector-borne viruses (Figure 2).[140]

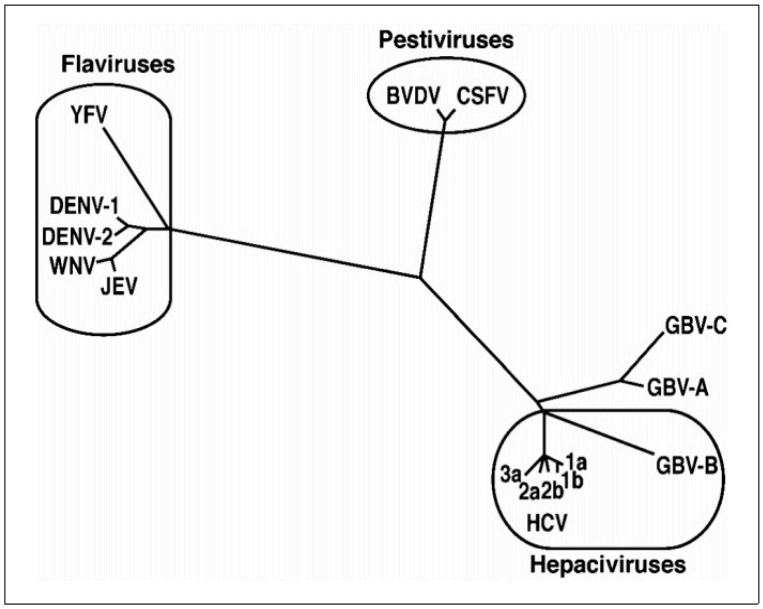


Figure 2. Phylogenetic tree of Flaviviridae family based on analysis of NS3 helicase regions. Some members of each genus are shown as examples – Flavivirus genus: yellow fever virus (YFV), dengue virus 1-4 (DENV-1 to DENV-4), West Nile virus (WNV), Japanese encephalitis (JEV); the Pestivirus genus: bovine viral diarrhea virus (BVDV), classical swine fever (CSFV); Hepacivirus genus: hepatitis C virus (HCV); and the unclassified viruses GBV-A and GBV-C. © 2007 Field Virology, 5th Edition.

Those vector-borne viruses that share the genus *Flavivirus* with DENV include a large number of significant human pathogens including Japanese encephalitis virus (JEV), West Nile virus (WNV) and yellow fever virus (YFV)[108] Beyond humans, the flaviviruses have a variety of both invertebrate and vertebrate hosts, the latter including humans, birds, rodents, bats, and nonhuman primates.[235] Geographically diverse, the complete number of *Flavivirus* species is

uncertain; most estimates range from 56[105] to 70[70] although a statistical method developed by Pybus et al. (2002) using phylogenetic node heights to estimate the true number of taxa in a clade came up with an estimate of roughly 2,000 flaviviruses still awaiting identification.[176] The origin of the Flavivirus genus is a matter of conjecture. Some reviews on flavivirus evolution suggest two possibilities: 1) that the mosquito and tick transmission clusters each diverged independently from an ancestral non-vector virus group[49] or 2) that the evolution progressed from non-vector to tick and then to mosquito.[83,129]

All flaviviruses have common group epitopes on the E protein. The consequences of such serological similarity are two-fold: first, there is abundant evidence of immunological cross-reactivity. Examples include DENV and Zika virus (ZIKV) where the evidence strongly supports an infection enhancing effect [32,174]; DENV, JEV and YFV, where cross-immunity may be preventing YFV dissemination in Asia[80]; and JEV and DENV, where there is evidence for both neutralization and enhancement at present.[6,224] Such immunological interactions have broad implications for public health and vaccine development.

A second implication of flavivirus immunological similarity is that it often results in extensive cross- reactions in serologic tests. In an earlier era, when flaviviruses were categorized into antigenic complexes and subcomplexes, based upon classic serological criteria, clear-cut serologic diagnosis of flaviviruses were always problematic.[78] Fortunately, advances in phylogenetics have lent clarity to organization of the genus into clusters, clades and species.

Based on molecular phylogeny therefore, the flaviviruses are now divided into two unique groups: the vector-borne viruses transmitted by arthropods and those viruses that have no known vector.[108] The latter is made up of viruses that are transmitted either directly between

vertebrates or between invertebrates, and replicate solely within these hosts. The arthropod-transmission cluster on the other hand, further divides into another two monophyletic groups based around their respective transmission vectors: viruses that are mosquito-transmitted, and those that are transmitted by ticks. (Figure 3.)[108,115,129]

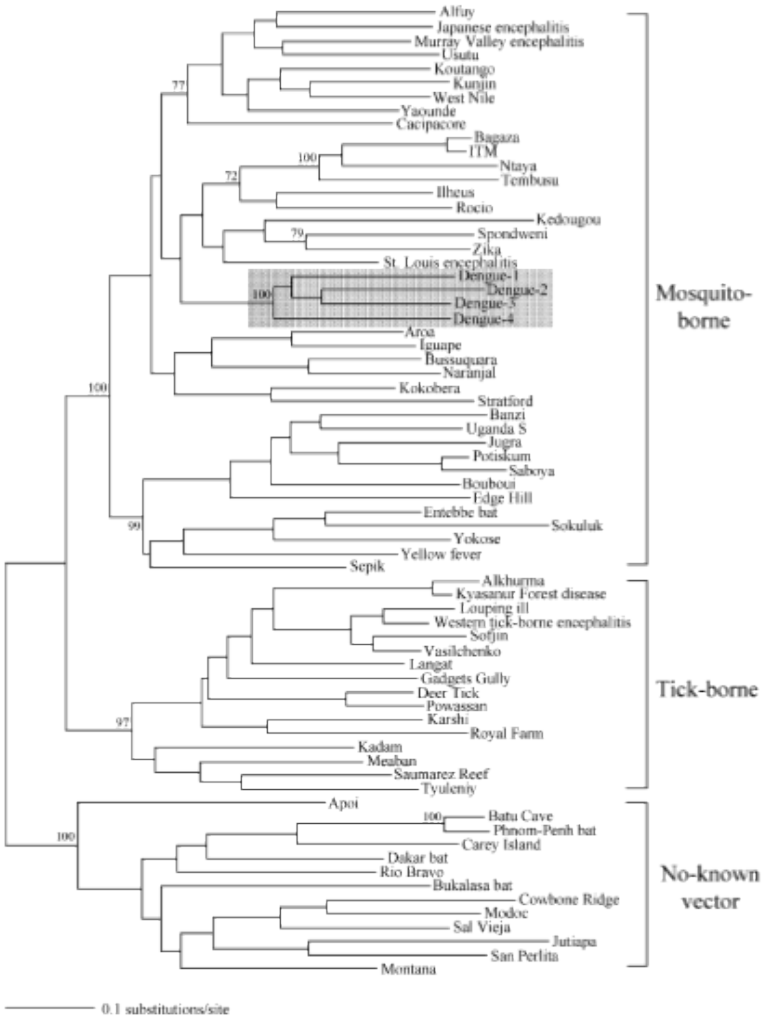


Figure. 3. Maximum likelihood (ML) phylogeny of Flavivirus genus based NS-5 gene sequences (990 bp) from 70 viruses. Because of major differences in base composition between the three major groups of viruses (tick-borne, no-known vector, vector-borne—Jenkins et al. 2001), the trees for these groups were estimated separately and then joined in a final phylogeny in which all branch lengths were re-optimised. The tree is mid-point rooted for purposes of clarity only and all horizontal branch lengths are drawn to scale. The four dengue viruses are shaded. From Holmes and Twiddy 2003.

DENV is notable in existing as four antigenically defined serotypes (DENV-1–DENV-4). With only 62-67% amino acid identity amongst the polyproteins of the four serotypes, they have no more genetic similarity than some flavivirus "species."[19,107] Infection by any one of the four serotype produces long-lasting immunity to that serotype, but only temporary cross immunity to the other three.

Thus, a person living in an endemic area could theoretically have as many as four DENV infections during her or his lifetime, one with each serotype.[78] Such heterotypic infections are significant, as there is abundant evidence that subsequent infections with a heterologous DENV serotype may in fact increase the risk for more serious disease through a process called *antibody dependent enhancement* (ADE).[100] At present, there is no clear explanation as to why the virus exists as four distinct serotypes or evidence to determine the origins of such an arrangement. It has been suggested that the separate serotypes represent four initially geographically separate viral populations that have only recently come together or that they could be the outcomes of independent cross-species transfer events from the various non-human primate species that harbor DENV.[107] The most likely explanation however is that their diversification was driven by ADE as a means of reducing protective cross-reactions in the primate immunological response.[64] Separate mathematical models developed by Esteva et al. (2003) and Adams et al. (2006) support this last hypothesis as both suggest that under any conditions of complete cross-immunity, a primary infection reduces the force of infection of subsequent heterologous serotypes.[3,62] Whereas, if heterologous infection resulted in subsequent partial cross-immunity or immune enhancement that this would in fact increase the force of infection and thus increase the probability that a second serotype would establish itself in the population. On the other hand, a recent paper in the journal *Science* by Katzelnick et al. reports on research demonstrating that geographically and temporally diverse DENV isolates clustered only loosely by type, that in fact many were as similar antigenically to a virus of a different type as to some viruses of the same type.[120] Further, they found that primary infection antisera did not neutralize all viruses of the same DENV type any better than other types did up to two years after infection and did not show improved neutralization to homologous type isolates. Apart from the obvious implications for

vaccine research and surveillance, these results also appear to strengthen the case for a more balanced view of dengue pathogenicity. It confirms the role of prior immunity to a heterologous serotype as being of primary importance but acknowledges that "neutralizing responses to the particular DENV lineages circulating in a population modifies the magnitude and severity of epidemics caused by subsequent infecting lineages."[120] It has been long suspected for example that the severity of dengue symptoms may vary markedly in response to the order of serotype infection, for example DENV-1 followed by DENV-2. More recent research however suggests a more complicated scenario in which disease severity in response to secondary infection is more related to DENV lineage than simply heterologous serotype.[3,170]

Even to the extent that the degree of serotypic "clustering" is up for debate, the stable configuration of DENV into four serotypes is nonetheless notable given its tremendous genetic diversity. As with all organisms, such diversity is a result of evolutionary forces: those which introduce genetic variation, such as mutation, gene flow and recombination, and those that change the relative proportion of variants in populations over time, i.e., natural selection and genetic drift.[19] In regards to those factors that introduce variation; DENV is highly mutagenic, given a RNA-dependent RNA polymerase that typically generates one error per round of genome replication.[56,107] Additional genetic variation is also contributed through gene flow as DENV is transported, often over large distances, by both human hosts and vectors[107]. And recombination may also contribute to DENV genetic diversity; although its extent and overall significance in increasing genetic diversity in natural populations of DENV is uncertain, it is well documented that it can occur. [219,226,230,252]

However, it has to be emphasized that while mutation, gene flow and recombination

introduce genetic variation; genetic diversity ultimately results from the survival of specific phenotypic traits. Moreover, survival of specific phenotypes – whether through the random chance of genetic drift or non-randomly through adaptive selection – results in changes in the frequency of the underlying genotypes, which ultimately happens with the fixation of substitutions. [16,19] With this in mind then, an important question regarding DENV evolution is whether genetic clustering, whether as serotypes or "genotypes" (defined below) reflects some level of natural selection or is simply a result of drift. If selection does play a role then could such selection possibly lead to variants with greater epidemic potential[87,108] or strains resistant to future vaccination programs?[107,250] Numerous papers have debated the role of selection in driving DENV evolution and whether positive, negative or neutral selection is of greater importance. A tentative consensus appears to have formed around the idea that much of the phylogenetic structure within DENV populations consists of lineages in which silent or synonymous nucleotide changes (mutations) predominate after vigorous purifying selection.[106,228] While the broad diversity of DENV lineages may arise partly due to positive selection on certain sites of the DENV genome under specific conditions[20,21] the more significant factors may include local evolution by genetic drift and sporadic gene flow of DENV strains into different geographic areas. In some studies, local microevolution has been interrupted by lineage replacement through importation, such as been shown with the competitive displacement of the American genotype of DENV-2 by the Asian/American.[182,244] In some cases where localized evolution of dengue has been examined, for example, in schools in rural Thailand, rates of evolution were quite low and dominated by genetic drift.[114] In other cases, DENV microevolution occurred in situ with little influence from importation, due to both selection and genetic drift.[20,21] The examples given above generally involve geographic partitioning. However, in DENV, as with other arboviruses,

host-alternation can also impose significant bottlenecks that contribute to genetic drift and act as drivers of evolution. Demographic fluctuations can be severe in vector populations, particularly in those areas with seasonal rainfall or cold spells. In addition, each blood feed results in stochastic sampling of a small number of viral genomes. This is followed by limited infection and traversal of mosquito midgut cells, followed by virus dissemination and invasion of the salivary glands; all these steps are expected to result in strong population drops.[58,136] Indeed one study, using whole-genome deep-sequencing of human- and matched mosquito-derived DENV samples to track single nucleotide variants (SNVs) within each sample, found that >90% of SNVs were lost upon transition from human to mosquito.[206] In addition to the stochastic sampling associated with host alternation, negative selection could also be expected given that a fit genotype in one host may not be fit in the other and indeed a reduction in the rate of adaptive evolution was recently demonstrated experimentally in a related arbovirus, Venezuelan equine encephalitis.[43] On the other hand, a set of experiments in host alternation, using DENV grown either in human cells or insect cells or in both, saw results that were more equivocal. Not surprisingly, DENV passaged in either single host cell line exhibited fitness gains in that line and fitness loss in the bypassed cell line. However, most DENV passaged in alternating cycles exhibited fitness gains in both cell lines.[233] Thus, both in the case of geographic isolation and in host-alternation there is still no clear sense as to the evolutionary drivers that predominate and their ultimate contribution to DENV pathogenicity and/or epidemic potential. Certainly, as populations of humans and *Aedes aegypti* continue to expand, opportunities for viral circulation will increase in turn. This creates ever more opportunities for co-infection of mosquito and human by multiple serotypes of virus and potentially sets the stage for genetic events that could lead to the emergence of more virulent or transmissible strains as well as strains of greater

vaccine resistance.[250]

Before leaving the topic of DENV serotypes, it must be noted that an additional fifth serotype has recently been discovered. Announced in 2013, the fifth serotype was discovered in the routine screening of viral samples obtained from a 37-year-old farmer admitted in hospital in Sarawak state of Malaysia in 2007. The virus was originally assumed to be spillover from the circulation of sylvatic DENV-4 among local primates and local *Aedes nivalis* forest mosquitoes. Subsequent isolation and genetic sequencing demonstrated however that the virus was phylogenetically distinct from the three previous forms of sylvatic DENV-4 and bore some similarity with DENV-2. Further testing through inoculation of DENV-5 into macaque monkeys previously infected with the other four DENV serotypes and allowed to recover resulted in the production a significantly different set of antibodies, confirming that the new virus was a new serotype and not a variant of DENV-4. Interestingly, while the outbreak caused by the strain was associated with only a single hospitalized case, suggesting that disease caused by this DENV-5 strain is mild, the viral titer of secondary infections in the macaque was four times higher than other serotypes.[165,169] Previous comparisons of sylvatic strains with human adapted DENV in two surrogate human model hosts: monocyte-derived, human dendritic cells, and mice engrafted with human hepatoma cells, found that while sylvatic strains replicated at a lower titer, no overall difference between sylvatic and human-adapted strains was noted.[234] This present spillover event thus appears to confirm that the adaptive barrier for emergence of sylvatic dengue is relatively low and raises questions about the adequacy of tetravalent DENV vaccines as well as the larger question as to whether even more yet undiscovered DENV serotypes may remain in the jungle.[232]

Within each DENV serotype, there is further genetic diversity in the form of clusters of variants or "genotypes." Essentially defined by Rico-Hesse in 1990, through close comparison of a 240 base pair fragment from the *E/NS1* gene region in both DENV 1 and 2, she delineated a number of genotypes, each defined as "a group of dengue viruses having no more than 6% sequence divergence."[108,181] All four serotypes of DENV have genotypes and often these follow different geographic distributions (Figure 4). For example, in DENV-2 the genotypes named "Asian 1" and "Asian 2" were only found in Asian populations. This however, may be an artifact as continued expansion and mixing of infected hosts and vectors may bring genotypes far from their putative geographic range. And indeed, some genotypes are found in multiple geographic localities; e.g. the "Cosmopolitan" genotype within DENV-2.[107,182,225]

Regarding the origins of DENV, the historical and epidemiological evidence give reason to believe that DENV existed in Asia as long as 1,000 years ago, although probably in a sylvatic form.[240] Indeed, the existence of sylvatic cycles of DENV transmission in both Africa and Southeast Asia involving nonhuman primate hosts and gallery forest vectors other than *Aedes aegypti* or *Ae. albopictus* suggest a zoonotic origin for the virus. Phylogenetic analysis suggests that each of the four serotypes independently passed over to humans, possibly more than once, but eventually establishing endemicity within humans some 125–320 years ago subsequent to sufficient human populations size.[228,255] However, there has been some disagreement as to which continent might be the more likely locality of viral emergence.[185,193,210] Given that DENV-1, DENV-2 and DENV-4 serotypes have been found in Asia[193,194] while DENV-2 was the sole serotype to be found in Africa until the 1990s,[185] the relative lack of susceptibility to DENV infection of the ancestral *Ae. aegypti formosus* from Africa,[51] and the weight of phylogenetic analysis.[227,240] the evidence seems to favor Asia as the more likely ancestral source.

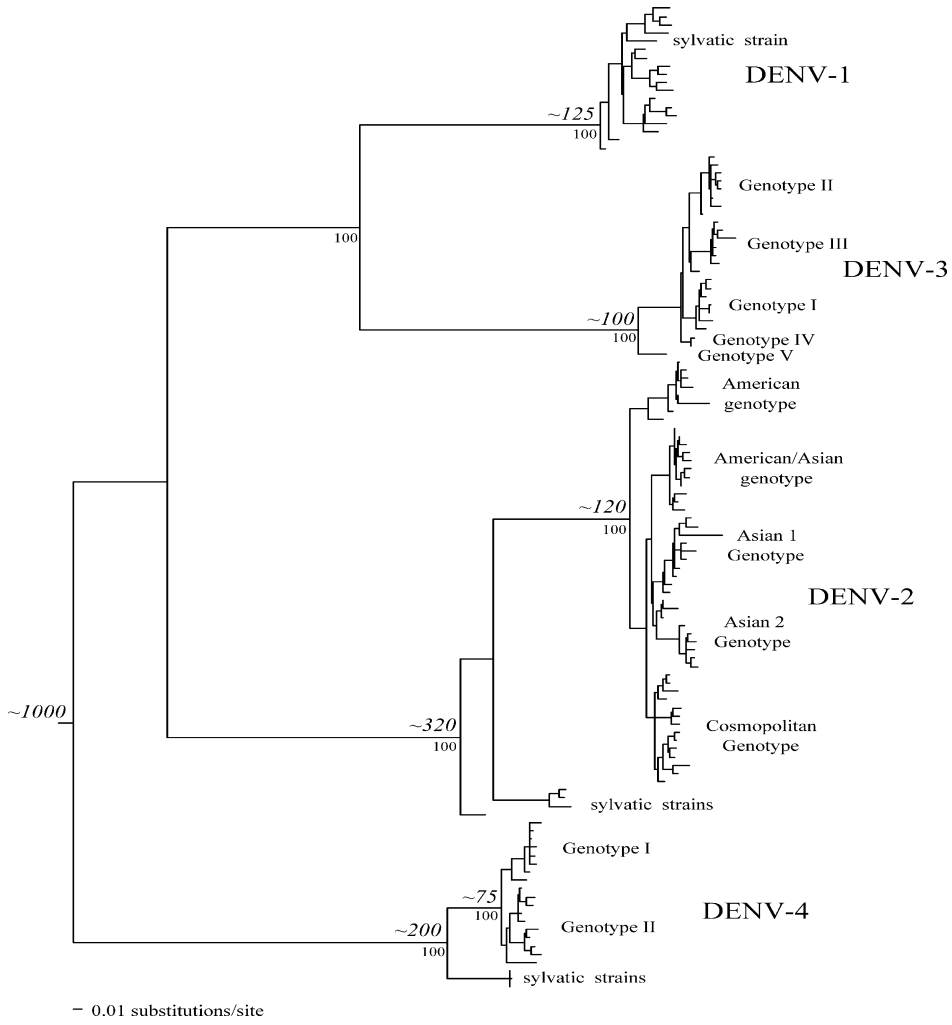


Figure 4. Serotypes and genotypes representing genetic DENV diversity. Tree generated through maximum likelihood using 120 Envelope gene sequences (1485 bp). Approximate divergence times are shown in italics above key nodes. All horizontal branch lengths drawn to scale. From Holmes et al. 2003.

History - Dengue Emergence and Resurgence

Examining the actual historical record for the emergence of dengue as a disease is problematic, given the difficulties in distinguishing its symptoms from those resulting from other diseases such as chikungunya, measles, influenza, typhoid, leptospirosis, or malaria.[183] Thus, there is no certainty when dengue was first recognized as a clinical disease. The most often cited first appearance is that found in a Chinese encyclopedia of disease symptoms and remedies,

originally published during the Chin Dynasty (265 to 420 A.D.) and revised thereafter. Called "water poison," the disease was thought to be connected with flying insects associated with water.[78] We have records of other outbreaks with dengue-like symptoms in the French West Indies in 1635 and in Panama in 1699.[24] If these were in fact dengue outbreaks then distribution of the virus was already widespread even before the 18th century, when the first known pandemic of dengue-like illness began.

The appearance of a considerable number of reports from the late 18th century of simultaneous dengue-like epidemics in Egypt, India, the West Indies, and the southern United States[30,78,183] certainly confirm the cosmopolitan nature of dengue distribution by this date. Although again, the possibility of diagnostic confusion with other diseases, particularly with chikungunya[30] means that any such conclusions require caution. In any case, by early 1800 dengue was widespread throughout urban tropical coastal cities of the world due to the global expansion of commercial shipping.[250] The shipping vessels supporting expanded trade facilitated transport of vector breeding habitat along with humans, thus supplying a complete transmission cycle.[79] With both the virus and mosquito vector introduced to coastal destinations around the world, dengue epidemics generally began to occur at 10–40 year intervals.[164]

Halstead suggests that the term "dengue" was introduced into English medical literature from the Spanish West Indies during a 1827-1828 Caribbean epidemic of a disease producing rash with arthralgia. "Dengue" is in turn a Spanish corruption of the Swahili "Ki denga pepo", meaning "seizure caused by an evil spirit."[92] As mentioned above, much confusion probably occurred between outbreaks of dengue and chikungunya with any febrile disease accompanied by rash being described by such terms as "Knokkelkoorts" from Jakarta in 1779 and "Breakbone

fever" from Philadelphia in 1780.

Transmission of DENV by the mosquito vector *Aedes aegypti* had to wait until description in the early 20th century[13,42] with discovery of a filterable, infectious agent in human blood[9] and the infection with this agent of human volunteers[205] and mosquitoes[208] following shortly thereafter. Definitive identification of dengue was finally accomplished through isolation during the Second World War by two independent groups; Kimura and Hotta in Japan in 1944[110,126] and Sabin and Schlesinger in 1945 in the United States.[196]

The waging of the Second World War in Asia and the Pacific marked a profound turning point in the emergence of dengue as a public health problem of worldwide scope.[78,130] War disrupted normal water infrastructure, necessitating more personal water-storage, created bomb craters and spread refuse and debris through formerly pristine environments. All of these newly created mosquito-breeding opportunities occurred simultaneously with the increased transport of mosquitoes and their eggs into new geographic areas accompanied by movements of immunologically susceptible troops between regions of ongoing dengue endemicity. The result by the end of the war was not just to spread the virus and its vector over an area much greater than it had inhabited heretofore but also to bring different serotypes into contact with one another.

Following the Second World War there were initially reasons for optimism in regards to dengue. For example, large-scale dengue epidemics in Central and South America appeared to be under control as a result of a vigorous and large successful campaign to eliminate the YFV and DENV vector *Ae. aegypti.* Unfortunately, these vector control programs were halted in the early 1970s; by the 1980s incidence had increased, and by 1995 *Ae. aegypti* had returned to pre-

campaign levels in the Americas once again.[24,80,81] This same time period saw rapid human population growth throughout the tropics with a concomitant demographic shift from the country and into cities along with lagging infrastructure and unplanned development. All of these factors combined to create ideal habitat for *Ae. aegypti* and continued the introduction of novel DENV serotypes into immunologically susceptible human populations. Increasingly, all four serotypes came to be maintained in hyperendemic co-circulation in large cities throughout the tropics and subtropics of the world. It is during this same period of time and in the largest cities of Southeast Asia that the more serious manifestations of DENV infection first began to break out with increased frequency.[78,148] The first well documented outbreak of what would eventually be known as dengue hemorrhagic fever occurred in Manila in 1953/1954, and was followed by a larger outbreak in Bangkok in 1958.[92,104] Thus, the failure of mosquito control efforts in the latter part of the 20th century in combination with growing urbanization and transportation have contributed to the re-emergence of dengue to the scale of a global pandemic.

Dengue Epidemiology

Recent decades have seen a tremendous growth in dengue incidence and severity, marked by more frequent and severe epidemics. Previous to 1970 only nine countries experienced epidemics of severe dengue; by 1995 that number had increased more than four-fold and dengue is probably endemic in more than 100 countries in Asia, the Americas, the western Pacific, Africa and the eastern Mediterranean regions.[90,249]

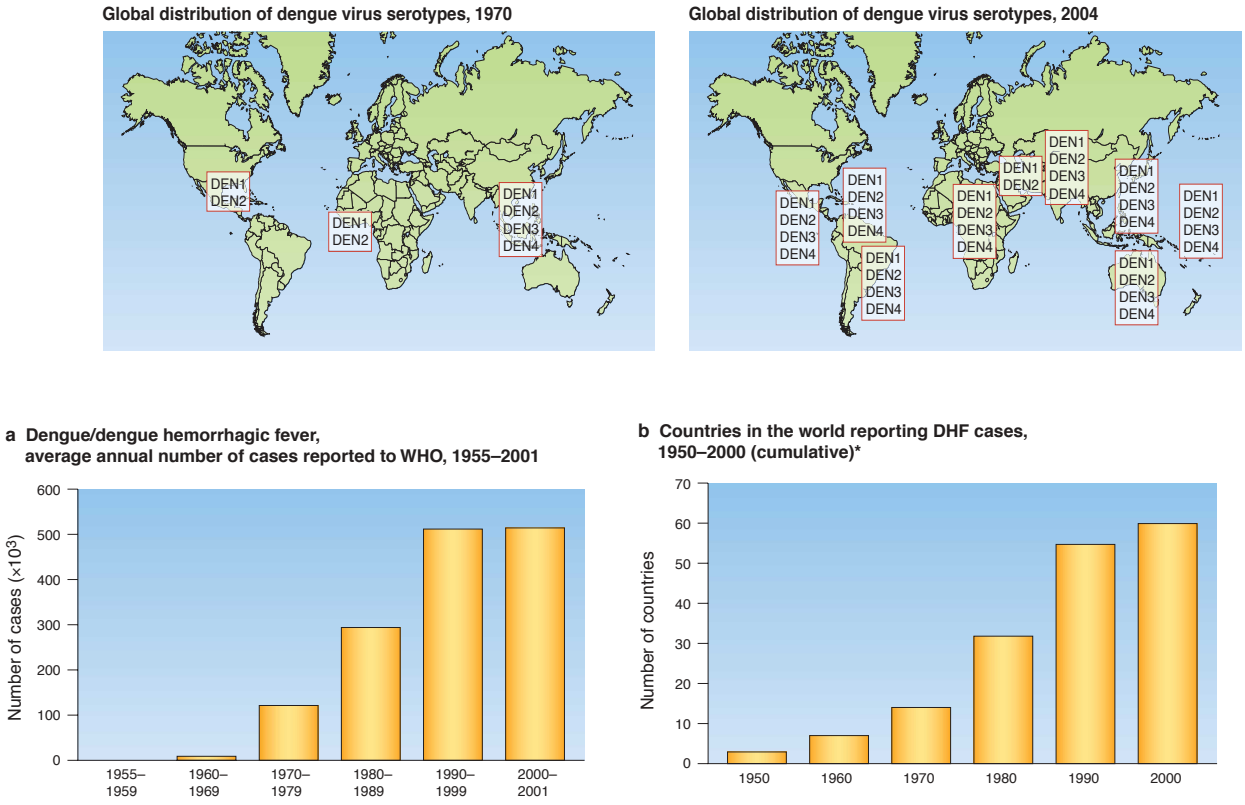


*Figure 5. Top Row: Comparison of DENV serotype distribution from 1970 to 2004. Bottom row: emergence of dengue and DHF 1950 - 2000, by incidence (**a**) and by country (**b**). All images MacKenzie et al. 2004.*

In terms of disease burden, many authors cite World Health Organization estimates of 50–100 million infections annually worldwide with 500,000 persons needing hospitalization from DHF, and more than 15,000 deaths.[183] More recent estimates however, have revised the number of infections significantly upwards to 390 million DENV infections per year, of which 96 million manifest some degree of disease severity (an estimate three times that of the World Health Organization).[22] Dengue is the most rapidly spreading mosquito borne disease in the world today with at least half of the world's population living in regions at-risk for dengue disease transmission.[249] For example, dengue disease incidence appears to have increased in Africa (although previous under-reporting may be a contributing factor),[5] and outbreaks are increasingly reported in areas formerly free of the virus such as Hawaii, the Galapagos islands,

Easter Island, Hong Kong, Buenos Aires, Florida, southeastern France, and Madeira Island.[90,248] In those countries where dengue has long been present, as in Southeast Asia, age shifts are occurring,[48,88,172,218] causing reevaluation of dengue as a "childhood disease" and prompting changes in surveillance and treatment.

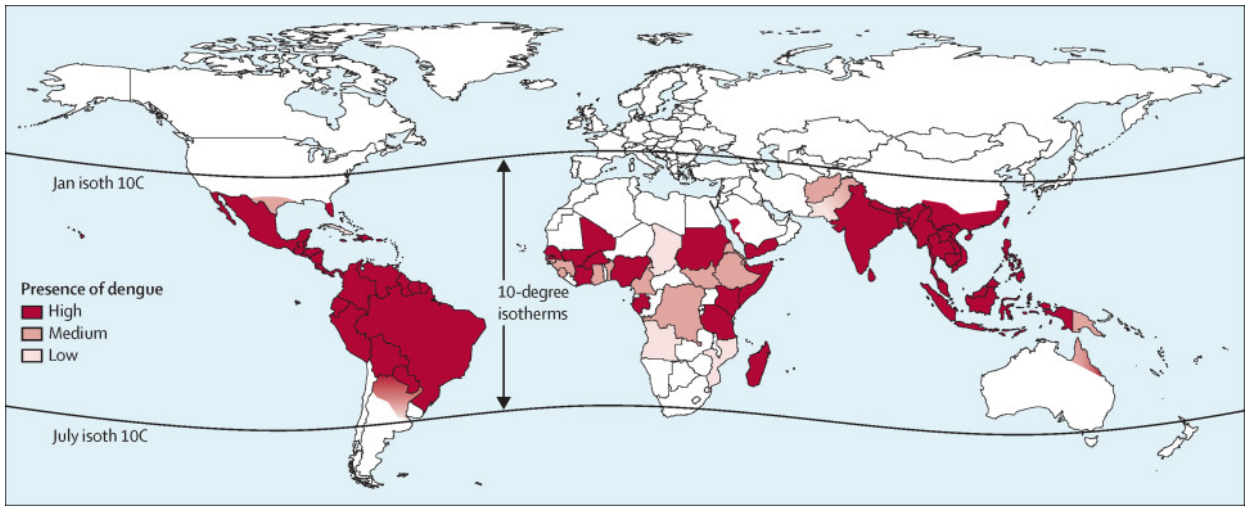


Figure 6. Global disease burden of dengue, 2014. From Guzman and Harris 2015.

The economic impacts of dengue are considerable and have a particularly pernicious effect on countries challenged with still emerging economies and developing health-care systems. Nevertheless, accurately estimating this impact is difficult; while case fatality rates are easily measured, dengue is a disease with a generally low rate of mortality but high rate of morbidity. Since most studies have focused on individual epidemics they do not take into account the larger indirect burden of the disease on a population resulting from lost productivity and time away from work or school owing to illness. Nonetheless, some estimates can be given. Estimates included in a review by Gubler in 2002 include: US $6 and $16 million in medical costs and control measures for a 1977 epidemic in Puerto Rico; US $12 million in medical costs alone for 1994 outbreak, also in Puerto Rico; and US $103 million for a 1981 Cuban epidemic,

of which nearly half was for mosquito control. A study in Thailand, estimated the annual economic burden of severe dengue in that country to range between US $31.5 and $51.5 million.[79] A survey of the economic and disease burden of dengue in 12 countries in SE Asia: Bhutan, Brunei, Cambodia, East-Timor, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand, and Viet Nam; estimated the annual economic burden was US $950 million or about US $1.65 per capita. Using a World Bank developed non-monetary composite index called 'disability-adjusted life years' (DALYs), the survey estimated 214,000 DALYs; equivalent to 372 DALYs per million inhabitants.[202]

Unfortunately, the increased burden of dengue has increasingly been joined by the emergence of other arboviruses such as chikungunya, West Nile and Zika virus.[173,50,59] Not only does this increase the complexity of clinical diagnosis but in the case of Zika, evidence is growing that exposure might prompt serological cross-reactivity with possible infection enhancement.[50,122] While much of the news around dengue is gloomy, some optimism may be gained from the seriousness with which dengue and other arboviral diseases are now accorded; it was not so long ago that dengue was referred to as a "neglected tropical disease." Certainly recent decades have seen an explosion of research and institutional attention.[192] Full-length genomic sequences were completed for all four serotypes of DENV in the early nineties.[83] And a particularly notable breakthrough has been the recent release of the first dengue vaccine. Developed by Sanofi-Pasteur, their tetravalent live attenuated vaccine, Dengvaxia, is composed of DENV structural proteins fused to a yellow fever 17D vaccine backbone. Dengvaxia passed its Phase III efficacy trials in 2014[209] and as of 2016 the vaccine has been approved for use in more than six countries.[247] (A further discussion of this and other dengue vaccines in

development follows in this Introduction.)

Other promising technologies are emerging from recent developments in vector control, which are discussed in the next section.

Mosquitoes and Transmission Ecology

To understand the reemergence of dengue as the world's single largest arboviral disease, the role of its mosquito vector must be understood. DENV is transmitted to humans by mosquitoes of the *Aedes* genus, although species (and even different populations) within the genus vary in their degree of vector competence. Transmission begins when the mosquito acquires DENV while feeding on the blood of a person infected with the virus. Following ingestion of viremic blood, extracellular virus arrives into the mosquito midgut and must bind to undefined receptors on the cellular surface of the midgut epithelium. If the virus can successfully infect and replicate within midgut epithelium cells then the viral infection spreads within the epithelium and disseminates from the midgut to secondary tissues. With secondary amplification, these new progeny virus are shed into the hemocoel (the cavity in which the hemolymph circulates, part of the open circulatory system of invertebrates), where DENV can subsequently disseminate and infect secondary tissues, including the salivary glands (and sometimes reproductive tissues for vertical transmission to offspring). Once sufficient virus replication has occurred in the salivary glands, virus is released into salivary ducts and upon the next probing/feeding event, the virus may be transmitted to a new uninfected vertebrate host via the saliva of the infected mosquito.[31] However, transmission is complicated by the mosquito's innate immune system and a number of tissue barriers associated with the midgut and salivary glands, *i.e.*, the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection

barrier (SGIB), and salivary gland escape barrier (SGEB).[66] It is the combination of these obstacles and host cell receptivity for the virus that ultimately determines vector competence.

Another important factor in transmission dynamics is the time period in which DENV incubates and goes through repeated cycles of amplification; there are two periods, one in the mosquito and the other in the human host (Figure 7). The period within the mosquito is termed the extrinsic incubation period (EIP), and is the time between when a mosquito takes a viremic blood meal and the time when the mosquito becomes infectious. Note that after EIP, the infected mosquito is capable of transmitting the virus to susceptible individuals for the rest of its life. Also, EIP is strongly dependent on temperature and thus influenced by variations in seasonal weather and climate. The intrinsic incubation period (IIP), by contrast, takes place within the host and is the time between a human being infected and the onset of symptoms due to the infection. A meta-analysis of previous EIP/IIP studies found that best-fitting temperature-dependent EIP model estimated that 95% of EIPs are between 5 and 33 days at 25°C, and 2 and 15 days at 30°C, with means of 15 and 6.5 days, respectively. The mean IIP estimate was 5.9 days, with 95% expected between days 3 and 10 days.[35]

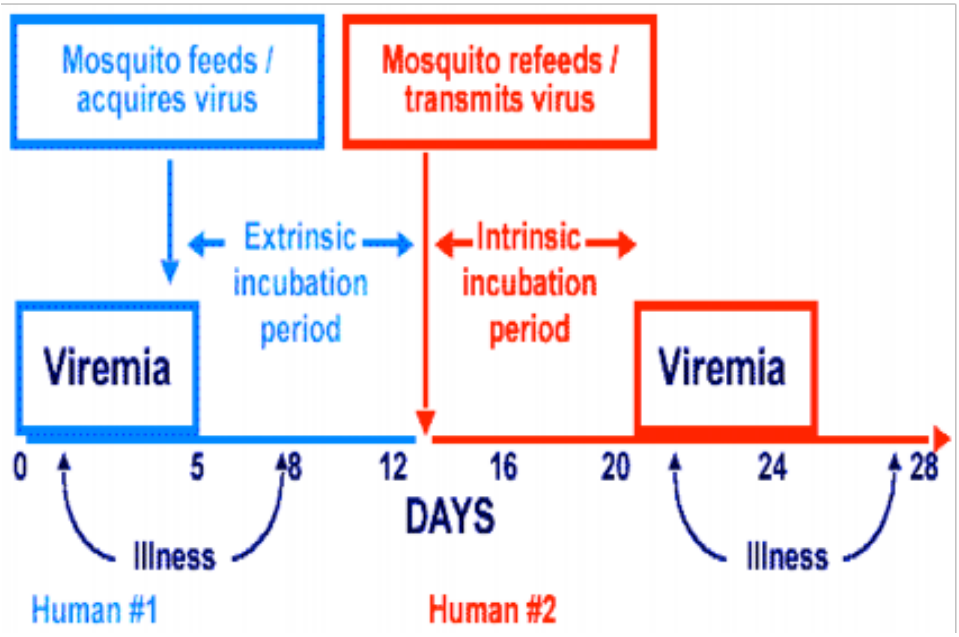


Figure 7. DENV intrinsic and extrinsic incubation periods.
(http://www.cdc.gov/ncidod/dvbid/dengue/slideset)

DENV is one of the few medically important flaviviruses that has an exclusive transmission cycle between mosquito and humans with no zoonotic hosts, i.e. urban transmission cycle.[245] While a sylvatic (non-human primate) cycle does exist and may occasionally spill over into human populations, and is believed to be ancestral to the urban cycle, adaption of DENV to the human host and its vector to the human environment is so thorough that the sylvatic cycle is no longer required for maintenance.[232,240]

While a number of mosquito species of the *Aedes* genus have been shown to be capable of transmitting DENV, *Aedes aegypti*, the YFV mosquito, is generally considered the primary vector although *Aedes albopictus*, the Asian tiger mosquito has an extensive range and is widely implicated with instigating dengue outbreaks as well. In addition, more geographically localized species such as *Aedes polyenesiensis*, and *Aedes tabu* may also play an important role in specific outbreaks. Nevertheless, *Aedes* (Stegomyia) *aegypti* is the major dengue fever vector everywhere

it occurs throughout the tropical and subtropical world.[186] The reasons for this are many but the essential factor is the unusual evolutionary adaptions the species has made to associate with humans. Almost exclusively anthropophilic, *Ae. aegypti* prefers to feed on human blood[175,214] and is rarely associated with any habitat other than human dwellings. Adults prefer to rest within sheltered dark spaces inside houses, such under beds and within closets[186] and as larvae and pupae, *Ae. aegypti* prefer breeding in artificial containers: generally household water containers such as barrels or cisterns, flower pots which are productive throughout the year, and various trash such as cans, bottles, etc. Other important behavioral factors are the species' preferred feeding times of dawn and dusk[186] when humans are less likely to be behind netted beds, its imperceptible bite, and its shyness, which tends to make it more prone to biting multiple individuals in order to obtain a blood meal thus spreading the virus more widely.[250] Finally, *Ae. aegypti* mosquitoes are competent for DENV, i.e., capable of infecting epithelial cells of this species' midgut wall and replicating to a high titer of 10^6 minimal infective doses (MID) per mL[83] and surmounting such physiological obstacles such as the midgut barrier, in order to disseminate to the salivary glands where the virus can be passed onto a new human host. [86] Surprisingly however, *Ae. aegypti* mosquitoes appear to have a relatively low susceptibility to oral infection, i.e., virus titers in the blood of the human host must exceed 10^5–10^7 virus particles per mL for infection and transmission to be sustained. It has been suggested in this regard that *Ae. aegypti* may act as a selection filter for maintaining viral virulence at a relatively high level, since only those virus strains that replicate in humans at a sufficiently high rate of efficiency are capable of producing the high viremias necessary for transmission by this mosquito.[157] Interestingly, *Ae. albopictus* is overall more susceptible to DENV midgut infection. Nonetheless, rates of virus dissemination from the midgut to other tissues are significantly lower in *Ae.*

albopictus than in *Ae. aegypti*. This factor, as well as the more anthropophilic host preferences of *Ae. aegypti*, may explain why those localities where *Ae. albopictus* predominates over *Ae. aegypti* have never experienced a typical explosive dengue epidemic with severe cases of the disease.[132]

In those areas where large populations of *Aedes* mosquitoes persist, such as large, urban areas located in the tropics, one or more DENV serotypes are typically maintained endemically whereas in smaller or more isolated localities, where host herd immunity can build up, epidemics often result from the introduction of novel serotypes.[77] DENV transmission shows a high degree of temporal and spatial heterogeneity based on seasonal and geographic variations in rainfall, relative humidity and temperature. *Aedes* survival is longer during the rainy season due to the high humidity, while temperature affects the maturation of mosquito life stages. Higher temperatures produce smaller females, which are forced to take more blood meals to obtain the protein needed for egg production.[116] This of course increases the number of humans likely to be bitten by a single female and increases the probability of transmission. Temperature also determines the amount of time that ingested virus takes to reach the mosquito salivary glands ready for transmission as higher temperatures accelerate the extrinsic incubation period (see above for further discussion of EIP).[96,243]

The high statistical correlation of household water storage containers with disease incidence[68,71,223] is so well established that vector-control efforts are focused specifically on their removal. In some countries the presence of water storage containers may be so significant a predictor of *Ae. aegypti* infestation, that container abundance is found to mitigate any seasonal variations.[146] Therefore, elimination or at least control of mosquito egress has always been one of

the highest priorities for minimizing the risk of DENV infection. Unfortunately, container control has a mixed record of success at best. One difficulty is that of surveillance. The localized presence of water storage containers renders the use of remote sensing ineffective except perhaps in more temperate regions[61] and simply gaining access to properties to survey the containers can be time consuming, expensive and even hazardous.[146] Furthermore, an increasing number of studies has demonstrated that larval abundance can be a poor predictor of adult abundance since container abundance is an inexact surrogate for larva abundance. This is because container indices do not address productivity, that is, the number of *Ae. aegypti* adults produced over time.[178] Thus, even a single container can supply enough adults to cause significant DENV transmission. Finally, effective container control typically demands a high level of community participation, difficult to achieve, particularly in poor, urban areas where the challenges of daily life leave little time for the vague promise of more effective mosquito control.

In fact, it is arguable that dengue has been successfully prevented exclusively through vector control on only three occasions. The first of these was the highly successful, vertically structured paramilitary eradication campaign directed by the Pan American Sanitary Board from 1946–1970 that almost succeeded in eradicating *Ae. aegypti* from the Western hemisphere.[79,198] The second was also a rigorous, top-down, military-like vector control campaign in Cuba that was implemented in 1981 and relied upon intensive insecticidal treatment followed by reduction of available larval habitats and also included rapid diagnosis of dengue and treatment.[69,172] The third program was in Singapore, and was based on entomologic surveillance and larval source reduction (i.e., reducing the availability of *Aedes* larval habitats) and included public education and law enforcement, the latter to discourage intentional or unintentional mosquito propagation. This program succeeded in achieving a 15-year period of low dengue incidence before seeing

resurgence in the 1990s.[172] Thus, none of these programs was sustainable over the long-term.

Nonetheless, new technologies in vector control have made an appearance with the potential to change the vector control equation. Genetic modification of *Aedes aegypti* with insertion of a dominant lethal gene (*RIDL*) developed by Oxford, UK–based Oxitec, offers a means to diminish this vector's population levels through release of sterile males.[4] Another approach, being studied by researchers at Monash University in Melbourne, Australia, has been to adapt the endosymbiotic bacterium *Wolbachia* from *Drosophila* to *Ae. aegypti.* This appears to both shorten the lifespan of the mosquito and block transmission of DENV.[67,239] Both technologies have been tested in various field trials in various tropical locations, and results from both of these technologies are encouraging if preliminary with further trials ongoing.

Ultimately, the transmission and incidence of dengue is a complex, multi-factorial process in which numerous factors affect disease incidence as much or more than vector abundance. Host immunity (both on the individual and population level), genetic variations in susceptibility in humans and mosquito and variations in virulence among different strains of DENV are just some of the other factors that must be considered.

Dengue Pathogenesis

In spite of decades of research into the causes of dengue pathology, we still have more questions than answers. It is clear that pathogenesis of dengue disease, both mild and severe, is determined by many factors, including those in the host, vector, and environment. Host immunity (both on the individual and population level), vector abundance, genetic variations in susceptibility in humans and mosquito and in virulence among different strains of dengue are just some of the

elements that must be considered in attempting to understand epidemic behavior. Research into dengue pathogenesis has been greatly hindered by the lack of an effective animal model. As non-human primates are the natural hosts of sylvatic strains of DENV they can be infected by DENV and they do develop viremia. However, primate infections result in much lower viremic titers than those found in humans and never show signs of hemorrhage or shock.[78] Several murine models of DENV infection have been recently described but results are still extremely preliminary and their applicability to human pathology remains controversial.[190,253] In spite of these challenges, there are two leading theories as to the cause of morbidity from DENV infection, neither of which is mutually exclusive.

Secondary heterotypic infection and the risk of severe dengue

As previously mentioned, DENV has four genetically distinct serotypes. Infection with any one serotype offers relatively long-lasting immunity against reinfection by the homologous serotype but only temporally limited cross-immunity to the other three, generally lasting only 6 months.[196] After this time individuals infected with one serotype are fully susceptible to infection by any of the other three serotypes. [99] Moreover, secondary heterotypic infection appears to be a significant risk factor for severe dengue disease [98] although how the immune response to DENV protects against or contributes to severe disease is incompletely understood and the mechanisms suggested are controversial.[53] In general, antibodies play an important role in immune protection against viral infection through neutralization by binding to virus particles, blocking their ability to bind to host cells and aggregating virions for elimination. However, in the case of DENV, while cross-reactive antibodies produced in response to a primary DENV infection recognize and do bind to newly infecting virus of a heterologous serotype, the concentration of those antibodies

fall below the threshold for neutralization. Instead, these antibodies appear to form antibody-virus complexes, which are bound to and internalized by cells expressing Fcγ receptors (FcγR), i.e., cells of the monocytic/macrophage lineage. Thus, these "sub-neutralizing" antibodies appear to promote entry of DENV into FcγR cells in the process called ''antibody-dependent enhancement'' or ADE[100] a mechanism observed in other flaviviruses including YFV, WNV, TBEV and JEV.[52]

A key epidemiological observation is that while serotypes may have rarely co-existed in the past, increased urban development and transportation has resulted in greater areas of the world becoming hyperendemic with multiple DENV serotypes, such as in Southeast Asia in the sixties and South America in seventies. As hyperendemicity has increased, so have rates of heterologous secondary DENV infection and with them higher rates of severe dengue with hemorrhage and shock.[75] Further support for this theory came from clinical observations in Southeast Asia in the 1960s and 1970s that demonstrated that dengue fever without complications occurred in foreigners who were non-indigenous to the area while much more severe dengue occurred in indigenous children.[103] The theory of ADE explains that such severe disease in infants is a result of the transfer of DENV-specific maternal IgG antibodies across the placenta. As these anti-DENV antibodies diminish to lower levels, they predispose the infant to ADE by facilitating enhancement of a newly acquired primary DENV infection.[103] Further evidence comes from passive administration of DENV-reactive monoclonal or polyclonal antibodies into IFN receptor-deficient mice[256] as well as in non-human primates[73] resulting in an increase of viral burden and disease severity.

Over time, the ADE model has been further expanded to recognize that binding of DENV

antibody-antigen complexes to myeloid or mast cell Fcγ receptors may affect host immunity in a number of ways: increased release of cytokines such as IL-10 results in the skewing CD4+ T-cell responses.[102] Alternatively, ADE may promote degranulation of vasoactive molecules that enhance capillary leakage.[216] In mice, transfer of enhancing concentrations of antibody appears to promote ‘‘cytokine storms’’ and vascular leakage; again a result of interactions of the Fc region of antibody with Fcγ receptors.[12]

In spite of such evidence, there are numerous documented cases of severe dengue with hemorrhagic and/or shock symptoms occurring after primary infection[41,163,200] as well as cases in which, even upon secondary infection, no hemorrhagic fever or shock manifests.[158,244] The specific mechanism by which ADE leads to severe symptoms is still in question as well. While ADE may enhance viral uptake, it is not clear that higher viremia is by itself a cause of more severe symptoms. For example, while average peak virus titers are higher in patients with severe dengue than in patients with dengue fever, these titers are several logs below peak levels by the time plasma leakage occurs.[236] Conversely, many patients who have high virus titers do not develop plasma leakage.[134,137] Such observations suggest that high viremic titers resulting from ADE may not be the only mechanism responsible for the pathogenesis associated with DENV infection.

DENV strain variation and virulence

In common with other organisms, DENV populations demonstrate genetic change as both a cause and consequence of evolution over time. As discussed in the section **Dengue Phylogenetics and Evolution**, evolution results from various forms of genetic variation, such as mutation, gene flow and recombination, being modified by those forces that change the relative

proportion of variants in populations over time, i.e., the random chance of genetic drift or non-randomly through natural selection.[19] What is less clear is whether such genetic change in DENV might lead to the phenotypic expression of such traits as increased virus replication and viremia, severity of disease (virulence), and greater transmissibility. As with other aspects of dengue research, correlating genetic changes in the DENV genome to specific phenotypic changes is difficult. The lack of good animal models for *in vivo* testing has been mentioned before. Epidemiological correlations with genetic difference are problematic because of the frequency of silent transmission and the variability of risk associated with uneven distribution of mosquito habitat and the resulting rates of transmission.[217] An additional problem is that overall rates of virus isolation from patients with DENV infection are very low, particularly in the case of severe dengue with hemorrhage or shock. In most cases, by the time a patient begins to exhibit severe dengue, viremia is no longer detectable, probably because of the high levels of antibody present within the body at the time may inhibit isolation.[87,168,190]

In spite of these challenges, it has been clearly demonstrated experimentally that DENV do vary in phenotypes related to virulence of the virus. For example, serial passage of DENV strains through tissue culture or suckling mice brains have produced viruses with attenuated virulence in humans[196] and differential propagation has produced viruses with changes in temperature tolerance, plaque size, mosquito infectivity, mouse neurovirulence and the tropism of strains of DENV for various cell lines.[101,190]

Good epidemiological evidence for natural variation in virulence is scarce since one of the attributes of the most useful epidemiological cases is that they should have occurred in remote and isolated areas where it can be shown that the outbreaks are due to infection from a

single DENV serotype and as a result of primary infections. The latter factor is important in order to distinguish the effects of strain variation from ADE resulting from secondary infection. The 1974 outbreak in Tonga (described below in the section **Case Epidemiology**) that serves as a natural experiment in DENV evolution and epidemic behavior is considered one of the classic examples because it is one of the very few cases in which these requirements are met. However, a number of other cases are frequently cited. They include an outbreak of DENV-3 in central Java, Indonesia in 1978, which showed dramatic attenuation, compared with an epidemic of the same serotype just two years earlier.[87] Another example is the failure of successive epidemics in Iquitos, Peru – DENV-1 in 1990 followed 5 years later by DENV-2 – to result in severe symptoms in spite of substantial secondary infection.[244] Our ability to analyze and compare outbreaks advanced significantly with the development of techniques allowing for partial, then whole genome sequencing and the use of these sequences to generate phylogenetic trees of evolutionary relationships among the viruses responsible for the outbreaks. Important early examples of such genetic epidemiology include an examination of the genetic relationships, distribution and evolution of DENV-1 and DENV-2 by Rico-Hesse (1990), which included one of the first sequences of sylvatic dengue, the delineation of geographically independent DENV-3 subtypes and their evolution by Lanciotti et al. (1994), and a phylogenetic comparison by Chungue et al. (1993) of DENV-3 isolated from patients suffering severe or mild forms of dengue fever in French Polynesia.[41,133,181]

One of the most thoroughly investigated cases for natural variation in DENV virulence is a shift in Central and South America from mild dengue fever to severe epidemics coincident with the replacement of the American genotype of DENV-2 with a seemingly more virulent Southeast Asian genotype of serotype-2.[181] Since the late 1960s, DENV-2 in the Americas had only been

associated with classic dengue fever with no epidemics of DHF/DSS, despite co-circulation with other serotypes, in contrast with South-East Asia where severe dengue had been endemic since the 1950s.[77,182,244] Beginning in the early 1980s however, a DENV-2 strain of higher pathogenicity originating from South East Asia began replacing the American subtype throughout Latin America with an attendant rise in dengue disease severity.[81] Whole genome comparisons between the low-virulence American DENV-2 strain and high-virulence Asian strain revealed a number of structural differences throughout, particularly in the UTR of the latter.[135] Further experimental support for this shift to an Asian DENV-2 of greater virulence has come from reverse engineering of the mutations that distinguish the two genotypes demonstrating lower viral output by the American.[45] Further work showed greater susceptibility to infection by DEN-2 viruses of the SE Asian genotype than to those of the American genotype[8] and higher rates of replication by the SE Asian genotype in both human dendritic cells and mosquitoes.[44] It should be noted that these investigations are relevant to the present study in as much as the DENV responsible for the South Pacific epidemic sweep were of the American genotype.[213]

Other experimental support for epidemiological observations of viral virulence has included efforts to identify genetic markers of virulence and to characterize their phenotypic expression. For example, Mangada et al. (1998) did complete genome sequencing and characterization of 8 DENV-2 strains isolated from patients exhibiting different disease severities during an epidemic season in northeastern Thailand in 1993.[151] While they found that the strain associated with shock symptoms showed the greatest divergence from other isolates, they were unable to identify any specific molecular markers associated with virulence, infectivity or cytopathic effect. In summary, there is as yet no evidence for a specific molecular basis for

virulence and/or epidemic potential, if only because we still have much to learn about the structure of DENV itself and its functions.

Thus, while much progress has been made in elucidating the causes and mechanisms behind dengue pathogenesis, we are still far from any conclusive and comprehensive explanation. Both viral and host immune factors appear to play an integrated role in this process. Available evidence supports the hypothesis that severe dengue disease can more often be observed with some viral strains than with others and that it can occur in the absence of sequential infection. At the same time, there is abundant evidence that the risk of severe dengue is increased with secondary infections and that host immune responses must play a major role in capillary leakage. It may be the case that strain variation and ADE work in tandem to generate greater severity. For example, a comparison of low passage DENV-2 isolates from Thailand found evidence that strains associated with hemorrhagic symptoms had a tendency to cross react with more of a panel of DEN-4 monoclonal antibodies than those strains associated with classical dengue fever.[160] Halstead also noted many years ago that different strains within serotypes 'differed markedly in their reactions with dengue antisera'.[95] Both observations suggest that some DENV strains from the same serotype could cause more severe disease than others because they have a greater ability to be enhanced by heterotypic antibodies. Thus, apparent increases in strain virulence might actually result from the virus generating a less robust immune response leading to enhancement.[107,160]

Such a dynamic might work in conjunction with or even be the cause of a phenomenon frequently noted in dengue literature; that severity of outbreaks seems to correlate with a particular sequence in serotype or genotype exposure. For example, a prospective

epidemiological study in Rayong, Thailand found that all children exhibiting shock had secondary infections and the specific sequence determining the highest risk of shock was a primary infection with DENV-1 followed by DENV-2.[197] Interestingly, in a table published in 1980 that listed ten 'simultaneous or sequential epidemics involving two or more dengue virus types but not associated with epidemic dengue shock syndrome' only two were DENV-1 followed by 2 and in those two cases the epidemics followed each other by more than 30 years.[93] Yet, a clinically mild outbreak of DENV-1 in Cuba in 1977, followed by DENV-2 in 1981, affected more than 300,000 persons, causing over 10,000 severe cases with 158 deaths.[25] But on the other hand, the very same pattern of DENV-1 in Iquitos, Peru in 1990 followed 5 years later by DENV-2 was notable precisely because it did *not* result in severe symptoms.[244]

In an intriguing side note to the 1981 Cuban DENV-2 epidemic, Kouri et al. (1989) observed that the responsible strain appeared "to have become more virulent with successive passages through the human host."[69] As evidence they noted that while the case fatality rate would have been expected to drop over the course of the epidemic due to improved case management, instead Cuba's case fatality rate increased. They further argued that if one accepts a combined extrinsic and intrinsic incubation period of 15–20 days, then over the course of the Cuban 1981 outbreak the DENV-2 would have had time for approximately three human passages, which they claim as a possible explanations for the increased severity and case fatality rates.[127] The concept of DENV gaining greater virulence as it goes through repeated "passages" through human hosts during epidemic outbreaks is interesting in as much as it offers a mechanism to explain the emergence of greater virulence. Yet, as previously noted, there is little evidence that strong positive selection plays a significant role in DENV evolution, in comparison to the role of importation leading to lineage replacement or localized genetic drift[107,255] although

some evidence for localized adaptive evolution does exist.[20,21] On the other hand, the importation of strains that then replace previous lineages means that adaptation must have occurred in a different location. Similarly, given the co-circulation of all four serotypes through most of the tropics and the rapid global movement of people, increased mixing of viral strains is inevitable. It has been suggested that this might possibly result in increased competition among DENV strains, "selecting for those that attain the highest levels of viremia, and hence maximum infectiousness."[107]

In any case, it is most likely that both viral and host factors are relevant to determining the risk of severe dengue disease but the specific interactions and relative importance of all these factors in determining the expression of clinical disease has yet to be established.

Clinical Features and diagnosis of Dengue and Severe Dengue

Infection with DENV results in clinical manifestations ranging from asymptomatic to mild febrile illness to severe shock and death. Of the more serious illnesses, two major syndromes have been defined, dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The spectrum of illness is similar regardless of which of the four serotypes of DENV caused the infection, although there is some evidence that severe and fatal hemorrhagic disease is more often associated with DENV-2 and DENV-3 infections.[78] In most tropical countries, any patient presenting with high fever with a history of mosquito exposure or living in an endemic area should be immediately suspected of DENV infection. Nonetheless, clinical diagnosis of dengue is problematic as symptoms can vary over the course of the illness and during the febrile phase, dengue resembles other arboviral infections, as well as measles, rubella,

enterovirus infections, adenovirus infections, and influenza.[207]

Regardless of clinical outcome, infection typically begins abruptly, with an incubation period lasting an average of 4–7 days (<14 days). Specific symptoms are frequently dependent upon the age of the patient. For example, in those areas where DENV is endemic, infections frequently result in undifferentiated febrile disease particularly in infants and young children. Serological studies have found that up to 90% of infected individuals were asymptomatic or minimally symptomatic.[27,37] In those cases where fever does manifest, it is often characterized by nonspecific flu-like symptoms.[153]

Classic dengue fever is a self-limiting febrile illness characterized by abrupt onset of high-grade fever (temperature of 38.9°C–40.6°C) associated with a variety of symptoms including headache (frequently retro-orbital), lumbosacral pain, conjunctival congestion and/or facial flushing. These initial symptoms can be followed by generalized myalgias or arthralgias that can worsen in severity over time. The constellation of symptoms of severe and incapacitating body ache, back pain, and arthralgia often is called "break bone fever."[121] Roughly 50% of patients experience a transient, generalized macular or mottled rash within the first or second day, while a secondary rash, maculopapular or morbilliform and nonirritating, can appear coincident with or soon after defervescence, on the torso initially and then spreading centripetally to the face and limbs.[171] Fever may last 1–2 days, may persist 6–7 days, or may have a biphasic (saddleback) course, although the latter is not the norm.[183] Laboratory abnormalities may include thrombocytopenia, leukocytopenia, and a rise in liver enzymes along with a variety of hemorrhagic manifestations including petechiae and epistaxis, and a positive tourniquet test. The latter test can be easily performed using a simple tourniquet or blood

pressure cuff and has been reported to detect about four-fifths of patients with DENV infection.[251]

Severe dengue or dengue hemorrhagic fever/dengue syndrome (DHF/DSS) describes DENV infections with more severe symptoms that typically begin with the sudden onset of a high temperature. This lasts 2–7 days, with accompanying chills, flulike constitutional symptoms, and a flushed face. As the fever subsides, patients may either recover or progress to a state of plasma leakage, which may in turn be transient or may progress to hypovolemia and profound shock with elevated hematocrit and hypoproteinemia, which if untreated, may progress to death within hours.[246] Children with DHF frequently present with a sudden rise in temperature (>39°C and occasionally up to 40–41°C) along with facial flush, and at higher temperatures febrile convulsions may occur, particularly among infants. Those children with shock are often somnolent, exhibit facial petechiae, and show perioral cyanosis.[121] Clinical evidence of accompanying plasma leakage includes serous effusion found postmortem, pleural effusion in radiographs, hemoconcentration, and hypoproteinemia.

Hemorrhagic manifestations of severe dengue can include skin hemorrhages such as petechiae, purpura, and ecchymoses; bleeding from mucous membranes (epistaxis, gingival bleeding); and bleeding from the gastrointestinal, vaginal, and urinary tracts. As with pleural effusion associated with shock, these hemorrhagic symptoms usually occur after the fever subsides, with the gastrointestinal tract being the most common site of bleeding.[78,121]

In patients that progress to shock, there are a number of signs of circulatory failure: the skin becomes cool, blotchy and congested; circumoral cyanosis is often observed; pulse becomes rapid. Initially lethargic patients may become restless; acute abdominal pain is frequently

observed. Many patients recover spontaneously or after fluid and electrolyte therapy. Patients with more severe plasma loss are in danger of profound shock leading to death without appropriate therapeutic management. The duration of shock is usually short; the patient may die within 8–24 hours or recover rapidly following appropriate volume-replacement therapy. With early diagnosis and prompt replacement of plasma, the severity of DHF/DSS can be substantially ameliorated.

In terms of laboratory tests for dengue diagnosis, there are two general classes: the primary presumptive test, of which the most important is complete blood count. This can confirm the presence of increased hematocrit (hemoconcentration), atypical lymphocytosis and thrombocytopenia. The second class being confirmatory of dengue infection, which may be important in those cases where symptoms suggest the possibility of confusion with another pathogenic agent. Of the latter a variety of tests are available including reverse transcription PCR, viral isolation, and NS1 antigen rapid kits, but the most common are serological tests (typically ELISA) and focus on measuring either IgM or IgG.[251]

Treatment for dengue is only palliative; there is no specific anti-viral treatment available for dengue. Nonetheless, early diagnosis and aggressive fluid replacement therapy with good nursing care can decrease case fatality rates to 1% or less.[78] Fever is controlled with acetaminophen, with other nonsteroidal anti-inflammatory agents to be avoided due to their anticoagulant properties and risk of Reye syndrome in children. In most cases, patients testing positive for DENV infection do not require hospital admission or intravenous fluids unless they present with severe vomiting, dehydration, bleeding, altered mental status, clinical deterioration, or other evidence of severe dengue.[121] In patients exhibiting mild hemorrhagic symptoms or

shock, normal saline or lactated Ringer's solution can be used, but plasma or plasma expanders may be necessary in those with severe cases.[78]

The majority of DF patients recover, even in cases of hemorrhage or profound shock.[78] Convalescence is usually short, with surviving patients recovering within 2–3 days, generally without sequelae, although some convalescence may be prolonged, with generalized weakness, bradycardia, ventricular extrasystoles, and mental status changes that can include lethargy, somnolence, and feelings of depression.[121]

Diagnosis of dengue has gone through significant recent changes. Patients previously diagnosed according to World Health Organization guidelines either had DF or DHF. DF was defined as a febrile illness with at least two clinical findings; none of which were particularly specific, thus necessitating laboratory or epidemiological evidence for a confirmed diagnosis. The definition of DHF consisted of four clinical criteria: fever, a hemorrhagic tendency (spontaneous bleeding or a positive tourniquet test result), thrombocytopenia (platelet count, 10^5 cells/mm^3), and plasma leakage shown by pleural effusion, ascites, or 20% hemoconcentration.

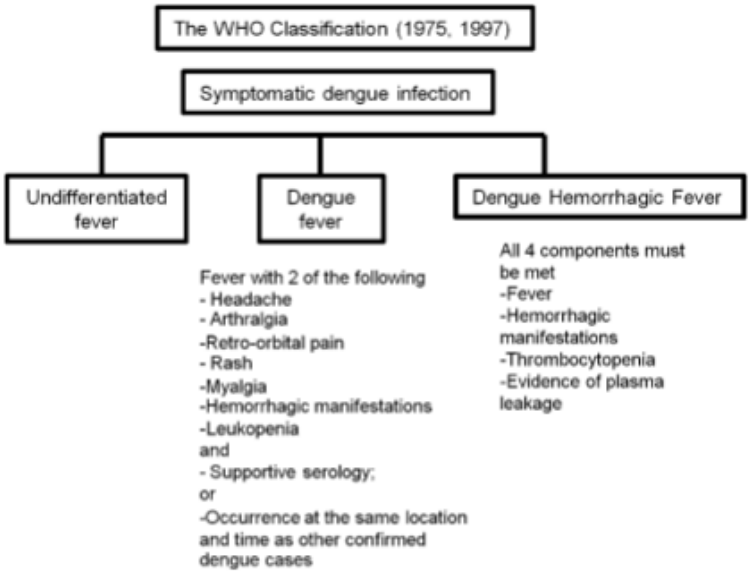


Figure 8. Dengue classification according to the World Health Organization guidelines issued in 1975 and 1997. From Srikiatkhachorn et al. 2011

However, the large numbers of patients with severe dengue disease exhibiting symptoms not matching those of the WHO system prompted calls for more inclusive case definitions.[83] Other criticisms included questions whether the case definitions, which were primarily developed using pediatric data from cases in Southeast Asia, were applicable to other regions and populations.[211] The recent revision of the WHO dengue classification scheme states, "For the purpose of clinical management, WHO classifies dengue illness as (i) dengue with or without warning signs for progression towards severe dengue and (ii) severe dengue."[248] Patients who recover without major complications are classified as having dengue, whereas those who have any of the following conditions are designated as having severe dengue: plasma leakage resulting in shock, accumulation of serosal fluid sufficient to cause respiratory distress, or both; severe bleeding; and severe organ impairment.

The changes made in classification have not been without controversy. For example, an opinion paper by Halstead (2013) argued, "pathogenesis research should be conducted as much as possible on carefully defined categories of human disease response. This requires splitting, not lumping."[97] A group of authors defended the new guidelines, writing; "DF/DHF/DSS was too complicated to use in clinical or public health settings, yet was not sufficiently precise for detailed pathogenesis studies." And that "D/SD brings clarity, clinical and epidemiological utility, and the potential for development of more precise definitions of clinical phenotype for pathogenesis studies."[63] A systematic review comparing the 1997 and 2009 WHO dengue case classifications, using 12 eligible studies (4 prospective), including ten expert opinion articles, was published by Horstick, et al. 2014.[109] They summarized their findings as "The 2009 WHO classification has clear advantages for clinical use, use in epidemiology is promising and research use may at least not be a disadvantage."

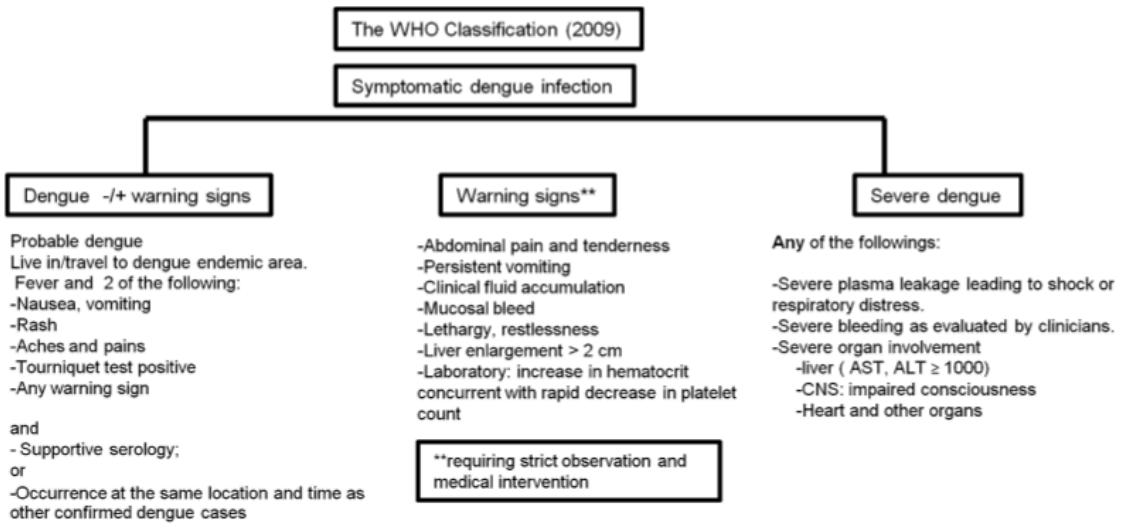


Figure 9. Dengue classification according to the World Health Organization guidelines issued in 2008. Dengue is classified as dengue with or without warning signs and severe dengue. From Srikiatkhachorn et al. 2011

Useful summaries of relevant clinical features of dengue infection include Gubler 1997, Gubler 1998, Kalayanarooj et al. 1997, Kaushik et al. 2010, Oishi et al. 2007, and WHO 2007.[77,78,119,121,171,246]

Dengue Vaccines

No specific anti-viral treatment is available for dengue. Aggressive fluid management to correct hypovolemia has reduced the case fatality rates of severe dengue to 1% or less but this is only palliative.[78] Historically, prevention against dengue disease has focused on vector control and elimination but unfortunately, there is little evidence of long-term sustainable success for such an approach[172] and even when such efforts have been successful, the primary impact appears to lie in suppressing mosquito population numbers rather than lowering the incidence of dengue disease.[2] Thus, given the increasing scope and severity of dengue, efforts towards the development of a protective dengue vaccine are critical. And in theory, a vaccine for dengue should be readily achievable. DENV has no significant animal reservoir (while some spillover from sylvatic DENV has been reported[165] there is no evidence that sylvatic DENV plays any role in maintaining transmission).[232] Furthermore, DENV does not cause chronic infections such as with HIV or hepatitis C. Indeed, following infection with any one serotype, homotypic immunity appears to be quite long-lived if not life-long.[238] Nonetheless, DENV has particular characteristics, detailed below, that present a challenge to the development of an effective and safe vaccine, many of which are corollaries to the advantages just enumerated.

For example, the lack of an animal reservoir ironically presents a significant obstacle inasmuch as the clinical development of candidate vaccines is severely hampered by the lack of a relevant animal model. Mice are one of the most commonly used animal models in clinical

studies but wild-type mice exhibit little to no replication of DENV isolates. Moreover, non-human primates exhibit few signs of severe dengue illness such as fever or shock.[34,259]

A second obstacle is that an effective vaccine needs to prevent infection with all four DENV serotypes. While natural infection does induce long-lasting homotypic immunity, heterotypic immunity, while variable in duration, generally lasts only 6 months and thus only temporary.[201] Development of vaccines with a tetravalent formulation that retains the immunogenicity of all four components has proven difficult, requiring the use of more complicated, multiple-dose immunization regimens.[247] A third obstacle, and one connected to the second, is the possible complications of immune mediated enhancement of disease. Secondary natural DENV infection presents a sixfold higher risk of severe disease compared with primary infection,[27] which is hypothesized to be due to heterotypic ADE. Thus, for a vaccine to be protective, it has to induce both strong and long-lasting neutralizing antibodies against all four DENV serotypes simultaneously yet not be so over-attenuated as to be unable to induce a protective immune response or indeed, to possibly enhance infection. The unique risk associated with dengue vaccines is that their attenuation or inactivation could possibly lead to subneutralizing concentrations of antibody and present a risk of enhancing disease rather than prevention.[159] For example, vaccine induced ADE appears to have played a role in a trial of an experimental live-virus vaccine containing DENV-2. Volunteers in that trial previously given yellow fever virus vaccine exhibited higher antibody titers against DENV-2 than naïve patients and had evidence of higher and more frequent viremia.[199] A fourth obstacle is a relative lack of data to predict the efficacy of candidate DENV vaccines in preventing severe dengue. Experimental challenge studies in humans that were conducted during and shortly after the Second World War made no effort to test the efficacy of candidate vaccines against symptoms of

severe dengue, such as DHF and DSS, simply because such pathologies were unrecognized prior to the late 1950s. Subsequent studies of candidate vaccines have analyzed efficacy only in experimental animal models, none of which faithfully reproduce the DHF syndrome seen in humans. As a result, selection of the most promising DENV vaccine candidates has necessarily relied on comparisons of vaccine- induced immune responses against observation of the protective immunity resulting from natural DENV infections.[191] Nevertheless, a lack of reliable immunological markers distinguishing fully protected individuals from those at elevated risk has always meant that such observations are provisional at best.[192]

Table 1. Dengue vaccines in clinical development (adapted from Sinha 2014)

Developer	Product description	Stage
Sanofi Pasteur	Dengvaxia®. Live attenuated tetravalent vaccine (Yellow Fever 17D/DENV chimeric viruses)	Licensed
GlaxoSmithKline/ Walter Reed Army Institute of Research	Live attenuated vaccine passaged through primary dog kidney cells and fetal rhesus lung cells	Phase 3
National Institutes of Allergy and Infectious Diseases/ Butantan Institute	Live attenuated vaccine (targeted mutagenesis of DEN viruses and DENV/DENV intertypic chimeric virus)	Phase 3
Takeda Pharmaceuticals / Inviragen	Live attenuated DNA tetravalent vaccine (attenuated DEN2 PDK-53 virus and DENV/DENV intertypic chimeric viruses)	Phase 2
GlaxoSmithKline/Oswaldo Cruz Foundation/Walter Reed Army Institute of Research	DENV1, purified inactivated virus vaccine	Phase 1
US Naval Medical Research Center/Vical	Vaxfectin DNA Vaccine (TVDV), expression of prM and E membrane proteins. Formulated with and without Vaxfectin adjuvant	Phase 1

In spite of these impediments, efforts towards dengue vaccine development have been in place since the 1920s, involving attenuating DENV in blood with ox-bile or grinding infected *Ae.*

aegypti mosquitoes in a salt solution and chemically pure phenol and formalin.[154,208] During the Second World War, military physicians in the United States and Japan began efforts soon after the virus strains were isolated. Their studies demonstrated that DENV could be attenuated in humans after serial passage in suckling mouse brains and could elicit protective immunity against DENV challenge.[110,196] Halstead (1980) cites investigations into developing live attenuated DENV-2 and DENV-4 vaccines that began in 1971 at the Walter Reed Army Institute of Research and the University of Hawaii, respectively.[92] Walter Reed tested their vaccine candidate on human volunteers, derived from DENV-2 isolated from the serum of a patient with DF in Puerto Rico on 19 August 1969.[14]

Sanofi-Pasteur Dengvaxia®

While there are a number of candidate DENV vaccines in development (Table 1) the first to be successfully licensed is Dengvaxia®, otherwise known as chimeric yellow fever dengue-tetravalent dengue vaccine (CYD-TDV), developed by Sanofi-Pasteur. This recombinant chimeric live attenuated tetravalent vaccine uses a yellow fever 17D vaccine strain as a backbone for which the PrM (pre-membrane) and E (envelope) structural genes are replaced with those from each of the four DENV serotypes[195] (Figure 10a).

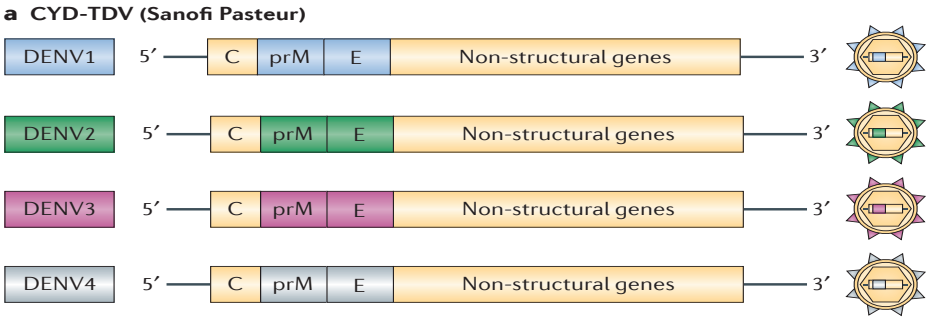


Figure 10a) CYD-TDV structure: a yellow fever virus 17D vaccine strain backbone (in yellow) with prM and E gene segments from DENV 1-4. From Screaton et al. 2015

Sanofi-Pasteur tested the vaccine extensively in large trials in southeast Asia and Latin America among cohorts of adults and children who were either immunologically naive against DENV and other flaviviruses before vaccination or who had some degree of pre-existing flavivirus immunity due to vaccination against YFV or JEV or natural exposure in endemic areas.[89] Based on these tests, the WHO Strategic Advisory Group of Experts (SAGE) on immunization recommended in early 2016 that Dengvaxia be used in countries with high DENV endemicity based on the vaccine's safety and expected public health benefit; particularly when used in high transmission intensity regions with approximately 70% seroprevalence at 9 years of age. On the other hand, based on independent modeling[65] it appears that in very low transmission intensity settings (seroprevalence 10% at 9 years) vaccination of 9 year-olds was likely to increase dengue hospitalization rates. Thus, the recommendation of WHO is that the vaccine CYD-TDV not be used when seroprevalence is below 50% in the age group targeted for vaccination nor in children under 9 years of age.[247]

Based on the WHO's projection of a 10%–30% reduction in symptomatic and hospitalized dengue illness over 30 years, as of 2016 Dengvaxia is now commercially available in the Philippines and Indonesia and approved for use by Mexico, Brazil, El Salvador, Costa Rica, Paraguay, and other countries.[60,254]

CHAPTER 2: EXPERIMENTAL BACKGROUND

Case Epidemiology

The studies described in this dissertation are based on DENV-2 isolates from a series of outbreaks that occurred in the South Pacific in the early 1970s, which were notable – for the purposes of this study – in abruptly shifting from a distinctly virulent character to one that was quite attenuated[85]. Because the epidemiology of these outbreaks was relatively well documented, and serological and clinical data are available for all isolates used in this study, a close examination of this epidemiological evidence seems warranted and is described below.

Outbreaks of dengue were known to occur among the Pacific islands, if only on an occasional basis, such as in the late 1800s and early 1900s in such countries as Australia, New Caledonia, and Fiji. Later, dengue appeared in Fiji, Tonga, and both islands of Samoa during the 1930s[47] .

However, the frequency and size of dengue outbreaks increased sharply across the Pacific with the Second World War, as movements of troops and materials brought increased human population densities as well as expanded vector distribution. Large epidemics of dengue erupted among troops of both armies[76] and there were major epidemics in at least twelve of the nineteen South Pacific territories.[47] Retrospective serological surveys in many of these areas indicated that these epidemics were largely due to the DENV-1 serotype. All available evidence indicates that following the war there were no dengue epidemics for the next 20 years[41.]

The next appearance of dengue began with a mild but widespread epidemic in French Polynesia in 1964 affecting several thousand people (approximately 50% of the population at the

time) due to DENV-3. No major hemorrhagic manifestations were seen. DENV-3 was responsible for another outbreak in Tahiti in 1969, even more mild than that previous.[47]

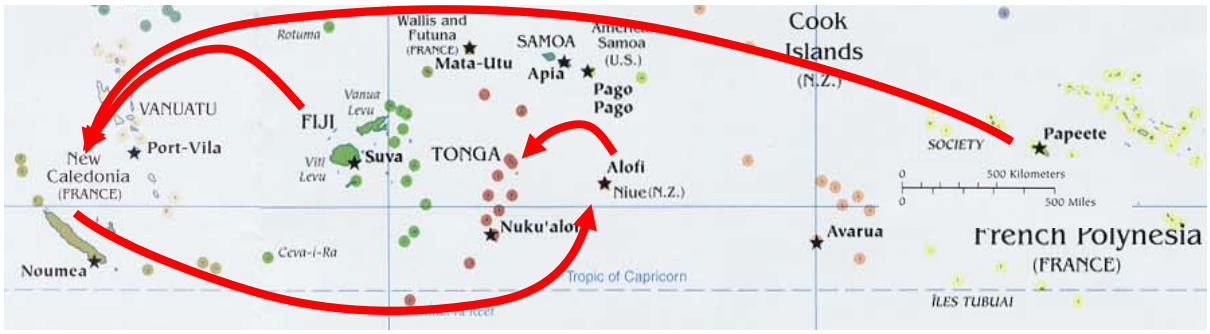


Figure 11. Map of those South Pacific islands - Fiji, New Caledonia, Niue, Tahiti and Tonga - from which DENV-2 was isolated and analyzed in this study. From: U.S. Central Intelligence Agency, 2007.

Fiji

The DENV-2 isolates with which this dissertation concerns itself were derived from a specific set of outbreaks that swept across islands of the South Pacific in the early 1970s (Figure 11). There is some evidence to suggest that the DENV strains involved in these outbreaks were genetically related to outbreaks in Puerto Rico that took place between 1969 and 1973. Thus the virus could have been introduced into the South Pacific from the Caribbean (or vice versa)[220,181]. The South Pacific epidemic sweep began with nearly simultaneous outbreaks in Fiji and Tahiti and smaller neighboring islands beginning in early 1971. The first clinical case in Fiji was diagnosed in March, although subsequent serological tests of young people in the surrounding area suggested that initial infections had occurred several weeks before the diagnosed case. Sporadic cases were reported over the next few months but only in May 1971 was formal notification made of a dengue outbreak. The number of cases remained high until the end of August, and then declined to a minimum of three cases in January 1972, whereupon cases increased again each month to a second peak of 79 cases in April. Notification ceased in April

1973 with a total of 3,413 cases[150].

While many cases showed the classic dengue fever syndrome, a majority of cases were quite mild with serological tests indicating additional large numbers of subclinical infections as well. Only three individuals (all adults) demonstrated hemorrhagic manifestations although none were ever proven to be due to DENV infections. Hemagglutination inhibition (HI) antibody titers in all cases were much higher to DENV-2 than to DENV-1. Seventy-three percent of notifications were from people over 20 years of age, in spite of this cohort only constituting 26% of the population, and 35.4% of these individuals had anti-DENV antibodies before the onset of the epidemic. The age distribution of the outbreak suggested that pre-existing antibodies might have played a role in enhancing subsequent infection. Regarding mosquito populations, with the exception of one neighboring island, dengue outbreaks only occurred in those areas where the *Aedes aegypti* mosquito was present.

Tahiti

Concurrently with the epidemic in Fiji, another outbreak, also of the DENV-2 serotype, took place on the island of Tahiti. The first clinical diagnoses in Tahiti were made in May 1971 with a peak of cases in late June/early July and lasting until September of the same year. Estimates based on hospital and employment records indicate that at least half of the Tahiti's population of approximately 80,000 persons was affected, with the majority affected being adults. More than 50 isolates were obtained, though only one from a DHF case, all of which were DENV-2.

Although most patients exhibited symptoms of classic dengue fever such as headache, muscle pain, morbilliform eruption, etc., there were a small but significant number of patients

who exhibited DHF of unusual severity.[158 188] Of these hemorrhagic patients, 33 experienced symptoms serious enough to warrant hospitalization and 3 died. All were older, all but three had antibodies showing a previous DENV infection, i.e., a secondary antibody response, and the DHF was remarked as being "distinctly hemorrhagic" as opposed to manifesting as dengue shock syndrome. Because, as mentioned above, DENV-3 outbreaks were known to have occurred in Tahiti in 1964-1965 and then again, on a smaller scale, in 1969, the DHF was felt to be compatible with the theory of sequential infection, also known as ADE.

New Caledonia

An outbreak of DENV-2 occurred on the island of New Caledonia from October 1971 to May 1972 affecting approximately 40% of the population, which at the time was around 60,000 persons. Hemorrhagic cases were described as "frequent"[143] with one death of a young male believed to have had a primary DENV infection some years before.

Niue Island

In early 1972 a DENV-2 epidemic that exhibited much greater manifestations of DHF severity took place on Niue Island[15]. Located roughly 300 miles east of Tonga and 350 miles southeast of Samoa with an area of about 100 square miles, Niue Island at the time was relatively isolated, receiving one plane a week and one boat a month. The first case of DENV-2 was laboratory-confirmed in March 1972 although several febrile illnesses suffered by Europeans in February were retrospectively suspected to have been dengue. The largest number of cases occurred March – June and tapered off until September. Out of a total population of approximately 4,600 persons, (all of whom but ~ 150 were Polynesian) the number of people seen by the local

department of health and confirmed as having DENV was 790. There were 12 deaths, seven of which were among children between the ages of 2–14 years. Most patients exhibited signs and symptoms associated with classical dengue infection: a fever (sometimes of "saddle-back" configuration), prostration, headache, backache, retro-orbital pain, and in some cases, a morbilliform rash. However, a small but significant number of patients exhibited DHF/DSS with such symptoms as abdominal pain, hepatomegaly, hematemesis, epistaxis, ecchymosis, shock and coma. Unusually, in a number of patients ecchymosis progressed to ulceration.

Given the severity of the outbreak on Niue Island, a retrospective study was carried out in August 1972. Six hundred and thirteen individuals were queried as to the state of their health since February 1972 and whether they believed they had had dengue. Of these 275 answered affirmatively (44.8% of the total 613 individuals), with 70 having experienced hemorrhagic manifestations (25.4% of cases, 11.4% of the total 613 individuals). A serological study carried out at the same time collected sera from all 8 surviving severe cases who had not left Niue Island, from all available household contacts (4 years of age or older) of the severe and fatal cases (73 total), and from a random sample of persons of various ages who lived in different parts of the island, for a total of approximately 500 samples. HI and plaque-reduction neutralization tests were carried out for both DENV-1 and DENV-2.

The results showed that the outbreak was caused exclusively by DENV-2. The data further showed that all infected persons under the age of 25 had contracted a primary dengue infection during the recent outbreak while most infected persons over the age of 30 experienced an infection of the secondary type. The latter correlated with epidemics of DENV-1 known to have taken place on many Pacific islands during the Second World War. In regards to clinical

outcomes, no striking differences were observed between those individuals experiencing primary infection versus those experiencing secondary either with respect to the percent reporting illness or the percent of illnesses with hemorrhagic manifestations. Such results prompted the authors of the original report to conclude that the observations on Niue were not compatible with the theory of sequential infection and ADE.[15]

Any possible role in regards to vector was also considered. Although *Aedes aegypti* was likely to have inhabited Niue Island at the time of the outbreak (it had not been recorded beforehand but was found subsequently), it was far less prevalent than the endemic species *Aedes cooki*. Since there is no surface water on the island, the probable source for the mosquitoes were cisterns kept for household water supplies. There was no noticeable increase in the number of mosquitoes either before or during the outbreak.

To summarize the status of the DENV-2 outbreaks on those South Pacific islands during the period from 1971 through 1972, i.e., Fiji, Tahiti, New Caledonia and Niue, all had gone through explosive epidemics typified by severe disease and high rates of virus isolation.

Smaller in scale and less documented were outbreaks characterized by mild disease manifestations and poor virus isolation rates as seen in Samoa and a few smaller islands during this same period of time.[84] A sense of the differences in outbreak character between islands can be seen below (Figure 12).

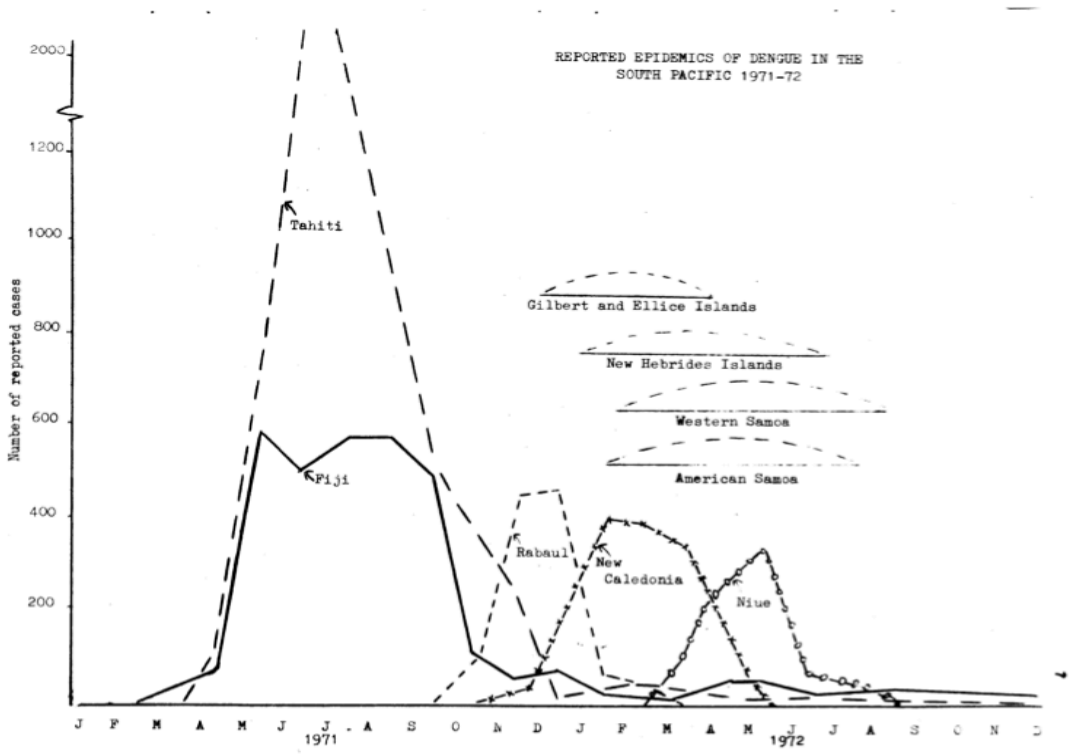


Figure 12. Graphic comparison of the number of reported DENV-2 cases on each island per year in 1971 - 1972. From: The South Pacific Commission Dengue Newsletter. July, 1974 v. 1.

Kingdom of Tonga

The Kingdom of Tonga remained unaffected by the epidemics taking place on neighboring islands in 1971–1972. Made up of more than one hundred islands, of which about thirty are inhabited, the total population of Tonga in this period was estimated to be approximately 90,000. The nearest neighboring territories are the Samoa islands, roughly 450 miles to the north, Fiji to the west and Niue Island to the east, both about 300 miles distant. Given the progression of

epidemics on all of these neighboring islands, public health officials in Tonga had reason to anticipate an epidemic of corresponding severity on their island. In addition, high rainfall over the fall and winter of 1973 had led to greater than usual populations of *Aedes aegypti* as well as *A. tabu* thus heightening concern.[85]

Beginning in August 1973, human plasmas collected in the course of a filariasis survey were tested for dengue HI antibodies in order to detect silent infections. In general, individuals over the age of 40 showed seropositivity against DENV-1 while younger persons did not. However, there were two or three individuals in their late 20s with antibodies against DENV-2. As these individuals had never left Tonga this finding suggested the arrival of DENV-2 on the island before August 1973.[47] Nonetheless, no clinical signs of DENV infection appeared until late December 1973/early January 1974. Subsequent serological studies in March confirmed the presence of DENV-2. With a large dengue outbreak seemingly imminent, it was decided to take advantage of the circumstances to gather data on the duration and magnitude of viremia in effected individuals. Accordingly, records of cases at the major hospital were reviewed for dengue-like illnesses and house-to-house surveys carried out in a local village and two local schools.

Surprisingly, no major outbreak occurred. The incidence of cases built up slowly to a noticeable level in mid-February, with the number of people seeking medical attention at the hospital peaking at 165 in March and 127 in April. Case numbers fell sharply thereafter, with dengue ceasing to be recognized by the end of October. Among those individuals who were either hospitalized or seen as outpatients, almost all had remarkably mild clinical manifestations of short duration and the majority had primary infections. Incidence of hemorrhage was

extremely low. Of those few patients with secondary infections, all were over 40 years of age.[85]

Given the difficulty of determining numbers of those infected and rates of transmission, surveys consisting of interviews and blood samples were conducted in a local village that appeared to be a focal point of infections in April as well as in local schools.[47] Of the 412 villagers interviewed, around 20% had a history of dengue-like illness within two months of the survey with the highest rates of reported illness among persons 5–19 years old. Among 100 school children surveyed, 8% of primary and 13% of secondary school children reported having dengue-like illness or fever in the previous two months.[85]

Isolation of the virus was attempted in 18 patients and all successful isolations were of DENV-2. However, even under conditions that ought to have been ideal, both the isolation rate and virus content of positive sera were quite low. Some patients were identified on day of onset with mild illness and followed for as long as 8 days, with blood samples taken daily and mosquitoes allowed to feed on them. Nevertheless, the majority of these patients, confirmed with DENV-2 infections by seroconversion, had undetectable levels of viremia. When virus was detectable, viremia was at a low titer (≤ 106 MID_{50}) and of only 1–3 days duration.[78] Thus, viremia levels among those tested were found to vary broadly in both magnitude and duration. Retrospective serological assays using plaque-reduction neutralization demonstrated that, with the exception of one individual with no neutralizing antibodies, all patients less than 40 years of age had antibody patterns compatible with DENV-2 while those tested who were over 40 had patterns suggestive of DENV-1 infections.

In summary, this study derives from epidemiological observations of a series of outbreaks of DENV-2 in the South Pacific in the early 1970s, which had a virulent character

initially but then appeared attenuated upon their emergence in Tonga.[85] The outbreaks began, after an absence of almost 30 years, almost simultaneously in both Fiji and Tahiti in early 1971,[158, 150, 188] then spread to New Caledonia and Niue Island in 1972,[143, 15, 188] and then Tonga in late 1973.[85] The outbreaks in Tahiti, New Caledonia, and Niue Island all spread rapidly, infecting from 40 - 50% on New Caledonia and Tahiti and up to 90% on Niue. These outbreaks were of severe classical dengue fever and associated with high rates of viremia. Rates of DHF on these islands were also high, especially on Niue Island where there were 12 deaths, and were recorded as being of a more distinctly hemorrhagic form as opposed to manifesting as dengue shock syndrome.[15, 47] Yet in contrast, the outbreak of DENV-2 in Tonga was extremely mild with almost no DHF and associated with very low viremia.[85]

It is instructive to contrast this DENV-2 outbreak in Tonga with a subsequent outbreak on the same islands in 1975 but this time involving DENV-1. The latter virus' progression through the population of Tonga was explosive; the incidence of hemorrhage higher, and there was a case fatality rate of 12 persons (compared to none previously.) Among the few (n = 11) serum samples collected from this outbreak, DENV-1 was isolated from 4 of 6 patients with primary infections, including one 26-year-old female fatality with DHF. DENV-1 was also isolated in one of the four patients with secondary infections. Thus, primary and secondary infections were roughly equal.[85]

To review then, all islands affected by dengue outbreaks from 1971 to 1974 and for which serological testing was conducted and/or isolates collected had outbreaks of DENV-2. Further, all islands experienced both primary and secondary infections, the latter as a result of previous dengue infections no more recent than 1950. However, with the possible exception of

Fiji, no particular difference was seen in disease severity between the primary and secondary infections.

Those islands from which DENV-2 were isolated previous to Tonga experienced sudden outbreaks that showed a rapid increase in the number of people affected, resulting in 40–50% of the populations becoming infected, and which were characterized by disease of unusual severity. In Tonga however, DENV-2 exhibited near-silent transmission as it circulated around the island for almost a year among a population that was fully susceptible without being detected, resulted in less than 1% of the population exhibiting retrospective serological evidence of infection, and could only be characterized as remarkably mild with the virus seemingly attenuated. And again, a significant difference between these outbreaks was their relative rates of virus isolation. Because the public health systems in Tahiti and New Caledonia were so overwhelmed with the number of sick patients, they were actually unable to draw as many serum samples as might have been preferred. Nonetheless, virus isolation rates were extremely high. By contrast, in Tonga where preparations had been made for a large outbreak, patients showed low viremia and virus isolation success rates were very low. Indeed, in most cases, DENV infections were identified by plaque reduction neutralization antibody tests rather than by clinical symptoms. Interestingly, a subsequent outbreak of DENV-1 in 1975 was dramatically greater in severity, as judged by symptoms, numbers of cases, and virus isolation rates. Table 2 summarizes these differences.

Table 2. Epidemic DENV-2 in the South Pacific (adapted from[84,213])

Country	Year	Nature of outbreak	Severity of disease	Est. case numbers	Virus isolation rate (%)
Tahiti	1971	Explosive	Severe	40,000 (50% of pop)	> 75
Fiji	1971	Explosive	Severe	3,400 acute; 20,000 subacute	> 75
New Caledonia	1972	Explosive	Severe	25,000 (40% of pop)	> 75
Niue	1972	Explosive	Severe	2,070 (45% of pop)	NA
American Samoa	1972	Smoldering	Mild	30	57
Tonga	1974	Silent	Mild	24 hospitalized; 100+ subacute (~ 0.05% of pop)	33
Tonga (DENV-1)	1975	Explosive	Severe	40,000 (50% of pop)	45

Possible factors in the variation in South Pacific DENV-2 epidemic severity

The transmission and incidence of DENV, and the epidemic response to infection represents a complex matrix of interactions among multiple populations of hosts, vectors, and pathogens. Thus, in order to better understand the apparently dramatic attenuation observed in the outbreak on the island of Tonga versus those on the other islands of the South Pacific epidemic sweep, it is necessary to delineate the various factors that may have played a role.

Epidemiology

The epidemiology of the series of outbreaks constituting the South Pacific epidemic sweep of DENV-2 from 1971 to 1974 has been reviewed in the previous section. It bears emphasizing however, that much of the unique character of this series of outbreaks is due to the unusual degree of geographic isolation of the human populations affected. All the islands described, except Tahiti, were relatively inaccessible at the time and, again with the exception of Tahiti, had

not experienced DENV for 25–30 years.[85]

Entomology

The possible role of mosquitoes in the differential expression of DENV-2 between islands warrants discussion. As previously mentioned, while other *Aedes* mosquitoes have been shown to be competent for the DENV, *Ae. aegypti* is considered the primary vector of transmission.[132]. While no information is available from the 1971 outbreak on Tahiti, *Aedes aegypti* was found in all areas of confirmed infection in Fiji with the exception of the neighboring island of Rotuma. There, only the endemic species *Aedes rotuma* was detected.[150,47] In New Caledonia *Ae. aegypti* mosquitoes were found to be abundant in all areas where dengue was occurring[143] while in Niue, although *Ae. aegypti* had not been reported previously, it was found subsequently and was assumed to be present during the outbreak.[47, 15]

A number of *Aedes* species, including *Ae. aegypti*, and *Ae. tabu*, were known to be present on Tonga during both the 1974 DENV-2 and 1975 DENV-1 outbreaks although overall numbers were lower in 1975 and while each species' relative numbers varied between Tongan island groups, these differences did not correspond to epidemic intensity.[85] Attempts to infect both *Ae. aegypti* and *Ae. tabu* by feeding on dengue patients were successful in only one case and subsequent examination of salivary glands found no presence of DENV-2. Interestingly, infectivity evaluations using the Tonga strain as a basis for vaccine development also found that the strain failed to infect the midgut or head of *Ae. aegypti* while they noted that DENV-2 New Guinea prototype strain had been shown to be highly infectious.[23]

Thus, evidence for vector influence on differences in epidemic behavior is lacking. *Ae.*

aegypti was found on almost all islands in which dengue outbreaks occurred. While vector competency has been shown to vary between species, as well as between different geographic populations of the same species, no correlation has been established between these variations and clinical outcome, nor have subsequent dengue outbreaks with different serotypes been observed to follow the same geographic pattern.[186]

Serology and host genetics

Much of the pathophysiology associated with DENV infection is thought to be a direct consequence of immune response.[17] It is worth asking therefore whether the dissimilar outbreak epidemiologies were owing to variations in host response, due for example to differences in exposure history on the various islands or to possible genetic differences.

Beginning in the 1960s with pioneering seroepidemiological studies by Halstead and colleagues in Thailand there has been growing attention paid to the increased risk of severe dengue disease, such as DHF, following secondary infection with a novel serotype; that is, a new DENV infection in an individual who had previously experienced one or more DENV infections of a different serotype.[94] Exposure to the initial infection generates antibody recognition but there is evidence to suggest that structural differences between DENV serotypes are sufficiently large that the cross-reactive antibodies are poorly neutralizing and even enhancing. Thus through the process of ADE, antibodies generated by during primary infection may promote entry of DENV into host cells during secondary infection leading to greater viral burden and more severe disease.[98]

While sequential infections with heterotypic serotypes are routine in hyperendemic

countries, the South Pacific at this time was a special case. As has been mentioned repeatedly, this area was unusually isolated, not just from other parts of the world but also from other islands in the region. Inter-island traffic at this time was infrequent. In addition, and in consequence of its long isolation, most of these islands – with the exception of Tahiti – had no record of prior dengue outbreak for the past 20–30 years. Accordingly, all islands, with the possible exception of Tahiti, had similarly low seropositivity rates from previous infections. Thus, there is little evidence for some contribution of secondary infection to the epidemiological pattern seen in the South Pacific sweep. As described in the section on Niue Island – site of perhaps the most severe outbreak in the South Pacific DENV-2 sweep – retrospective serology found that all infected persons under the age of 25 had contracted a primary DENV infection. While on the other hand, most infected persons over the age of 30 had an infection of the secondary type, the latter correlating with epidemics of DENV-1 that took place during the Second World War. Yet significantly, in regards to clinical outcomes, no striking differences were observed between those individuals experiencing primary infection versus those experiencing secondary infection, either in terms of the percent reporting illness or the percent of illnesses with hemorrhagic manifestations.[188,15] Similarly with Tonga, site of the least severe outbreak, clinical severity was not seen to correlate with secondary vs. primary infection, either in the 1974 DENV-2 outbreak nor, and perhaps more surprisingly, in the subsequent DENV-1 outbreak in 1975, which was much more severe both in terms of numbers of patients and severity of symptoms. In neither outbreak was there any evidence of correspondence between clinical severity and secondary infection.[85]

Other host factors that have been suggested to contribute to a higher risk of severe dengue include age, race/ethnicity, nutritional state, and underlying chronic diseases such as

asthma.[91] As to the latter possibility; there were numerous cases of patients with pre-existing conditions whose character might have increased their susceptibility to hemorrhagic symptoms. Yet no overall pattern of pre-existing conditions could account for the differences in disease severity seen between islands such that would explain the attenuation seen in Tonga. In regards to race/ethnicity – there is in fact some evidence for host genetic differences in dengue disease susceptibility – for example, it has been suggested that individuals of African descent may have greater resistance to DENV infection. [203,204] Yet again, there appears to be no obvious correspondence between differences in the host genetics of the South Pacific island populations affected and the pattern of DENV-2 outbreaks described. As previously mentioned, affected island populations were either primarily Polynesian (Tahiti, Niue, Tonga) and/or Melanesian (New Caledonia, Fiji). As an example, the population of Niue was the most affected in terms of severity of symptoms and proportion of people infected, whereas Tonga was the least affected; yet both islands are primarily populated by Polynesians. And again, patterns of ethnicity do not bear out upon subsequent dengue outbreaks as for example, the dramatically different epidemic outcomes on Tonga itself between the attenuated DENV-2 outbreak of 1974 and the 1975 DENV-1 outbreak which was associated with high morbidity.

Phylogenetics of the South Pacific DENV-2 Sweep

Introduction

In an article published in 1978, Dr. Duane Gubler stated,

> "The most striking feature of the 1974–1975 dengue experience in the Kingdom of Tonga was the contrast between the severity of the clinical manifestations and the explosive nature of the outbreak in 1975 as compared with 1974."[85]

After reviewing (and rejecting) various alternative explanations for this contrast he wrote,

> "The most likely explanation, therefore, for the difference in dengue manifestations in the 2 [years] is that in 1975 the dengue type virus circulating in Tonga was by nature more virulent than the dengue type 2 viruses present in 1974."[85]

Nevertheless, he was forced to conclude,

> "Since it is very probable that both dengue type 1 and dengue type 2 viruses spread from one island to another, the reason or reasons for the differing virulence of the infection on different islands remains a mystery." [85]

In hopes of resolving this mystery, and given the evidence for variations in DENV virulence (as reviewed above) we performed a comparative genetic analysis of the samples of DENV isolated from the outbreaks that made up the 1971–1974 South Pacific sweep. Because this genetic analysis served as the foundation for the phenotypic research described in this dissertation, it is worth summarizing in some detail.

Based on the epidemiological and serological data from these outbreaks (reviewed in the Epidemiology section above) and the lack of a reasonable alternative explanation for the attenuation seen in Tonga outbreak (see the section "Possible factors in the variation in South Pacific DENV-2 epidemic severity" above) it seemed reasonable to suggest that the attenuation seen on Tonga might be a result of genetic changes in a population of DENV-2 in the course of its transmission from human populations on neighboring islands.

Thus, genetic analysis was carried out with the objective of delineating the evolutionary relationships among the virus samples collected from the South Pacific series of outbreaks with the goal of establishing whether genetic variation among those strains led to the eventual attenuation observed on the island of Tonga.

With these samples in hand, the following investigations were performed, based on specific hypotheses with their rationale included, the approach taken, the method used, and the results acquired therein.[213]

Phylogenetic analysis of South Pacific evolutionary relationships.

Hypothesis: DENV isolated from the 1971–1974 South Pacific sweep have a high degree of sequence homology to the degree that they cluster together as a distinct clade within the genotypes of the DENV-2 serotype reflecting a single infectious origin.

Rationale: Contemporary observers of the 1971–1974 South Pacific dengue outbreaks[15, 85, 158] assumed that they were due to viruses undergoing *in situ* evolution rather than the consequence of multiple introductions into the region. Nonetheless, with no method of proving such a supposition, multiple questions remained, questions that whole-genome sequencing is designed to answer:

- What was the likely evolutionary and geographic origin of the DENV-2 responsible for the South Pacific sweep?
- Was there a single virus ancestor common to all the outbreak isolates or would their sequences reveal the existence of more than one source?

Approach: In order to examine the evolutionary relationships between the 20 South Pacific DENV isolates and more specifically, to provide them with a genotypic context and examine whether *in situ* evolution rather than multiple source introduction occurred during the South Pacific sweep, a phylogenetic tree was generated that included 54 publicly available sequences representative of all genotypes within serotype 2. Additional verification was provided through multiple node support analysis.

Methods: The 20 low-passage South Pacific sweep DENV-2 isolates (see Table 3, page 110) were used to infect C6/36 cells. Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen), reversed-transcribed with SuperScript III Reverse Transcriptase (Invitrogen), and amplified by PCR using PfuUltra II Fusion HS DNA polymerase (Stratagene), using primers designed for 2X coverage of the entire ORF. PCR-amplified DNA fragments were run out on 1% agarose gel and purified using QIAquick Gel Extraction kits (Qiagen). Purified DNA fragments were sequenced at the UH Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics sequencing facility, using an Applied Biosystems 3730XL DNA Analyzer. Sequence editing, alignment and assembly of completed genomes were done using Sequencher 4.7 (Gene Code Corp.) with alignments verified in Se-Al 2.0 (Rambaut, Oxford Evolution Group). To provide genotypic, geographic and temporal context, 54 publicly available sequences spanning all known DENV-2 genotypes and representing a comprehensive and diverse assemblage of DENV across

the serotype were added; this included whole genome nucleotide sequences from Zhang et al (2006)[257] and envelope gene sequences from Twiddy et al (2002)[225]. In addition, envelope gene sequences from sylvatic DENV-2 strains were used as outgroups to root the tree. All additional sequences were aligned with the South Pacific DENV isolates, as detailed above. Evolutionary relationships among isolates were inferred through two methods of tree building: maximum likelihood (ML) and Bayesian inference. For ML, sequence alignments were imported into PAUP* 4.0b10[215] and RAxML[212] for phylogenetic analysis. In order to verify ML tree topologies and node support Bayesian posterior probability values were generated for each node using MrBayes[113,187]. In addition, 100 ML bootstrap replicates were implemented in the RAxML BlackBox web server [212] as an additional measure of node support. Node support values and topologies generated from all three methods–PAUP*, MrBayes and RAxML BlackBox–were virtually identical.

Results: A key question regarding the outbreaks on each island was whether they might have been caused by strains that were genetically and evolutionarily unrelated. Given the geographic isolation of these islands, even greater at that time than today, a series of unrelated outbreaks would have been unlikely. Nevertheless, there was always the possibility that the dramatic differences in epidemic behavior seen between Tonga and the other islands were simply due to Tonga having experienced the introduction of a different DENV-2 strain from elsewhere. The ML-generated phylogenetic tree (Figure 13) unequivocally confirmed that a) the viruses isolated during the South Pacific epidemic sweep were of the same evolutionary lineage; b) these same isolates all belonged to the DENV-2 American genotype, which includes New World isolates dating back to the 1950s, and c) all stemmed from a single introduction into the region from the Americas. Both the Bayesian posterior probability and ML bootstrap support values provided an assessment of 100%

support for critical nodes on the tree, confirming both the robustness of the tree and the placement of the South Pacific isolates within the American DENV-2 genotype.

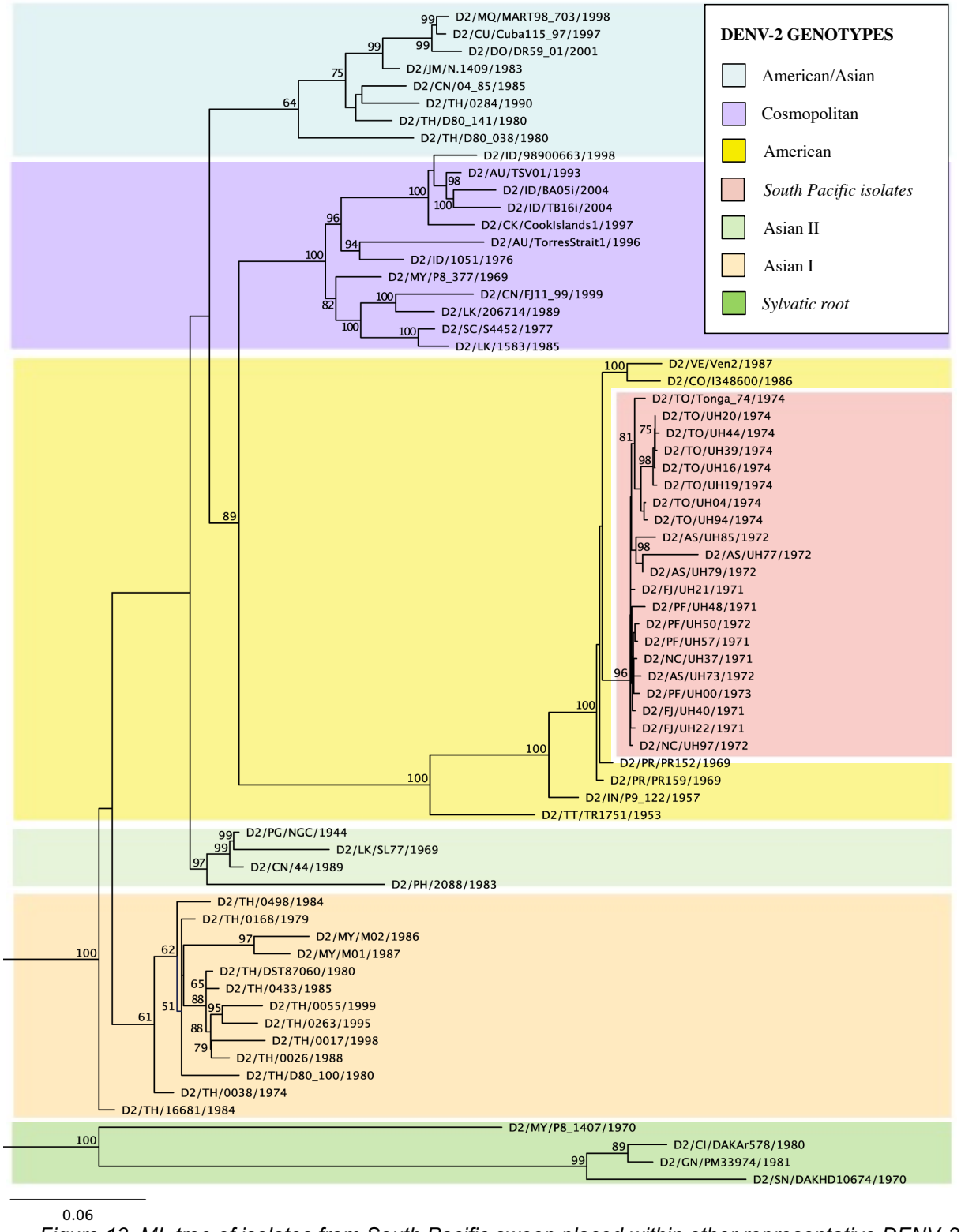


Figure 13. ML tree of isolates from South Pacific sweep placed within other representative DENV-2 genotypes, including whole genome (Zhang et al. 2006) and E gene sequences (Twiddy et al. 2002). E gene sequences from sylvatic DENV-2 strains used as outgroups to root tree. Node numbers are 100 replicate bootstrap values under the ML substitution model and derived from RAxML program.

Phylogenetic analysis of Tonga clade for signs corresponding to attenuation

Hypothesis: Genetic sequences derived from DENV isolated from patient blood draws on the island of Tonga are expected to form a distinct clade within a phylogenetic tree of the South Pacific isolates, and in so doing, demonstrate a distinctive genetic basis for the clinical attenuation unique to the outbreak on Tonga.

Rationale: Having ruled out variations in vector transmission efficiency, human immunity and secondary infection with a heterotypic serotype as possible causes of the attenuation observed on Tonga, Gubler et al. (1978) suggested that strain variation was the most likely factor to fit the evidence.[85]

Approach: Evolutionary relationships between South Pacific isolates were examined by generating a phylogenetic tree along with verification through multiple node support analyses. The goal, as in the previous experiment, was to answer the following questions:

- Would the clinical similarity seen among Tonga patients be reflected in the similarity of the sequences among the Tonga DENV isolates? Would viruses isolated from patients on Tonga show greater genetic homology with one another than they do with DENV-2 isolated from patients on the other South Pacific islands? Or, in other words, would the viruses isolated from dengue patients on Tonga show sequence homology great enough to form a monophyletic group on a phylogenetic tree?

Methods: Phylogenetic analysis was carried out as described above. As with the full serotype tree described above, evolutionary relationships among the South Pacific DENV-2 isolates were inferred using a ML method in the phylogenetic program PAUP*. However, this second tree consisted only of South Pacific isolates rooted with four American genotypes as an outgroup,

thus allowing for a test of the hypothesis of Tonga forming a unique and distinct clade apart from the other South Pacific sequences.

Results: The ML-generated phylogenetic tree consisting of only the 20 whole-genome South Pacific isolates plus the American genotype sequences for rooting (Figure 14) clearly revealed that the Tonga sequences clustered into a distinctly monophyletic clade lending support to the hypothesis that there is a genetic correlation to the clinical attenuation. In addition, this ML tree of (Figure 14) provides a host of evidence; including clustering of the Tonga clade with a 1971 Tahiti strain, the high shared-branch node probabilities, and the high percent identity between the Tahiti and Tonga strains, that the Tonga clade is clearly derived from earlier Tahiti strains and not the result of a separate infection event.

Amino acid substitutions in Tonga clade that correlate with patterns of attenuation.

Hypothesis: Samples of DENV isolated from patients on Tonga will show evidence of nucleotide substitutions leading to amino acid replacements shared across the clade with these amino acid changes as the basis for the distinct attenuated phenotype of Tonga DENV.

Rationale: As with other RNA viruses, DENV lacks the proofreading enzymes used by DNA-based organisms to enhance the fidelity of genome replication. Yet, genetic diversity in DENV is relatively low in comparison with other RNA viruses; DENV strains belonging to the same serotype vary by no more than 10% at the nucleotide level and 4% at the amino acid level. The constraints on DENV evolution probably reflect the need to preserve critical determinants involved in virus-cell interactions across two very diverse phyla (Arthropoda and Chordata).[157] Nonetheless, we know that minor amino acid changes can result in significant changes in viral epidemic potential as has been recently illustrated by the chikungunya virus where a single

mutation in the envelope protein gene resulted in enhanced infection and accelerated viral dissemination in *Ae. albopictus* mosquitoes, conferring a selective advantage over infection in *Ae. aegypti.* [222] Given our inference of an underlying genetic basis for the observed phenotype of attenuated virulence, genetic alteration of the protein sequence warrants investigation.

Approach: Nucleotide substitutions detected under phylogenetic analysis were mapped for amino acid changes.

Methods: The 20 whole-genome sequences derived from the South Pacific DENV-2 epidemic sweep plus American genotype sequences for rooting (see Figure 14) were analyzed using a parsimony approach implemented in the program MacClade.[149] Beginning with an assumption of minimum evolution, MacClade mapped the most parsimonious distribution of amino acid changes onto the branches of the ML phylogenetic tree made up of the 20 South Pacific isolates, noting amino acid changes that occurred on branches both antecedent and within the Tonga clade.

Results: Mapping of the distribution of amino acid changes onto the Tonga clade allowed for the identification of 3 distinct substitutions specifically associated with and acting as synapomorphies for the Tongan isolates; in **NS4A** which saw a change from an isoleucine to a methionine at residue 46 ($I46_{4A}M$) that defines the entire Tonga clade including the attenuated vaccine strain, Tonga 74, as well as a Tahiti strain isolated in 1971 and presumably sharing ancestry with the Tonga strains; in the **pre-membrane (prM)** gene region which had a histidine to arginine substitution at residue 54 ($H5_{4pM}R$) and defines all naturally isolated Tongan samples; and in **NS2A** which experienced a serine to glycine substitution at residue 83 ($S83_{2A}G$) and defines a subset of Tonga isolates (see Figure 14).

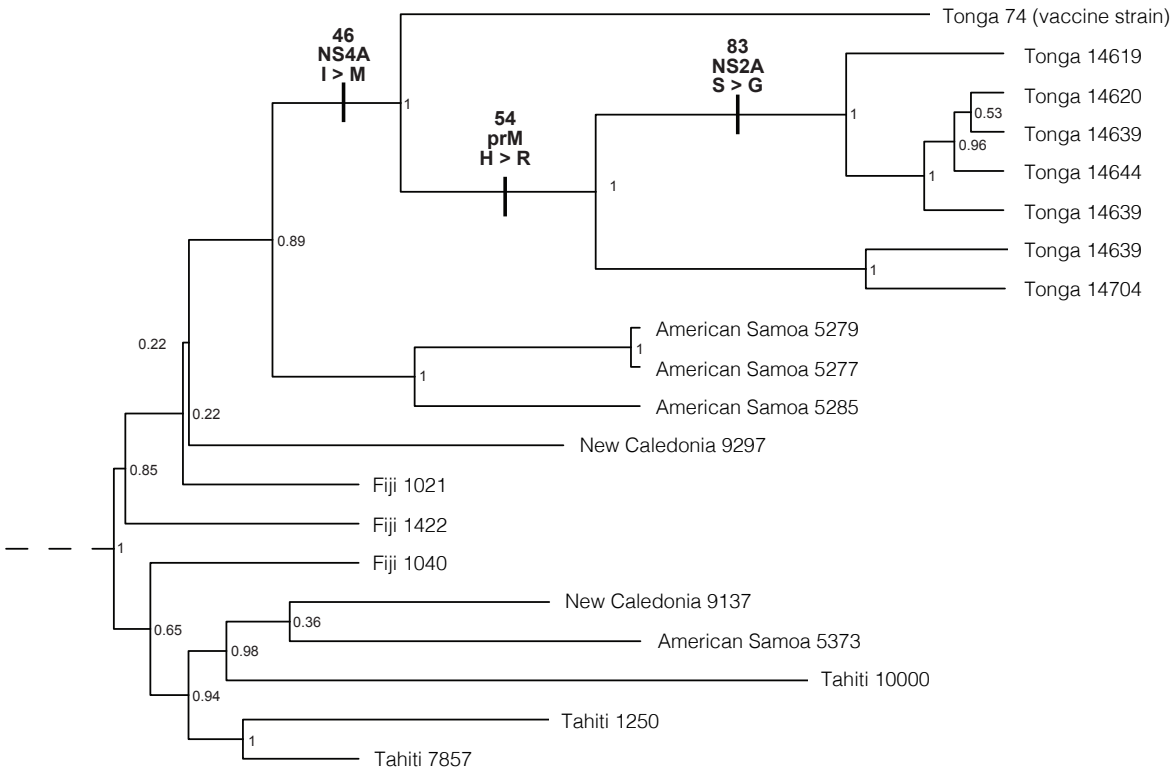


Figure 14. Phylogenetic tree of the South Pacific DENV-2 epidemic sweep illustrating the three amino acid changes defining the monophyletic Tonga clade.

Whether these amino acid substitutions can account for the attenuation observed among DENV-infected Tonga patients is open to question, and even if so, the mechanism by which substitutions could lead to attenuation is unknown; nevertheless, our attempt to correlate specific amino acid substitutions with the epidemic attenuation offers insights into DENV genes whose functions still remain under investigation.

NS2A and NS4A have both been implicated in the inhibition of the interferon-mediated antiviral immune response through interference of STAT1 function[162] albeit to a lesser extent than NS4B. Nonetheless, the antagonistic effects of these proteins did appear to be stronger when all three proteins were co-expressed. Working with WNV, Liu et al. (2004)[141] also found NS2A

to be a major inhibitor of IFN whose effects could be reduced 15- to 50-fold through a single amino acid substitution (Ala30 to Pro [A30P]). Interestingly, Umareddy et al. (2008) found that such inhibitory effects were strongly strain dependent (though not serotype-specific), suggesting a molecular basis for strain variations in epidemic potential.[229]

prM, a precursor to the M protein, is thought to promote infectivity of mature virions upon rearrangement of virion surface structure upon proteolytic cleavage.[36] Leitmeyer et al. (1999)[135] identified two amino acids in the prM protein - prM-28 (Glu3Lys) and prM-31 (Val3Thr) - that distinguished the more virulent Southeast Asian genotype of DENV-2 from the American and noted previous experiments[26] that demonstrated the induction of protective antibody in mice models upon exposure to the prM protein in combination with the membrane (M) protein.

The specific amino acid changes themselves bear examination. The change from histidine (H) to arginine (R) in the pre-membrane protein replaces an aromatic amino acid with a straight-chained molecule capable of forming multiple H-bonds. Both amino acids are polar but arginine is much more hydrophilic (it is the most hydrophilic of all amino acids) and while both amino acids have a positive charge, arginine is more positive, while relatively small shifts in physiological pH are capable changing the average charge of histidine.

In NS2A, while serine (S) is relatively neutral in terms of hydrophobicity, glycine (G) is decidedly hydrophobic. Perhaps more important, serine is a polar amino acid with a hydroxyl side chain while glycine is non-polar and structurally is the smallest of the amino acids.

Finally, the change from isoleucine (I) to methionine (M) in the NS4A protein replaces the most hydrophobic of all amino acids (I) with one considerably less so. Both amino acids are

nonpolar and neutral in charge although the substitution replaces a hydrocarbon side chain with a sulfur-containing one.[166]

Whether any of these amino acid substitutions would be likely to modify the proteins that they are found within is unknown although even small structural differences might have an important effect on protein secondary structure.

South Pacific isolates tested for recombination and/or positive selection.

Hypothesis: We hypothesize that genetic and phenotypic variation among the South Pacific isolates will be the result of nucleotide substitution, rather than recombination, and that any amino acid replacements are more likely to be fixed under conditions of genetic drift rather than positive selection.

Rationale: Recombination is the formation of new, covalently linked combinations of genetic material from either two separate genomes or between different sites on the same genome [55]. In the case of dengue, natural recombination is thought to result from the co-infection of a single individual human or mosquito host by different viruses, which may be of different DENV subtypes, serotypes or even more than one species of Flavivirus[252]. The existence of recombinant DENV and their role in DENV evolution remain controversial[1,180] and the existence of a recombinant among the South Pacific isolates would be unusual. However, since recombination between strains can conjoin sequences with distinct and individual lineages, it can profoundly affect the topology of a phylogenetic tree, increasing the danger of misinterpretation. Thus, the detection and quantification of recombination is a necessary task in genetic analysis. Similarly, given the stochastic nature of the DENV transmission event, seasonal fluctuations in vector populations, and the isolating effect of islands to generate numerous population bottlenecks, any

fixation of amino acid replacement is much more likely to be a result of drift rather than positive selection.[18] But again, as with the relative unlikelihood of recombination, even though positive selection is not expected to have played a role in the South Pacific sweep, the significance of positive selection and the relative ease of testing for it makes it worthwhile to carry out.

Approach: As a test of positive selection, the ratio of non-synonymous substitutions (d_N) to synonymous substitutions (d_S), i.e., d_N/d_S, is an accepted and frequently used surrogate [74]. Phylogenetic codon-based tests to detect recombination were used to test all South Pacific sequences.

Methods: To determine whether amino acid changes amongst the South Pacific isolates had been fixed by positive selection rather than random genetic drift, we estimated relative rates of nonsynonymous to synonymous nucleotide substitution (d_N/d_S) across coding portions of a multiple alignment that included the South Pacific isolates as well as the four American genotype outgroup sequences. We utilized two methods to assess the extent of adaptive evolution among the taxa in this alignment; PARRIS (rates across the alignment) and GABranch (rates on individual branches) (DataMonkey.org). Tests for recombination among the South Pacific DENV-2 isolates were carried out using the GARD detection method (also in DataMonkey.org). In the event that breakpoints were found, alternative phylogenies were explored by generating neighbor joining trees with bootstrap resampling (100 replications) in PAUP* for the multiple alignments of South Pacific sequences on either side of the breakpoint under the same model of evolution.

Results: Analysis of rates of nonsynonymous (d_N) to synonymous (d_S) substitution identified no significant positive selection pressure on amino acid substitutions throughout the South Pacific outbreaks or specifically on the Tongan clade. Tests for recombination tentatively identified an

incongruous Tahiti isolate which was removed from the list of compared sequences and thus played no role in further phylogenetic analysis.

Viral demographics: DENV-2 population changes across the South Pacific sweep

Hypothesis: We hypothesize that the evidence for genetic variation leading to attenuation will be revealed in changing DENV demographics. That is, we predict that the number of DENV will increase as the virus moves across the susceptible island host populations, but then as a result of attenuation, the introduction of the virus into Tonga will be accompanied by a drop in viral population numbers.

Rationale: We suspected that a source of evidence for viral genetic variation leading to the attenuation on Tonga might be found in tracking changes in DENV demographics. That is, we would expect that the number of DENV would be found to increase as the virus moved across susceptible island host populations but then as a result of attenuation the introduction of the virus into Tonga would be accompanied by a drop in viral population numbers. Given the lower viremia among Tonga patients remarked upon by Gubler et al. in their 1978 paper,[85] impairment of replication efficiency in the causative viruses might be a likely cause of the observed attenuation and this should be observable in demographic tests.

Approach: Coalescent based methods of phylogenetic analysis as implemented in the BEAST program provide a statistical means of comparing relative population sizes over time and these were carried out.

Methods: Effective population size for a virus like DENV is essentially equivalent to the effective numbers of infections that go on to produce subsequent infections. Virus effective population sizes (N_e) over the timespan of our study were estimated as a function of relative

virus genetic diversity amongst sequences isolated at different points in time, with most isolates in our study dated to month and day of sampling. Relative genetic diversity ($N_e t$, where t is the generation time, set to 2 weeks for DENV) is an indicator of effective population size under a neutral evolutionary process based on coalescent theory.

We used a Bayesian Markov Chain Monte Carlo (MCMC) inference framework in the program BEAST to independently estimate relative genetic diversity and N_e while incorporating uncertainty in the phylogeny by integrating across tree topologies.[57] Within BEAST we used the Bayesian Skyline model, employing a relaxed uncorrelated lognormal molecular clock model and a codon model of substitution.[72] Statistical uncertainty in mean N_e estimates is reflected in 95% Highest Probability Densities (HPD). MCC topologies were generated in the TreeAnnotator program and were identical to topologies generated by the other tree estimation methods.

Results: Our coalescent-based reconstruction of the demographic history of the American genotype DENV-2 in the South Pacific based on estimates of relative genetic diversity ($N_e t$) over time suggests that viral effective population sizes (N_e) experienced a decline representing a genetic bottleneck when introduced from the Caribbean. This was followed by an increase in diversity suggestive of exponential growth during first establishment in the South Pacific in 1971, followed by a brief decline starting around 1972 that did not reach former population lows and then what appears to be a period of slow or neutral growth beginning late 1973 (Figure 15).

These changes in population size are consistent with epidemiologic data of large initial case numbers in 1971 and 1972 – as in Tahiti, Fiji and New Caledonia – followed by diminishing case numbers in American Samoa in 1972 and Tonga in 1974.[158, 15,82,85]

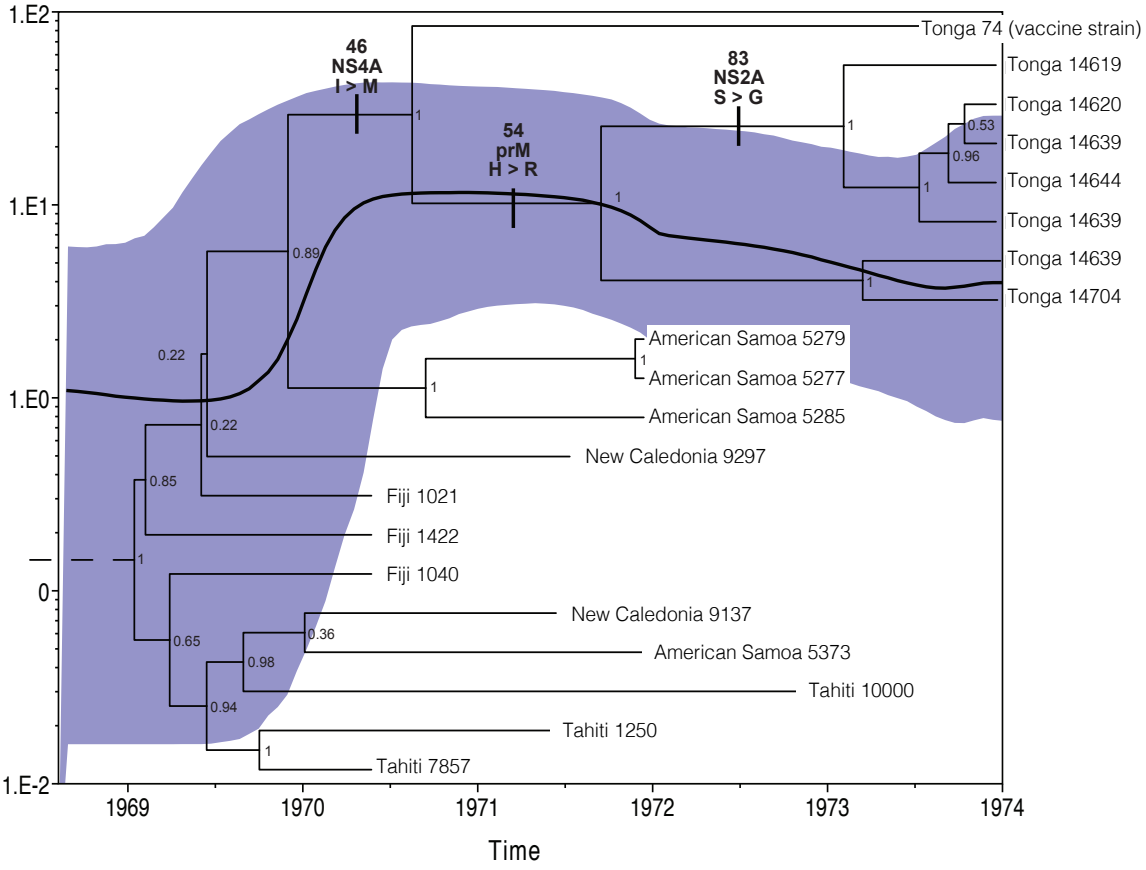


Figure 15. Maximum clade credibility tree of isolates from the South Pacific sweep on a temporal scale, aligned with the effective virus population size estimates (Bayesian Skyline plot) based on genetic diversity, both generated in BEAST [57]. E gene sequences from American genotype strain of serotype 2 used as outgroup to root the tree. Node numbers are posterior node probabilities. Bold values are inferred amino acid substitutions that may correlate with strain attenuation. Corresponding Bayesian skyline plot shows mean effective number of infections (Ne) over the same time scale (solid black line, left-hand axis) with shaded area representing 95% high probability densities.

Summary

This previous research involved carrying out whole-genome phylogenetic analysis of the DENV-2 isolates collected during this South Pacific sweep, comparing them with other publicly available DENV-2 sequences – all of which were representative of the temporal and geographic variation available within the DENV-2 serotype - in order to identify the genetic variations

within the South Pacific isolates, particularly those changes in amino acid composition that correlated with the attenuation of the DENV-2 outbreak in Tonga, and in so doing elucidate the relative contribution of virus strain variation to the epidemiology of the South Pacific outbreaks. Our phylogenetic analysis revealed:

- All South Pacific DENV isolates were found to fall within a single clade and a single genotype demonstrating their common evolutionary origin.
- All Tonga DENV isolates were found to cluster into a single monophyletic clade suggesting that there is a genetic correlation to the clinical attenuation seen among patients infected with DENV-2 on the island of Tonga as compared to other islands.
- 3 distinct amino acid substitutions were associated with the Tongan DENV-2 isolates and found in the prM, NS2A and NS4A proteins.
- No positive selection was detected in the South Pacific phylogeny suggesting evolution through genetic drift.
- A recombinant breakpoint was detected in the South Pacific DENV-2 phylogeny with the recombinant sequence suspected to have originated in Tahiti.
- Viral demographics show an increase in Tonga DENV-2 populations suggesting that attenuation was not accompanied by decreased transmissibility.

Thus, the results derived from the analysis in this case study strongly suggest a genotypic basis for the attenuation seen in the 1974 outbreak in Tonga. Having discussed this research as an introduction to that which is the focus of this dissertation, I now shift from analysis of the South

Pacific DENV-2 isolate's genotype to an analysis of their phenotype in order to determine whether the attenuation seen on the island of Tonga is due to the amino acid substitutions which has resulted in downstream altered function, and/or whether epidemic attenuation is due to reduced transmission or higher asymptomatic rates.

CHAPTER 3: OBJECTIVE & SPECIFIC AIMS

Introduction

The four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4) cause a wide spectrum of clinical disease, ranging from febrile illnesses to life-threatening hemorrhagic fever. Worldwide incidence has increased alarmingly to where DENV is now the most common mosquito-borne viral disease in the world.[22] A variety of known risk factors for serious dengue disease have been identified, such as viral strain variation, vector competency, and secondary infection by a heterologous DENV serotype, but the mechanisms by which DENV causes disease that could account for such variability in both clinical symptoms and epidemic intensity remains unclear. As discussed in the previous chapter, we have focused on a possible role for DENV strain variation in determining epidemic potential or virulence. To do this we focused on a simplified transmission arena and demonstrated a possible link between strain variation and epidemic severity by carrying out phylogenetic analysis of a DENV-2 sweep among South Pacific islands that took place in the early 1970s. We found that DENV-2 isolates from an outbreak on the island of Tonga revealed three unique amino acid substitutions in the prM, NS2A and NS4A gene regions that defined the Tonga clade by distinguishing them from all other DENV-2 isolates and may provide a structural basis for the distinctive epidemiological attenuation observed during the Tonga outbreak. That there is a molecular basis for such differences in epidemic potential is still unclear however. While a number of studies have attempted to identify specific structural differences that correlate with clinical and/or epidemic outcome[135,44,45,38] definitive evidence continues to be elusive.

Objective, Hypothesis and Aims

The **objective** of this dissertation research is to better understand the role of virus evolution in DENV epidemic dynamics and disease severity. Based on the apparent correlation between the apparent clinical attenuation of symptoms among DENV-infected patients on the island of Tonga with a unique set of amino acid substitutions that define the Tonga clade, we **hypothesize**: *evolutionary changes in the DENV genome can give rise to significant variations in disease severity and epidemic potential between different populations of DENV*.

In **Aim 1**, we will explore the relationship between genetic changes in DENV-2 epidemic strains and their phenotypic effects using *in vitro* models. Phenotypic differences between strains will be measured on the basis of viral productivity (number of virus produced, i.e. viral titer), infection efficiency (number of infected cells), and replication rate (rate of virus production per cell). In addition, the specific mutations characterizing the Tonga synapomorphies suggest additional measurements to examine the proportion of infectious virions and possibly the viral effects on host immune response. DENV-2 isolates from the entire South Pacific sweep will be compared for phenotypic differences in both human and mosquito cells.

In **Aim 2**, we will use *in vivo* models to investigate the effects of genetic changes on phenotypes relevant to the DENV mosquito vector such as infection and replication. At the conclusion of these studies, we will have expanded our knowledge of various DENV phenotypes, including replication and dissemination dynamics, examined variations introduced into such dynamics by genetic changes between strains, and developed new tools for further examination into DENV pathogenesis.

Significance

Given that DENV transmission takes place exclusively between the human host and *Aedes* vector, no animal model is available for direct examination of viral strain variation. This study is unique in that it takes advantage of a natural experiment in which many, if not most, of the confounding factors in dengue disease dynamics could be excluded except for virus evolution, thus allowing for a clearer examination of the effects of strain variation and genetic change on DENV epidemic severity. The correlation between genetic change and outbreak variation identified in DENV-2 isolates from Tonga thus provide a unique opportunity for comparing phenotypes of epidemic potential and/or virulence. Such phenotypic comparisons have the potential to elucidate the mechanisms by which DENV strains produce variations in disease outcome and have far-reaching impacts on DENV surveillance, vaccine development, and the study of viral evolution.

Specific Aims

Given the complexities and multifactorial nature of DENV pathogenicity, and in light of our previous research having provided strong evidence for viral genetic change as the basis for the attenuation of dengue symptoms seen in Tonga in 1974, this PhD research will focus specifically on the contribution of DENV strain diversity to virulence/epidemic potential.

This focus will clearly require a more full characterization of the DENV samples isolated from the islands of the South Pacific sweep. To understand what changed in the Tonga virus lineage that made it less virulent than it had been in Tahiti, Fiji, and the other islands of the South Pacific, we propose to compare the South Pacific DENV-2 isolates within both human and

mosquito cell lines as well as within live mosquitoes.

Thus, our long-term goal is to use insights gained from DENV evolution to better understand epidemic dynamics and DENV emergence. The objective of the proposed research is to ascertain if phenotypic effects from DENV genetic variation are responsible for epidemic intensity and disease severity. Our study is unique in that it takes advantage of a natural experiment in which many, if not most, of the confounding factors in dengue disease dynamics could be excluded, except for virus evolution, thus providing an opportunity for comparing phenotypes of epidemic potential and/or virulence. Such phenotypic comparisons could have far-reaching impacts on DENV surveillance, vaccine development, and the study of viral evolution.

Specific Aim 1

Determine the relationship between genetic changes in DENV-2 epidemic strains and their phenotypic effects using *in vitro* models.

Hypothesis: Unique genetic substitutions (synapomorphies) associated with attenuated DENV strains from the island of Tonga will reveal measurable phenotypic differences in comparison with strains from the other islands in the South Pacific sweep in *in vitro* assays. Approach: We propose to look for phenotypic differences among strains isolated from the South Pacific 1971–1974 DENV-2 epidemic sweep. Phenotypes to be measured include those of **viral productivity** (number of virus produced per unit volume such as plaque forming units per milliliter, i.e. viral titer) and **replication rate** (rate of virus production over time). Additional measurements may include the proportion of **infectious virions** (because prM mutation might result in more immature virus particles) and possibly the viral effects on host **immune response** (since NS2A

and NS4A mutations might result in diminished interferon suppression). Isolates from the entire South Pacific sweep will be compared for phenotypic differences, as correlates of epidemic potential, in both human and mosquito cells.

Specific Aim 2

Determine the relationship between genetic changes in DENV-2 epidemic strains and their phenotypic effects using *in vivo* models.

Hypothesis: Synapomorphies associated with attenuated DENV-2 strains from the island of Tonga will exhibit measurable phenotypes similar to those to be examined *in vitro*: viral productivity and replication rate.

Approach: We propose to infect genetically diverse colonies of *Ae. aegypti* with DENV-2 isolated from the 1971–1974 South Pacific sweep to compare their ability to infect, replicate, and disseminate. Using time course experiments, infections will be carried out through direct inoculation and measured by MID_{50} and qPCR.

CHAPTER 4: MATERIALS & METHODS

Samples

Sixteen low-passage DENV-2 samples were used in this analysis; all were obtained from the collection of Dr. Duane J. Gubler and acquired by him during the South Pacific sweep (Table 3). Viral passage history (Pass) refers to the number of passages in C6/36 cells; those passages followed by an asterisk indicate that virus was initially isolated in adult *Aedes* (specific species unknown). All sequencing was carried out with the passages as listed in Table 3, as was plaque assay, focus forming assay and SYBR Green qPCR. The Choy plaque assay, MID_{50} and TaqMan qPCR used samples with an additional passage in C6/36 and the MID_{50} assay used one further passage in *Ae. aegypti*.

Maintenance of cell cultures

C6/36 cells, an *Aedes albopictus* clone of whole larval tissue (ATCC, Manassas, VA), were maintained in DMEM (Gibco, ThermoScientific, Waltham, MA), 5% fetal bovine serum, $NaHC0_3$, non-essential amino acids, and sodium pyruvate in 28˚C incubator. Vero cells, derived from kidney epithelial cells from an African green monkey (ATCC, Manassas, VA), were maintained in M-199 media (Gibco, ThermoScientific, Waltham, MA), 5% fetal bovine serum, 4 mM L-glutamine, 100 X penicillin/streptomycin and 7.5% $NaHCO_3$ in humidified 5% CO_2 at 37˚ C. BHK cells, a kidney cell line derived from 1-day old Syrian golden hamsters (*Mesocricetus auratus*), were maintained in RPMI (HyClone, Bangkok, Thailand), 10% fetal bovine serum, plus L-glutamine, non-essential amino acids, penicillin and streptomycin in humidified 5% CO_2 at 37˚C.

Table 3. Epidemiological data on the isolates used in this study.

Strain ID	Pass[a]	Date of Onset	Isolation: Date/Time	Disease[b]	Age	Race	Sex	Travel History	Notes
Tahiti 10000	2*	4/12/1973	4/15/1973	DF	n.d.	n.d.	F	n.d.	
Tahiti 7848	2*		1971	DF	n.d.	n.d.	n.d.	n.d.	
Fiji 1021	4		1971	DF	n.d.	n.d.	n.d.	n.d.	
Fiji 1422	3		1971	DF	n.d.	n.d.	n.d.	n.d.	
New Caledonia 9137	2*	12/31/1971	12/31/1971	n.d.	60	C?	M	n.d.	
New Caledonia 9297	2*	1/26/1972	1/27/1972 8:15 A.M.	headache, joint pain	49	C	F	Born in France, in NC 1.5 years	
Samoa S-5277	2*	6/21/1972	1972	Febrile acute	50	P	M	Never out of Am. Samoa	
Samoa S-5279	2*	6/21/1972	1972	Afebrile acute	50	P	M	Never out of Am. Samoa	Repeat of S5277
Samoa S-5285	2*	6/21/1972	1972	Febrile acute	26	P	F	Never out of Am. Samoa	
Samoa S-5373	2*	6/22/1972	1972	Febrile acute	33	P	M		
Tonga 14616	2*	4/16/1974	4/16/1974 P.M.	Febrile, myalgia, arthralgia, no rash nor hemorrhage	16	P	F	Never out of Tonga	
Tonga 14619	2*	4/16/1974	4/16/1974 7:30 P.M.	Febrile	16	P	F	Never out of Tonga	Repeat of S14616
Tonga 14620	2*	4/16/1974	4/17/1974 8:30 A.M.	Febrile, myalgia, no rash nor hemorrhage	19	P	F	Never out of Tonga	
Tonga 14639	2*	4/17/1974	4/18/1974 2:30 P.M.	Fever, headache	16	P	F	Never out of Tonga	
Tonga 14644	2*	4/16/1974	4/19/1974 11:30 A.M.	Afebrile, headache no rash nor hemorrhage	16	P	F	Never out of Tonga	repeat of S14616; 5th blood
Tonga 14694	2*	4/25/1974	4/26/1974 10:15 A.M.	Febrile, hematemesis, melena, no other sign of bleeding	15	P	F	Never out of Tonga	

n.d. = no data, **DF** = dengue fever, **C** = Caucasian, **P** = Polynesian, **a** = Viral passage history, **b** = All patients were classified as having classical dengue fever (DF). ***** = First passage in adult mosquito

Virus replication

DENV-2 samples used in this research were passaged in C6/36 cells as follows: T-25 flasks were seeded with C6/36 cells in 9 mL of DMEM-10 and allowed to grow 2–3 days before replacing the media with a 200–300 µL inoculant of DENV-2 at a standard MOI of 0.1 in DMEM-2. Exceptions were made of viruses that had unusually low titers; i.e., Tonga strains 14619 and 14620 (MOI 0.01), and 14639 (which had an exceptionally low titer of 6.18E+03 pfu/mL and thus was infected with an MOI of 0.005) or for which very small volumes were available.

In some cases, due to prior bacterial contamination, virus was inoculated using 0.2 µM syringe filters for sterilization. Following incubation of cells with virus for 1 hour at 28° C, an additional 5 mL of DMEM-2 was added and the flask returned to incubate in humidified 5% CO_2 at 37˚C. Cell health was checked under inverted microscope every 2–3 days and pH adjusted (if necessary) with $NaHCO_3$ drops to revert to red color. Generally after 5 days, a sample of cells are scraped off and tested for successful viral infection using DFA. With confirmation of successful infection, all cells are scraped from flask, mixed with FBS at 23% (v/v) and spun down at 2,000 rpm for 5 min. 600 µL of super were aliquoted and frozen down to -80°C.

Direct Fluorescent Assay (DFA)

Cells were checked for successful DENV infection using direct fluorescent assay as follows: Using a plastic cell scraper to loosen C6/36 cells from bottom of flask, 200–500 µL of cell solution were pipetted from flask into a 1.5 mL tube, spun 5 min at approximately 1200 rpm to form pellet, then vortexed in 500 µL 1 X PBS to resuspend. Cell solution was then applied to glass slide, 10 µL/well, 2 wells/cell sample (with a negative control on each slide) and allowed to

air dry for 30 min, followed by immersion in ice-cold acetone within Copeland tray for 15–20 min at –20°C. 4G2 mouse monoclonal-antibody (mAb) was applied to each well at a dilution of 1:300, the slide placed in a moistened Petri dish humidity chamber in 37°C incubator for 30 min, immersed in PBS, then rinsed with distilled water. After a drop 1 μL of mounting media on each well, a glass slip was applied to the slide and it is examined with an epi-fluorescent microscope. Depending upon percentage of cell fluorescence: 0% - check again in two days, 10% - check again tomorrow, 60 – 70% - freeze infected cells tomorrow, 100% - freeze now at -80°C.

Plaque Assay

As an initial attempt to determine the viral titer of the samples of DENV-2 isolated from the 1971–1974 South Pacific sweep, plaque assays were performed as previously described. [11,155] Briefly, Vero cells were grown in 6-well plates in DMEM media supplemented with 10% fetal bovine serum (FBS) until almost confluent. Preparatory to infection, serial dilutions of viral supernatant are prepared, 10^{-1} to 10^{-7}, in cold DMEM media supplemented with 5% FBS and then each well is inoculated with 200 μL of the appropriate virus dilution, with all reps of each dilution on a single plate (most often with 2 reps per dilution). Inoculum was allowed to incubate for 1 hour at 37°C and 5% CO_2, followed by each well of infected cells being overlaid with 3 mL of 1% agarose. After approximately 7 days, a second 3 mL layer of 1% agarose plus 2% neutral red was added to each well, followed by another 24 hours of incubation. The resulting plaques were counted over a light box with PFU/mL calculated using the following formula:

(Average plaque count between reps) x (fraction of 1 mL) x (inverse of dilution)

Example: the average number of plaques at 10^{-2} dilution was 36 and at 10^{-3} was 12. The

amount of virus used to infect each well was 200 mL or 1/5 of 1 mL. Using the formula given: $(36 \times 5 \times 10^2 = 1.8 \times 10^4) + (12 \times 5 \times 10^3 = 6.0 \times 10^4) / 2 =$ Average pfu/mL $= 3.9 \times 10^4$.

Focus-Forming Assay

Because the results from working with plaque assays were unsatisfactory, we attempted to measure DENV infectivity titer using the **focus-forming assay** (FFA). Rather than relying on measurements of cytopathology as a surrogate of viral quantity, the FFA detects virus directly through use of horseradish peroxidase (HRP)-labeled anti-flavivirus antibodies. Briefly, the method is as follows; Vero cells (as with plaque assays described above) are grown to near confluence in 96-well plates in DMEM with 10% FBS. DENV samples are diluted in 2% FBS in DMEM from 10^{-1} to 10^{-7} and then 50 µL of each dilution is added replicate wells, and allowed to incubate at 37°C for 90 minutes with gentle rocking, followed by 150 µL of an overlay mixture of 2% methylcellulose plus 2X DMEM. After further incubation at 37°C in 5% CO_2 for 4 days, the overlay is removed, wells washed 5 times with PBS and 200 µL of methanol/acetone added to each well for 10 minutes. After monolayers are allowed to dry completely, 200 µL of antibody dilution buffer are added to each well and allowed to incubate at room temperature for 30 min, followed by 50 µL of primary antibody (4G2 antibody, 1:2,000 dilution) incubated at 37ºC for 1 hour, 2 washes with PBS, then 50 µL of secondary antibody (HPR- conjugated goat anti-mouse IgG, 1:2,000 dilution) to each well and for 1 hour at 37ºC, followed by 2 washes with PBS and 50 µL of TrueBlue peroxidase substrate (KPL, Inc., Gaithersburg, MD, USA) for 10 minutes at room temperature with rocking (making sure that blue foci are visible). Substrate is then discarded and the plate washed with ddH_2O. After thorough drying, the foci are counted and quantified in a digital ELISPOT reader (Cellular Technologies Limited, Cleveland, OH, USA).

Quantitative Polymerase Chain Reaction (qPCR)

Unlike PFA or FFA, qPCR avoids the complications resulting from viral variations in plaque-forming ability or growth in different cell lines by directly measuring viral RNA concentration. Thus, given our inability to titrate all DENV-2 isolates from the South Pacific sweep, particularly the DENV-2 isolated from patients from the island of Tonga, using either PFA or FFA, we carried out quantitative PCR using the SYBR Green method at the University of Hawaii at Manoa and the TaqMan method at Mahidol University, Bangkok School of Tropical Medicine. Regardless of the method, qPCR begins with RNA extraction as follows.

RNA Extraction

Viral RNA was extracted from South Pacific DENV-2 isolates using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) at the University of Hawaii at Manoa and the GenUP Total RNA kit (biotechrabbit GmbH, Hennigsdorf, Germany) at Mahidol University, Bangkok School of Tropical Medicine; in both cases following the manufacturer's instructions. In both cases, RNA was derived from the cell-free supernatant fraction of DENV-2-infected C6/36 in order to avoid incompletely transcribed/packaged RNA and the majority of immature virus particles. The extracted RNA was eluted in 60 μL of RNase-free water and stored at -80°C.

SYBR Green qPCR

Protocol for qPCR at University of Hawaii at Manoa was as follows: extracted RNA was converted to cDNA through reverse-transcription using iScript Reverse Transcription Supermix kit (BioRad, Hercules, CA) following manufacturer's instructions. Briefly, 5X iScript reaction mix and iScript reverse transcriptase were combined with nuclease-free water in a master mix on

ice, then aliquoted into 0.5 μL microcentrifuge tubes; one for each RNA template plus a negative control. Immediately after adding RNA template to master mix, tubes were loaded into a thermal cycler and run as follows: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and hold at 4°C. Finished cDNA was either quantified through qPCR immediately or stored at -20°C for later analysis.

Primer set used for qPCR is specific to the DENV 3'–noncoding (3'NC) region and was developed for detection of all DENV serotypes. The forward primer is DC10418 (10418-TTGAGTAAACYRTGC TGCCTGTAGCTC) and the reverse primer is CDC10590 (GGGTCTCCTCTAACCTCTAG TCCT-10564).[39] Viral titer was measured as genome equivalents/mL against the cDNA generated from the lab-adapted, high-titer DENV-2 strain DAKARA whose titer had been previously established through repeated plaque assay and FFA at $2.5x10^7$. DAKARA cDNA was serially diluted 5x (10^0–10^5) on ice to create a standard curve in sufficient quantities to run in duplicate. The cDNA of sample and standard was amplified using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA) for 40 cycles (5 sec at 95°C, 30 sec at 63.4°C, and 30 sec at 72°C) on BioRad CFX96 iCycler.

TaqMan qPCR

Rather than using the SYBR Green method, at Mahidol University, Bangkok School of Tropical Medicine, we used TaqMan to carry out qPCR, utilizing the KAPA PROBE FAST One-Step qRT-PCR Kit (Kapa Biosystems, Inc., Wilmington, MA). As a one-step quantitative PCR kit, a separate step of producing cDNA was avoided. Primers and probe are specific to the DENV-2 envelope region[117] with sequence and position as follows:

DEN-2 F	CAGGTTATGGCACTGTCACGAT	1605
DEN-2 C	CCATCTGCAGCAACACCATCTC	1583
DEN-2 probe	CTCTCCGAGAACAGGCCTCGACTTCAA	1008

Viral titer was determined through comparison with a standard curve established by a serial dilution of RNA from a strain of DENV-2 lab-adapted by Dr. Pornsawan Leaungwutiwong at Mahidol University and designated *D2 (16681), MK2-3, C6/36-1, Sm-2, C6/36-5, 20 Feb 13* The titer of this standard was established with repeated plaque assays at PFU $2.3x10^5$. Quantitative PCR run for 44 cycles (2 min at 95°C, 15 sec at 95°C, and 1 min at 60°C) on BioRad CFX96 iCycler.

Mosquito inoculation

Inter-thoracic inoculation of DENV into mosquitoes is a long-standing means of determining viral titer, particularly valued for its sensitivity, as well as a method for "rescuing" low-titer DENV isolates[86,189]. Working at Duke-National University of Singapore (NUS), we carried out both functions; beginning with the establishment of an initial seed bank of virus samples isolated from the South Pacific DENV-2 sweep, followed by their titration in order to compare with other titration methods.

All mosquitoes used in experiments were either *Aedes aegypti* or *Ae. albopictus* from a colony at the Duke-NUS Graduate Medical School. The original colony was established in 2010 with specimens collected in Ang Mo Kio, Singapore, and supplemented weekly with field-collected mosquito eggs which were collected from the Geylang area of Singapore, reared to adulthood, sorted by species and introduced into the laboratory populations (10% of colony), to

maintain genetic diversity. Generally, for reasons of safety, only male mosquitoes were initially inoculated. However, given their smaller size, survival post-inoculation was unacceptably low, so female *Ae. aegypti* were generally used.

In order to carry out inoculations, needles of a sufficiently small diameter to ensure minimal mosquito mortality are required. Needles were prepared by stretching borosilicate glass capillary tubes (internal diameter 0.4 mm; outside diameter 0.7 to 1.0 mm; 30 in length) through a Narishige PC-10 Dual-Stage Glass Micropipette Puller (Narishige Group, Tokyo, Japan) to draw to a fine point. The resulting needles were sorted and marked with 1 mm divisions using a rubber stamp for accurate and consistent inoculum delivery then mounted in a metal holder connected by flexible tubing to a large syringe (Figure 16).

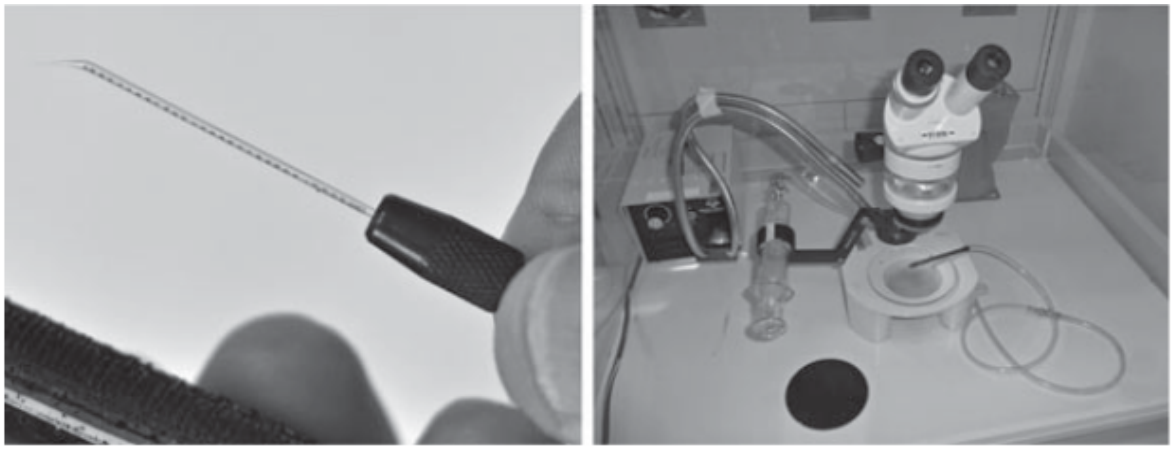


Figure 16. Glass needle stamped with 1 mm lines and mounted in syringe delivery system. Photo courtesy Current Protocols in Microbiology (2005).

To securely contain small numbers of deliberately infected mosquitoes, small "infection chambers" consisting of cardboard ice cream containers were used (i.e., white cardboard tubes with slide on cardboard caps, often called "Neptune cans") (Science Supplies WLE Corp., Fort Lee, NJ). These were modified for use by replacing the top caps with mosquito netting and

cutting a small door in the side of the tube for transferring mosquitoes in and out.

The inoculation of *Aedes* mosquitoes, whether for developing of seed pools or titration, requires extremely careful handling as the escape of even a single infected individual can present a considerable risk to those working in the insectary, as well as the possibility of escape into the outside environment. Therefore, all work was undertaken in dedicated mosquito insectary facilities rated at BSL-2 at both JABSOM and Duke-NUS, mosquitoes were kept in containment at all times (described in more detail below) with inoculations and mosquito sorting carried out in sealed glove boxes, all inoculation and virus handling was completely separated from mosquito rearing and particularly during inoculation, mosquito numbers were carefully accounted for.

Successful inoculation with minimal mortality requires that mosquitoes be immobilized during injection. While there are a number of methods for knocking down mosquitoes, we found it most convenient to chill containers of mosquitoes under crushed ice. Thus mosquitoes are aspirated from their rearing cage into a plastic collection canister, which was immediately plugged with cotton wool and then placed within zip lock bag and buried in ice for a minimum of 20 min to knock out (KO) mosquitoes. While waiting for mosquito KO a serial dilution of the virus was prepared within the inoculation room biosafety cabinet (BSC) and then transferred, along with the KO mosquitoes, into the inoculation room glove box. KO mosquitoes are transferred from collection canister to 50 mL blue top tube, then sorted by sex and roughly counted; approximately 60 females are transferred to another tube under dissecting scope and put back on ice, while males are returned to colony cage.

Within glove box, glass needle is inserted into syringe delivery apparatus, 20 µL of virus

dilution are pipetted onto parafilm, then drawn up into needle. Blue top tube with mosquitoes is taken off ice, and a single female shaken out onto dissecting scope stage and arranged to lie on back. With the needle carefully inserted into the neck membrane, 1 mm virus is injected (equivalent to 0.17 µL volume), and then mosquito is transferred to cardboard infection chamber (described above). After 10 mosquitoes are inoculated per isolate dilution, the chamber door is plugged with cotton ball and sealed shut with tape (Figure 17 and 18)

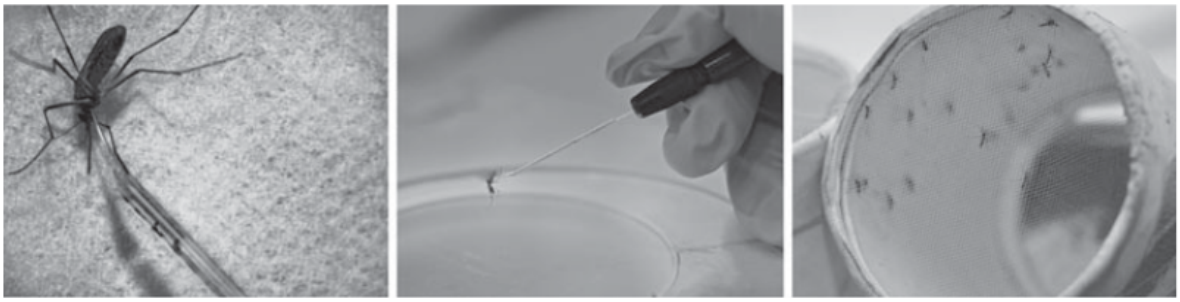


Figure 17. (Left) Virus inoculum of 0.17 µL is injected into the membrane anterior to the mesepisternum of the Ae. aegypti female. (Center). The inoculated mosquito is transferred from microscope stage using needle and placed in infection chamber for virus incubation. (Right) Inoculated mosquitoes are stored at 28◦C for 10 - 14 days to obtain optimal virus replication. Photo courtesy Current Protocols in Microbiology (2005).

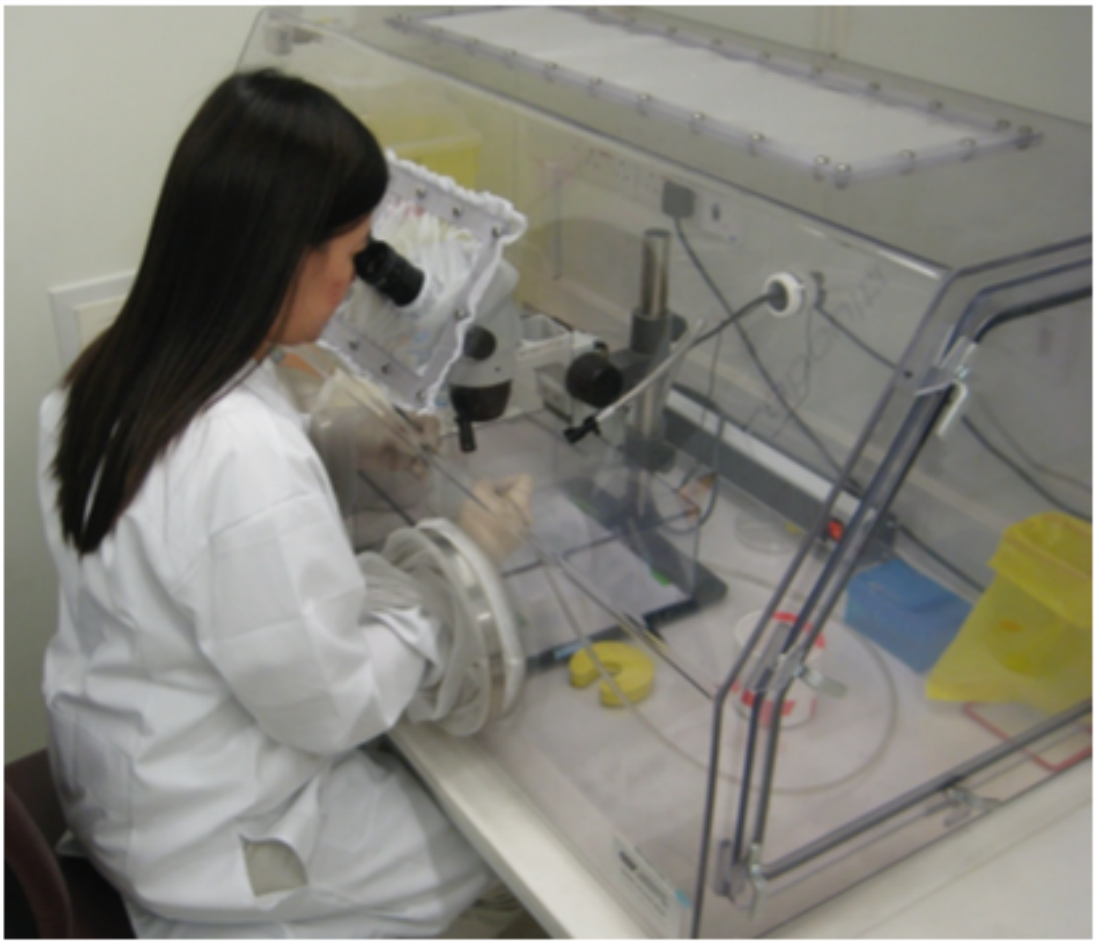


Figure 18. Mosquito inoculation system inside glove box in insectary at Duke-NUS Graduate Medical School, Singapore.

Four infection chambers (each for a separate viral dilution) can be placed into a BugDorm-1 cage (MegaView Science Co., Ltd., Tatchung, Taiwan) along with flasks of water and 10% sucrose with wicks to the chambers in order to maintain mosquito survival during virus incubation. Cages were then placed within a controlled-environment chamber at 27°C. Water and sucrose levels are checked daily and wicks replaced every 5 days to prevent bacteria and fungal build up. At 10 dpi mosquitoes are sacrificed by placing infection chambers in -80°C freezer for 15 min and then tested for infection with indirect fluorescent assay (IFA).

Testing that infection has taken place, whether through injection or oral feeding, in general relies upon crushing the head of the mosquito on a glass slide and staining these tissues with fluorescein isothiocyanate-tagged anti-DENV antibody; the principle being that the presence of virus in the head verifies that virus replication has occurred and led to dissemination throughout the body. [128,155] Head squash and antibody staining (usually as IFA) was carried out as follows: 12-well slides are labeled using dark **pencil**. Wells are numbered 1–6 on top and 7–12 on bottom. Both negative and positive controls are included on each slide if available (Any uninfected mosquito was used as a neg. control. Positive control mosquitoes were those inoculated with the high-titer DENV-2 virus DAKARA beforehand – generally – and stored at -80°C). Mosquitoes were immediately transferred from -80°C freezer, placed one per slide well and head removed using a razor blade. Bodies were stored in labeled microcentrifuge tubes at -80°C for future testing and possible virus isolation.

Each mosquito head was crushed within its well using a quick side-to-side motion with pestle, removing exoskeleton. After all heads are crushed slides are submerged in -20°C. acetone for 10 min then air-dried inside BSC. After placing slides inside a petri dish lined with damp paper towel, 25 µL of antibody is pipetted onto each well, the slides are incubated at 37°C, followed by a rinse 1x PBS and air drying in BSC; first primary antibody, then secondary with FITC. Finally, a 5-µL drop of mounting fluid is placed onto each slide well then gently covered with a glass cover slip and viewed under fluorescent microscope. The titer of infectious DENV measured by this mosquito inoculation technique gives a mosquito infectious dose at 50%, i.e., MID_{50}, as calculated by the method of Reed and Muench, which essentially calculates the minimum dilution of virus required to infect 50% of inoculated mosquitoes.[40,177,189]

Seed pools were produced as follows: 50 *Ae. aegypti* mosquitoes were inoculated per each South Pacific viral DENV-2 isolate (with interthoracic injections as described under MID_{50} but using no dilution of virus). Infections were confirmed at a minimum of 14 dpi through head squashes and IFA of 10 mosquitoes per virus pool. Upon verification of successful infection, 30 mosquitoes per pool were macerated using MP Biomedicals Fast-Prep (MP Biomedicals, LLC, Santa Ana, CA) in 1.25 mL of homogenization media. Viral supernatant was centrifuged to remove most mosquito body parts, with further purification using a 0.2 μm syringe filter, then aliquoted at 100 μL per vial. Remaining 20 infected mosquitoes per pool were stored at -80°C.

CHAPTER 5: INFECTIVITY TITERS OF DENV-2 ISOLATES DETERMINED *IN VITRO*

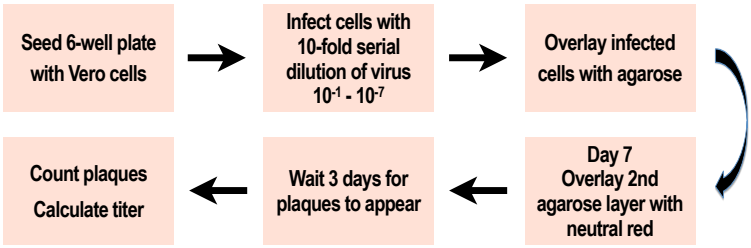
Introduction

None of the South Pacific DENV-2 isolates that we had carried out phylogenetic analysis on had ever been measured for their titer, or if they had, those records had been lost. Given the lack of infectivity titer data, sample measurements were taken from the cell supernatant of all isolates in our stock using plaque assays, focus-forming assays, and quantitative PCR.

Plaque Forming Assays

Plaque assays are a standard virological method and were used routinely in the Department of Tropical Medicine at the University of Hawaii by a number of investigators working with West Nile virus. Plaque assays can utilize a number of different cell lines to detect plaques; in our lab in Hawaii we used Vero cells, which work well with West Nile.

Details are provided in the Materials & Methods chapter but general method is represented in the flowchart below.



Focus-Forming Assays

While plaque assays are a longstanding method of virus titration, it has been found to be less than optimal for measuring DENV amounts, primarily because, as a number of papers have pointed out, DENV can be poorly cytopathic, and it can be particularly difficult to get good plaques from low-passage DENV isolates.[7,151] In addition, plaque formation can be restricted by the phenotype and passage history of the DENV strain and by the type and age of cells used in the plaque assay.[179]

An alternative titration method, the **focus-forming assay** (FFA) avoids this problem, since rather than detecting virus indirectly through cell lysis; FFA labels virus particles directly using a horseradish peroxidase-labeled immunoassay. An additional advantage of the method is that foci are high contrast, allowing for easier identification and even automation of counting thus increasing throughput. Given that the results from working with plaque assays were unsatisfactory (see Results section below), the FFA thus recommended itself to us for trial as possibly a more sensitive approach to measuring DENV infectivity titer.

Quantitative PCR

Other techniques available to estimate the concentration of DENV, in addition to plaque assay and FFA, include mosquito inoculation and qPCR. Each technique presents a different set of problems when attempting to compare titers between different virus strains. Viral infectivity assays measure a restricted phenotype and, therefore, may not accurately reflect the number of infectious particles if viral strains differ in their ability to form infectious plaques or to establish

infection in the mosquito by intrathoracic inoculation. Moreover, results may be difficult to reproduce because of variation among lots of cell lines or populations of colonized mosquitoes. QPCR may circumvent many of these limitations by directly measuring the concentration of viral RNA; however, it will also measure viral RNA that is not fully transcribed or packaged while replication is occurring in the cell.[125,231,241]

In any case, given our inability to titrate all of our DENV-2 samples using either the plaque or focus-forming assays, particularly those samples isolated from the outbreak on Tonga (see Results section), we decided to attempt qPCR; first, with the SYBR Green method at the University of Hawaii and later the TaqMan method at Mahidol University, Bangkok School of Tropical Medicine.

In both cases, measurements of viral titer using qPCR relied upon using the lab-adapted, high-titer DENV-2 strains as a standard. These strains had the advantage of having produced high and consistent titers in numerous plaque and FFA and allowed us to correlate qPCR cycle number with plaque forming units as genome equivalents per mL.

Because the qPCR assays carried out at the University of Hawaii and at Mahidol University were of two distinct types; SYBR Green and TaqMan respectively, were conducted at widely different times and worked with different samples of the South Pacific isolates, they will discussed in separate chapters; this chapter will deal exclusively with SYBR Green qPCR carried out at the University of Hawaii at Manoa while Chapter 11 will review TaqMan qPCR assays performed at Mahidol University, Bangkok School of Tropical Medicine.

University of Hawaii SYBR Green qPCR

QPCR always begins with RNA extraction; followed by a two-step process to convert RNA to cDNA and then determine the copy number of each of the stock samples of virus isolated from the South Pacific sweep (details can be found in Chapter 4: Materials & Methods).

In the initial qPCR trials we attempted to use a primer targeting the nonstructural gene 5 (NS5) but given that this showed a high rate of non-specific binding (exhibited as a secondary peak on the melt curve) no further trials were carried out. The primer ultimately chosen for the SYBR Green assay was adopted from Chien et al.[39] and targeted the distal end of the 3'–noncoding region of the DENV, which is highly conserved, even among different geographic isolates, while also effective at distinguishing DENV from other related flaviviruses.[111,112]

The amplification protocol used was adopted from one successfully used by my colleague Ms. Panpim Throngsipong, as follows:

FlaviMelt.tmo

Cycle	Cycle Descript.	Repeats	Step	Process	Ramp Rate	Dwell Time	Hold	Set - Point	Gradient	Range	Melt Curve	+ Temp
1	Initial Denaturatio	1	1		MAX	03:00	☐	95.0	☐		☐	
2	Anneal\Extend	40	1	Denature	MAX	00:15	☐	94.0	☐		☐	
			2	Anneal	MAX	00:30	☐	50.0	☐		☐	
			3	Extend	MAX	00:45	☐	72.0	☐		☐	
3	Melt Curve	70	1		MAX	00:10	☐	60.0	☐		☑	0.5
4	Cool	1	1		MAX		☑	4.0	☐		☐	
							☐		☐		☐	

Nonetheless, the protocol did require optimization, as our initial qPCR trials based on her

protocol were unsatisfactory. For example, when only using a dilution series of the qPCR standard (the lab-adapted DENV-2 strain DAKARA) the correlation coefficiency value was $R^2 >$ 0.98 and the amplification efficiency was within the ideal range of 90–105% with amplification curves well spaced. However, with the addition of unknowns, all efficiencies were much poorer and amplification curves appeared "retarded," i.e., the exponential phase was greatly diminished. After considerable attempts at testing the ratio of primer to template and increasing the annealing temperature above that originally recommended, I eventually optimized my qPCR to achieve an $R^2 = 1.0$ and amplification efficiency = 102.8% with the following protocol:

Cycle	Repeats	Step	Dwell Time	Hold	Setpoint	Melt Curve	+ Temp
1	1	1	00:30	☐	95.0	☐	
2	40	1	00:15	☐	94.0	☐	
		2	00:30	☐	63.4	☐	
		3	00:30	☐	72.0	☐	
3	70	1	00:20	☐	55.0	☑	0.5
4	1	1		☑	4.0	☐	

FlaviMelt

As mentioned above, our qPCR measurements relied upon using a lab-adapted, high-titer DENV-2 strain as a standard and this was DAKARA at the University of Hawaii at Manoa. Upon the suggestion of Dr. Moti Chapagain, a large quantity of DAKARA virus was grown in flasks of C6/36 and pooled. Samples from this pool were titrated using the focus-forming assay on four separate occasions resulting in an average titer of **2.76E+07** ffu/mL (SD = 0.17). RNA was then extracted from the same pool of DAKARA, reverse transcribed to cDNA, aliquoted into 0.5 mL microcentrifuge tubes, frozen down and stored at -20°C, with individual tubes being used for each qPCR run to avoid cDNA deterioration through repeated freeze/thaw.

To accurately calculate the amount of DAKARA cDNA to use in creating the serial dilution for the qPCR standard curve, we calculated as follows (further information can be found

in lab notebook 10 June 2011 and in file "Standards Calc.numbers"). For purposes of simplicity, the average titer of the DAKARA DENV-2 strain was **2.5E+07** pfu/mL or 2.5E+04 pfu/µL.

RNA extraction

$\left(\frac{140\ \mu L\ super}{tube}\right)(5\ \text{tubes}) = 700\ \mu L$ = total viral super needed

(2.5E+04 pfu/µL) $(700\ \mu L) =$ 1.75E+07 pfu total titer

So total amount of viral RNA extracted = (50 µL eluate/tube) (5 tubes) = 250 µL

Thus, extraction results in 1.75E+07 pfu/250 µL RNA

cDNA conversion

Use 5.74 µL RNA per reaction to make 20 µL cDNA: $\frac{1.75E+07\ \text{pfu}}{250\mu L} = \frac{x}{5.74\ \mu L}$

x = 4.02E+05 pfu / 20 µL cDNA *or*

x = 2.01E+04 pfu / µL *or*

x = 1.0E+05 pfu /5 µL

Thus, the DAKARA serial dilutions $10^0 - 10^{-4}$ were made with 5 µL cDNA in 45 µL ddH_2O.

To convert qPCR results into pfu equivalents, we created a spreadsheet to carry out the following calculations:

1. The qPCR software "BioRad iCycler iQ Real-Time PCR Detection System" produced a SQ Mean (average estimated Starting Quantity) for each duplicate unknown based on it's Ct value compared with that of the standard curve generated from the DAKARA dilution series.

   For example, SQ Mean of the isolate Tonga 14616 = **2.3E+02**

2. The SQ Mean is then multiplied by the ratio of cDNA produced to the amount of cDNA used in the qPCR reaction to get the equivalent amount of pfu.

(SQ Mean 2.3E+02) (Ratio of total cDNA to added cDNA 10) = **2.3E+03**

3. This is then divided by the amount of cDNA (equivalent to the original virus particles) by the quantity of RNA eluate used to make the cDNA.

2.3E+03 / 5.74 µL of RNA used to make cDNA = **4.01E+02**

4. Next we multiply the number of µL of RNA extracted by the number of plaque forming units per µL of extraction eluate.

(4.01E+02) (50 µL of RNA from extract) = **2.0E+04**

5. This calculation derives average pfu/µL by dividing the total pfu/µL of the viral RNA extracted by µL of virus stock.

2.0E+04 / 140 µL used in RNA extract = **1.4E+02 pfu/mL**

6. The final calculation converts average pfu/mL to average pfu/µL.

(1.4E+02) (1000) = **1.43E+05 pfu/µL**

Results

Plaque Assay

While the titer of available lab-adapted strains of DENV-2 (i.e., DAKARA and NGC 1056) could be readily measured through the plaque assay method, the majority of the South Pacific DENV-2 isolates simply did not generate visible plaques (Figure 19). The Fisher exact test statistic value was 1, thus *not* significant at $p < .05$, illustrating our plaque assay's poor utility.

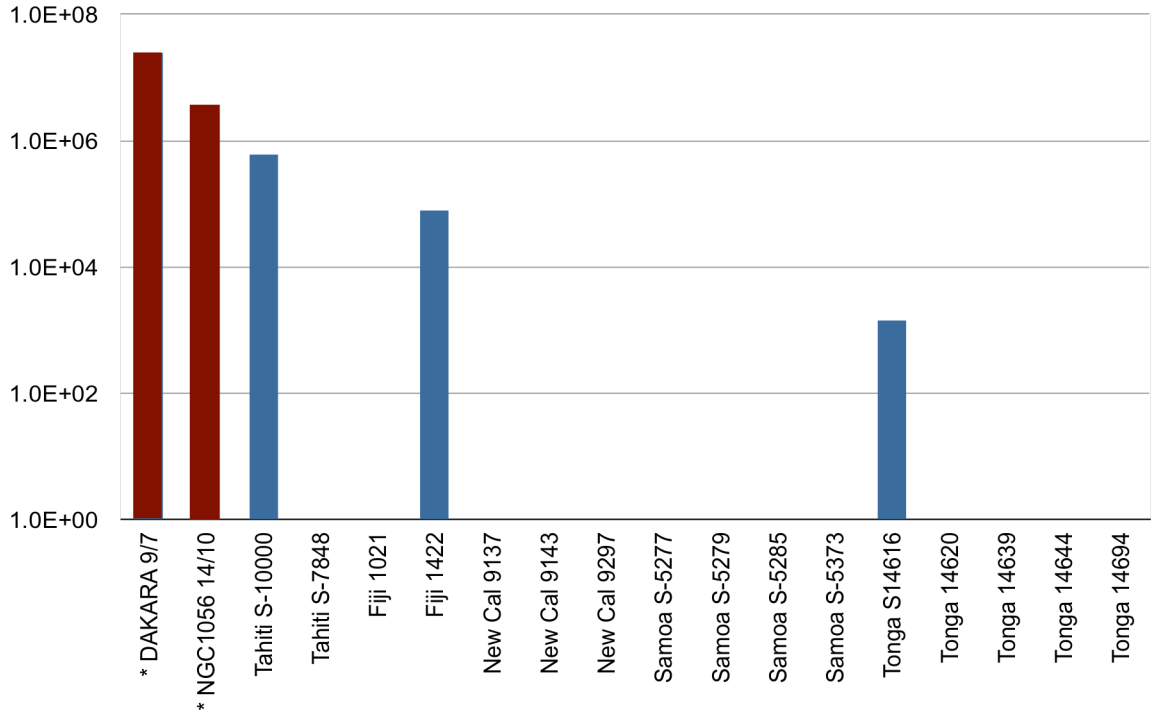


*Figure 19. Titers of stock South Pacific sweep isolates and lab-adapted DENV-2 standards (*) using **plaque assay**. Log scale.*

Focus-Forming Assay

Our results from focus-forming assays showed that while this assay was able to measure most of the South Pacific DENV-2 isolates, it was incapable - despite repeated attempts - of detecting the

majority of those samples isolated from the Tonga outbreak (Figure 20). Nonetheless, Fisher's exact test gives a p-value = 0.002747 showing that FFA was an effective measurement method.

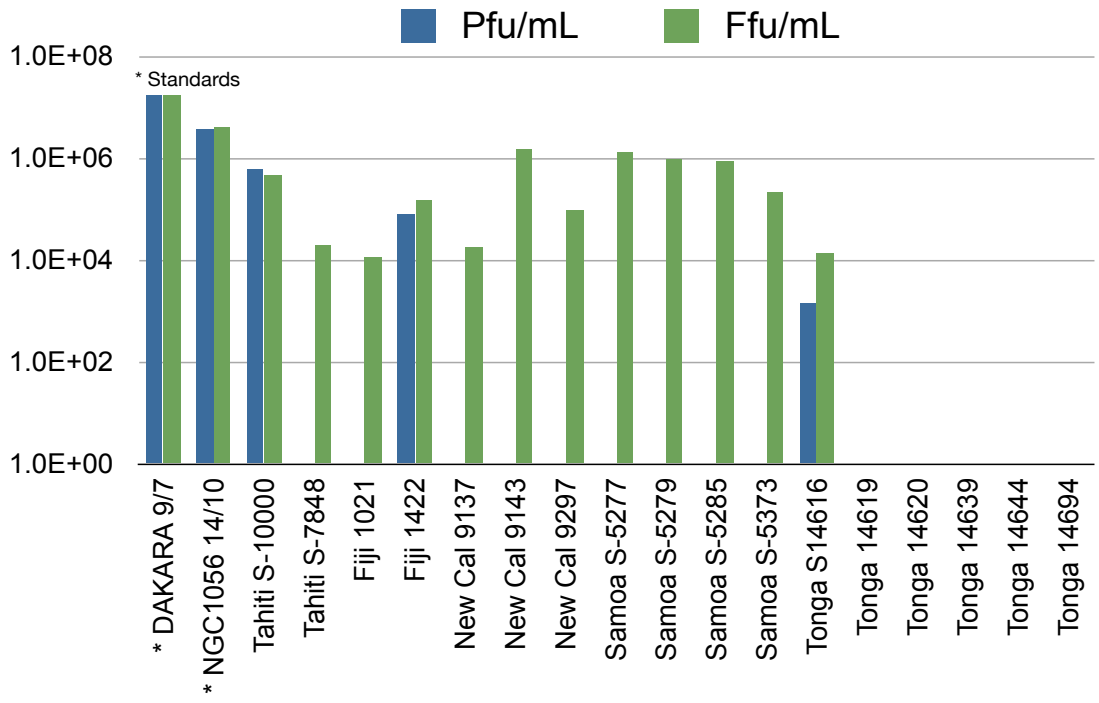


Figure 20. Titers of stock South Pacific sweep isolates and lab-adapted DENV-2 standards using both ***plaque*** *and* ***focus forming assay*** *(FFA). Note lack of bars for the majority of Tonga isolates. Log scale.*

Quantitative PCR

Results from SYBR Green qPCR tests conducted at University of Hawaii at Manoa demonstrated that all South Pacific samples, including those from Tonga, at the very least contained DENV RNA (Figure 21).

Choy Plaque Assay

Subsequent to our attempts, successful plaque assays for Tonga isolates 14619, 14620, 14639 and 14644 were carried out using BHK-21 cells by our colleague Dr. Ming Ju Milly Choy at

Duke-NUS Graduate Medical School, Singapore (Figure 21).

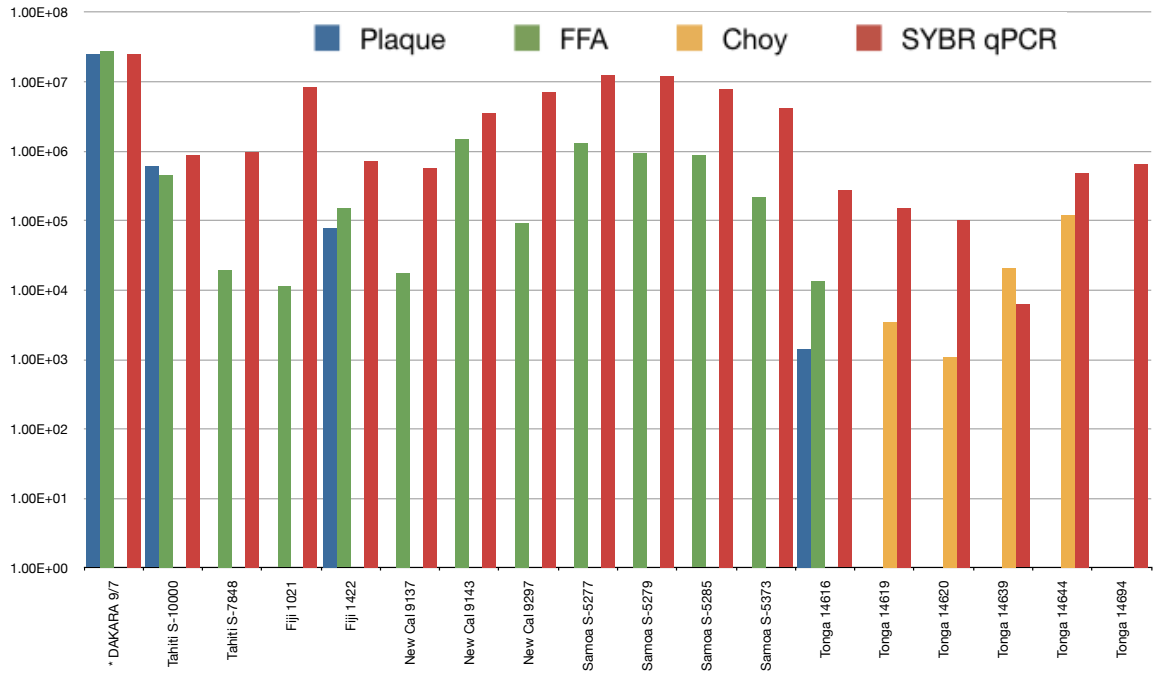


*Figure 21. Comparison of titers of stock South Pacific isolates and lab-adapted DENV-2 standards (*) using **plaque assays, FFA** and **qPCR**. Log scale.*

Discussion

Based on the negative results from the plaque forming and focus forming assays, we were beginning to have some doubts as to whether the Tonga isolates had survived passage. The qPCR assay however, demonstrated that all of the South Pacific samples, including those from Tonga, at the very least contained DENV RNA. As expected, absolute values for genome equivalents were much higher than that for the focus forming assays (Figure 21). In some cases, such as the isolate Fiji 1021, genome equivalents are almost three orders of magnitude higher than the titer measured in focus forming units.

On initial observation, the ability of a particular DENV-2 isolate to form plaques did not appear

to correlate with high titer. That is, the field-acquired isolates from the South Pacific DENV-2 sweep that did form plaques exhibited a variety of titers but in all cases less than the lab-adapted DENV-2 strains (Table 4 below shows the average pfu for each plaque forming strain from a minimum of two assays).

Table 4. Average PFU for each plaque forming isolate

DAKARA	NGC 1056	Tahiti 10000	Fiji 1422	Tonga 14616
2.46E+07	3.70E+06	6.03E+05	7.84E+04	1.43E+03

As noted in the Results section, we were unable to detect the majority of the Tonga DENV-2 isolates using either the plaque or focus forming assays (both of which used Vero cells). However, as mentioned above, Dr. Ming Ju Milly Choy at Duke-NUS Graduate Medical School, Singapore was successful at measuring the titer of some Tonga isolates using a plaque assay with BHK-21 cells. Her results, as illustrated in the chart in Figure 21, allow for comparisons with our other titration methods. Judging from her data as well as our own qPCR results, it is clear that the isolates from the Tonga outbreak exhibit a distinct difference in titer from other isolates, regardless of assay. Furthermore, if one refers back to the phylogeny of the South Pacific epidemic sweep (Figure 14) the Tahiti/Fiji/New Caledonia virus isolates are not dissimilar genetically – in the tree they are generally mixed together unlike the Tonga viruses which all lie within a single genetically distinct clade. Thus in comparing the two categories: Tonga versus Tahiti/Fiji/New Caledonia, one sees a distinct difference in titers, a difference that may possibly relate to the former's attenuated epidemic phenotype (Table 3).

CHAPTER 6: TIME-COURSE EXPERIMENTS FOR REPLICATION DYNAMICS *IN VITRO*

Introduction

To more accurately assess the phenotypic effects of the Tonga synapomorphies on virus replication rate in the mosquito vector, C6/36 (*Aedes albopictus*) cells were infected with 11 South Pacific isolates. Six-day viral growth curves according to genomic equivalents to pfu/mL by qPCR are shown below (Figure 22).

Results

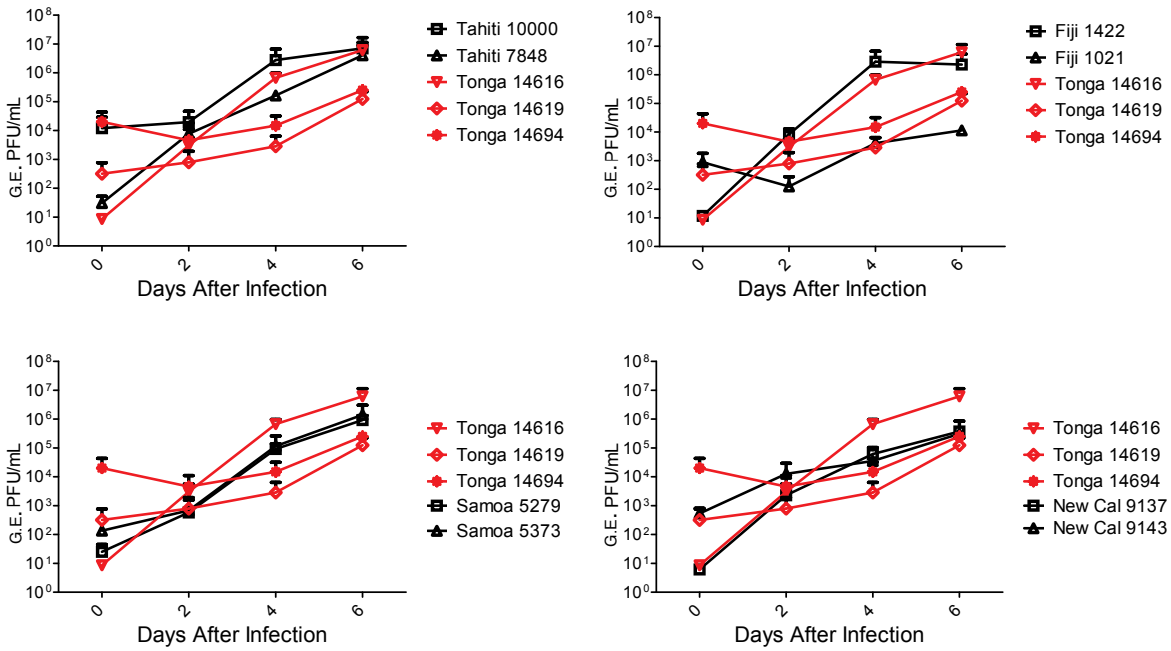


Figure 22. Comparative replication kinetics from an experimental infection of C6/36 cells using isolates from the South Pacific sweep. G.E. PFU/mL measured in genome equivalents from quantitative PCR.

Discussion

The experimental infection confirmed first, that in spite of the initial failure of plaque or focus forming assays in mammalian cell culture to detect the majority of Tonga isolates including 14619 and 14694, these isolates are indeed viable and infectious in mosquito cell culture. The detectable viral genome on day 0 of Tonga 14694 and other isolates is probably a result of inadequate washing immediately following initial infection (in an attempt to mitigate against poor monolayer adherence), particularly as both of their titers dropped on day 2. But aside from that decrease, the trend for all virus isolates is to show a daily increase in titer.

Second, it is interesting that of the three Tonga strains tested, Tonga 14616, the only Tonga strain initially capable of both forming plaques as well as being detectable by focus forming assay, showed a titer nearly as high or higher than that of the non-Tonga strains.

Nonetheless, taken together, the experimental infection showed no obvious correlation between Tonga replication rate/productivity and epidemic attenuation in as much as the Tonga isolates showed no consistent replication pattern among themselves nor in comparison with isolates from islands with more severe outbreaks. However, these results are quite preliminary. As mentioned, C6/36 growth and adherence of the monolayer across wells was inconsistent and this may have skewed the resulting viral titers. These studies will be repeated using this experience as a guide to improve future results.

Finally, since the beginning of this year we have passaged all the DENV-2 isolates from the South Pacific sweep in C6/36 cells. Most have been passaged at a standard MOI of 0.1 with the exception of those viruses that had unusually low titers and for which very small volumes

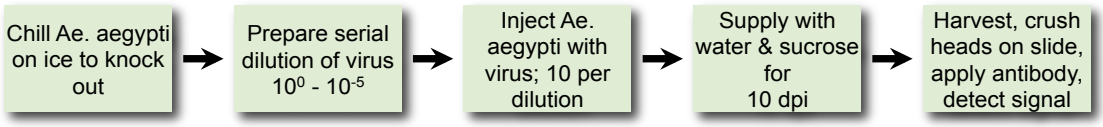
were available. These exceptions were all Tonga strains: 14619 and 14620 (MOI 0.01), and 14639 (which had an exceptionally low titer of 6.18E+03 pfu/mL and thus was infected with an MOI of 0.005). While only a personal anecdotal observation, we have been struck by the difficulty in growing the Tonga isolates, their consistently poor fluorescence using the Direct Fluorescent Assay to assess DENV-2 infections in C6/36 cells (data not shown) and their consistently low qPCR titers even after passage.

CHAPTER 7: MOSQUITO INOCULATION FOR MID_{50}

Introduction

Arguably the most sensitive quantitative assay for DENV and certainly one of the most affordable, mosquito inoculation is particularly helpful for "rescuing" DENV from samples taken from patients with low viremia, viral isolates with a very low titer, or viruses with low infectivity in cell cultures.[40,155] It also has the great advantage of requiring a small volume of sample in comparison with most other titration methods.[189] While inoculations may be a less "natural" model of natural infection than infection through oral feeding of blood containing virus, mosquitoes are generally more susceptible to infection with arboviruses from inoculation as opposed to feeding.[33,189] This may possibly be because inoculations bypass the mosquito midgut barrier.[66] In any case, positive results from this method may provide a useful comparison to negative results from oral infections. And again, as previously mentioned, inoculations have the advantage of requiring considerably smaller volumes of virus than that necessary for oral infections.

Titration through mosquito inoculation essentially involves the following steps:



Mosquitoes are collected from lab colonies, anaesthetized on ice, serial dilutions of virus are injected directly into the thorax of at least 10 mosquitoes per dilution, allowing viral infection to take place for 10–14 days and then a 50% threshold of a minimum infectious dose, i.e., MID_{50}

[40,177,189] is detected using head squash and IFA technique.[128] See the Materials and Methods section for more detail.

Our introduction to *in vivo* work with live *Aedes albopictus* mosquitoes commenced in August 2009 in the JABSOM insectary (University of Hawaii at Manoa, Honolulu, Hawaii, USA) under the tutelage of Dr. Brett Ellis. This involved learning and adapting methods for mosquito containment and safe-handling practices, waste management, collection of wild adults and eggs, mosquito care (including egg hatching, larval rearing, adult blood feeding), and harvest and preservation of eggs.

In November 2009, Dr. Shannon Bennett's lab carried out an experimental oral infection of *Ae. albopictus* using DENV-1 isolates from a 2001 DENV-1 outbreak in Hawaii. While the aim of this experimental infection differed from the current research project in as much as the infection was to examine *Ae. albopictus* gene expression, many of the methods used were the same as those to be used in this project and thus provided valuable experience. Briefly, wild-stock *Ae. albopictus* mosquitoes collected from across the island of Oahu were fed an infectious meal of human blood mixed with one of four Hawaii DENV-1 strains or a negative control with four replicates per treatment and an average of 50 blood-fed females per replicate. Infected mosquitoes were sacrificed in a time series up to 14 days and dissected into midgut, salivary glands and remaining carcass.

In September 2012, *in vivo* work for this doctoral research was shifted to Duke-NUS Graduate Medical School (Emerging Infectious Disease Program, Duke-National University of Singapore) where it was possible to work with both *Ae. aegypti* and *Ae. albopictus*. Our first priority was to establish and maintain genetically diverse populations of both mosquito species to

ensure that transmission dynamics closely mirror those under natural conditions. After consultation with the National Environment Agency – Singapore's government agency dealing with mosquito control – traps to collect eggs were set up in locations known to have high *Aedes* abundance. From January 2013 through August 2014, egg papers were collected on a weekly basis, eggs hatched and reared to adulthood, sorted by species, and introduced into the laboratory populations.

For all of the advantages of direct inoculation, the technique does require training and considerable practice. While some attempt was made at JABSOM using *Ae. albopictus*, real training began at Duke-NUS, primarily with the assistance of Milly Choy, Dr. Duane J. Gubler, and Dr. Ian Mendenhall, using both *Aedes albopictus* and *Ae, aegypti*. Initial practice only used PBS (no virus) and focused on maintaining mosquito survival 10–14 days post inoculation, then moved to using high titer DENV-2 isolates with known MID_{50} value. This trial inoculation required the preparation of a 6-step serial dilution of virus (undiluted to 10^{-5}) with 10 mosquitoes per dilution.

A few points should be noted: at first our inoculations were only carried out in male mosquitoes for reasons of safety but it eventually became clear that the survival rate of male *Aedes* was unsatisfactory and therefore inoculations were shifted exclusively to female *Ae. aegypti*. Indeed, mosquito survival post-inoculation was a persistent problem given the length of time necessary for DENV incubation (10–14 days) and we found that survival could be impacted by any number of factors: excessive age of individual mosquitoes, excessive time spent anaesthetized during inoculation, too much inoculant, or any interruption in mosquito's access to sucrose or water during the incubation period. Thus, many inoculation assays might be rendered

unusable after waiting more than a week for results. One strategy for compensating for this possibility was to inoculate 50% more mosquitoes than that needed for the assay. Such problems, along with the extensive training and practice necessary to inoculate a great number of mosquitoes within a reasonable amount of time, need to be carefully weighed against the technique's many advantages as a means of measuring viral titer. A further note is that while we had originally hoped to do oral infections in Singapore in order to make a comparison of dissemination rates of the South Pacific DENV-2 isolates in *Ae. aegypti,* we found working with colleagues at Duke-NUS that it was difficult to achieve successful oral infection even with high-titer, lab-adapted strains of DENV. Given time constraints, therefore oral infections were not pursued.

In any case, given the low titers exhibited by most of the Tonga isolates from the South Pacific DENV-2 sweep, it was thought to be beneficial to amplify the virus in mosquitoes through parenteral inoculation [189] with the goal of generating a **seed bank** of all DENV-2 isolates from the South Pacific sweep produced under consistent standards for further work. In addition to the higher titers achieved through this technique, it also has the advantage of avoiding artificial adaptation of the virus to cell culture. Seed pools were produced as described in the Methods section. The resulting seed bank of DENV-2 isolated from the South Pacific sweep was then assayed for titer using the parenteral inoculation/MID_{50} method described above with the results shown in Figure 23 and Table 5; both below in the Results section.

Results

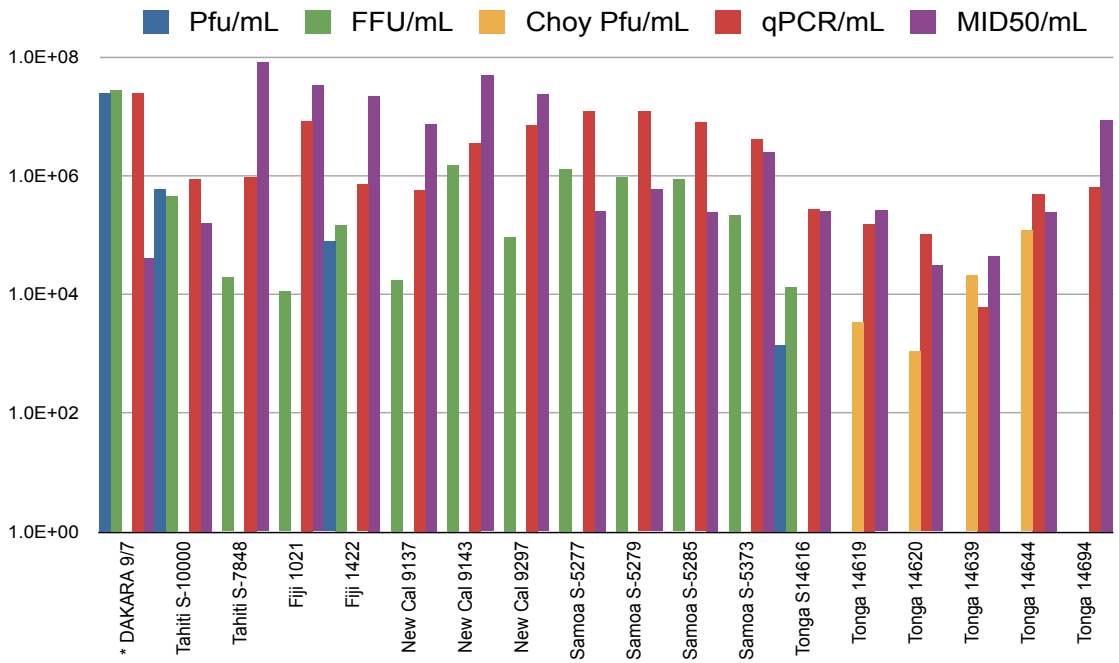


*Figure 23. Comparison of titers using **plaque assays**, **FFA**, **qPCR** and **MID$_{50}$** of stock South Pacific sweep isolates and lab-adapted DENV-2 standards (*). Log scale.*

Table 5. Comparison of titers using **plaque assays**, **FFA**, **qPCR** and **MID$_{50}$** of stock South Pacific sweep isolates and lab-adapted DENV-2 standard DAKARA. Isolates marked "negative" were tested but gave no results.

Source ID	Plaque	FFA	Choy	SYBR qPCR	MID50
* DAKARA 9/7	2.46E+07	2.76E+07		2.50E+07	4.10E+04
Tahiti S-10000	6.03E+05	4.55E+05		8.90E+05	3.31E+07
Tahiti S-7848	*negative*	1.94E+04		9.64E+05	2.22E+07
Fiji 1021	*negative*	1.13E+04		8.40E+06	1.24E+06
Fiji 1422	7.84E+04	1.49E+05		7.16E+05	8.05E+07
New Cal 9137	*negative*	1.77E+04		5.68E+05	7.51E+06
New Cal 9143	*negative*	1.51E+06		3.53E+06	4.86E+07
New Cal 9297	*negative*	9.30E+04		7.09E+06	2.34E+07
Samoa S-5277	*negative*	1.31E+06		1.24E+07	2.58E+05
Samoa S-5279	*negative*	9.47E+05		1.21E+07	5.88E+05
Samoa S-5285	*negative*	8.67E+05		7.90E+06	2.42E+05
Samoa S-5373	*negative*	2.17E+05		4.12E+06	2.48E+06
Tonga 14616	1.43E+03	1.34E+04		2.73E+05	2.58E+05
Tonga 14619	*negative*	*negative*	3.50E+03	1.53E+05	2.61E+05
Tonga 14620	*negative*	*negative*	1.10E+03	1.03E+05	3.09E+04
Tonga 14639	*negative*	*negative*	2.10E+04	6.18E+03	4.50E+04
Tonga 14644	*negative*	*negative*	1.20E+05	4.88E+05	2.48E+05
Tonga 14694	*negative*	*negative*		6.53E+05	8.63E+06

Our use of mosquito inoculation to measure titer in terms of MID_{50} was successful in as much as the technique was able to achieve measurable results with all samples, including those isolated from patients from the DENV-2 outbreak on the island of Tonga, although, not unexpectedly, those results varied from other titration methods previously used, see Figure 23.

Discussion

Given the sensitivity of MID_{50}, we expected higher titer numbers than those acquired from running plaque or focus forming assays but lower numbers in comparison with copy number from qPCR as the latter detects not just infectious virions but also noninfectious immature virions and defective virus particles. Thus, qPCR detects virions that for a variety of factors are unable to carry out infectivity, such as limited cell-binding capacity, inefficient cellular uptake of virions, or defects in membrane fusion properties, viral replication, or virus assembly; all of which can create PFU-to- particle ratios as great as 1:4,000.[231] Numerous studies have repeatedly shown just such a pattern although typically such comparisons have been between plaque assays and qPCR[10,46,179].

In the case of plaque assays and MID_{50}, both are measuring infective particles. And while comparisons have been fewer in number, in general they have shown MID_{50} results to be around 2 logs higher than that from plaque assays.[189]

One of the few studies to compare qPCR with MID_{50} was by Choy et al. 2013[40] and they also found a consistently higher, but variable ratio of qPCR-generated RNA copy numbers to infectious virus titer from MID_{50}. They found copy number/mL to be up to 5 logs higher than the same virus measured by MID_{50}. Our results are variable in that regard (Table 6 below). For

example, our experimental standard DAKARA was almost 3 logs lower in titer in MID_{50} in comparison with qPCR (and indeed all other assays) while other South Pacific DENV-2 sweep isolates showed a variety of results; some with higher MID_{50} values than from qPCR, others just the opposite. Intriguingly the Tonga isolates, as a group, appeared to show the least variation in titer between qPCR and MID_{50}.

Table 6. Log difference in titers between SYBR Green qPCR and MID50

Source ID	SYBR qPCR (G.E. pfu/mL)	MID50 (MID50/mL)	**Log difference** (G.E. pfu/mL: MID50/mL)
* DAKARA 9/7	2.50E+07	4.10E+04	-2.8
Tahiti S-10000	8.90E+05	3.31E+07	1.6
Tahiti S-7848	9.64E+05	2.22E+07	1.4
Fiji 1021	8.40E+06	1.24E+06	-0.8
Fiji 1422	7.16E+05	8.05E+07	2.1
New Cal 9137	5.68E+05	7.51E+06	1.1
New Cal 9143	3.53E+06	4.86E+07	1.1
New Cal 9297	7.09E+06	2.34E+07	0.5
Samoa S-5277	1.24E+07	2.58E+05	-1.7
Samoa S-5279	1.21E+07	5.88E+05	-1.3
Samoa S-5285	7.90E+06	2.42E+05	-1.5
Samoa S-5373	4.12E+06	2.48E+06	-0.2
Tonga 14616	2.73E+05	2.58E+05	-0.0
Tonga 14619	1.53E+05	2.61E+05	0.2
Tonga 14620	1.03E+05	3.09E+04	-0.5
Tonga 14639	6.18E+03	4.50E+04	0.9
Tonga 14644	4.88E+05	2.48E+05	-0.3
Tonga 14694	6.53E+05	8.63E+06	1.1

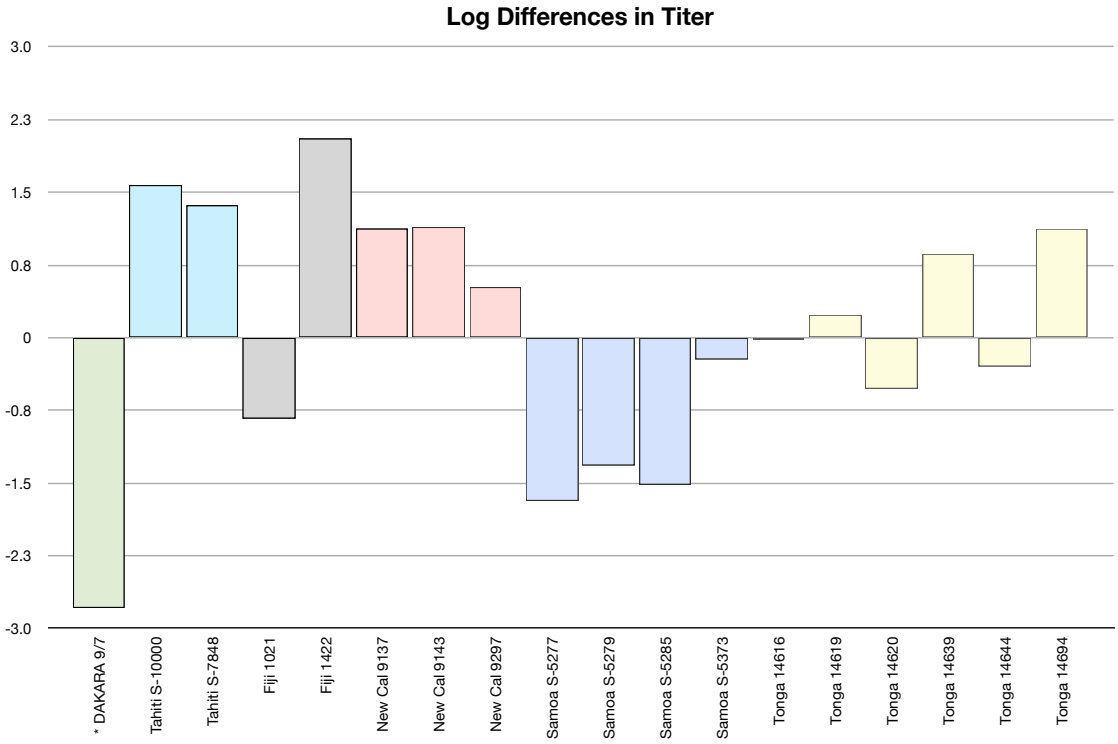


Figure 24. Log difference comparison of titers between qPCR (bottom) and MID_{50} (top) assays of stock South Pacific sweep isolates and lab-adapted DENV-2 standard DAKARA.

By the same token, our MID_{50} results appear to confirm observations from previous assays, that in general, DENV-2 isolated from the Tonga outbreak are lower in titer, as a group, than DENV-2 isolated from other islands during the South Pacific sweep. A notable exception is the Tonga 14694 isolate which has a distinctly high genome equivalent/mL value and one of the highest MID_{50} values of any isolate. While the titer of this specific isolate should be retested for confirmation, given that earlier tests showed no sign of positive selection[213] some degree of variability in titer among DENV-2 isolated from the 1974 Tonga outbreak should not be surprising.

As a further illustration of the low titer shared by the majority of Tonga isolates, we see in displaying MID_{50} results along the phylogenetic tree delineating genetic relatedness (Figure 25) that the majority of low titer DENV-2 isolated from the South Pacific sweep are concentrated

within the Tonga clade and as a group they demonstrate generally lower titer than those isolates from the other South Pacific outbreaks.

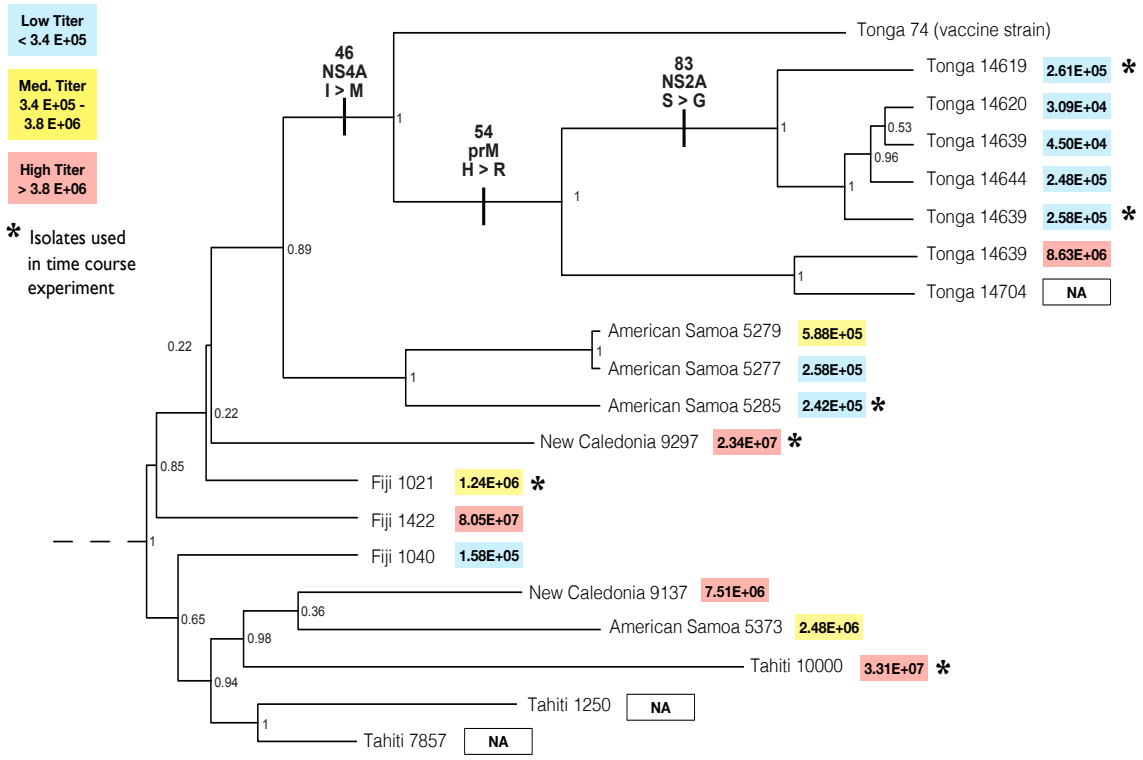


*Figure 25. **MID$_{50}$** titers of each isolate mapped onto phylogenetic tree for purposes of comparison across clades.*

CHAPTER 8: TIME-COURSE EXPERIMENTS FOR REPLICATION DYNAMICS *IN VIVO*

Time Course Experiment: I

An attempt was made to compare replication dynamics of DENV isolated from Tonga (which have consistently exhibited low titer) with DENV isolated from other South Pacific sweep islands which had more severe outbreaks and which consistently exhibit titers one and two logs higher.

Ae. aegypti mosquitoes were inoculated for this time course experiment with the following four representative South Pacific isolates (MID_{50} titers are included for reference):

Table 7. South Pacific isolates and MID50 titers used in *in vivo* time course experiments.

Tahiti 10000	New Cal 9297	Tonga 14616	Tonga 14619
3.31E+07	2.34E+07	2.58E+05	2.61E+05

MOI was normalized through the 100x dilution of high-titer isolates and viral dilutions used to inoculate 10 mosquitoes per isolate. Five mosquitoes were harvested per isolate on days 3–7 for a total of approximately 40 infected mosquitoes. Harvested mosquitoes were pooled, homogenized and purified of debris through centrifugation, then inoculated into *Ae. aegypti* for MID_{50} titration, which as described previously involves: serial dilution of the homogenate, inoculation of 10 mosquitoes per dilution (3 to 4 dilutions necessary per isolate, i.e., 30–40 mosquitoes per isolated). Inoculated mosquitoes held for 10 days, then harvested and assayed for infection using head squash and IFA.

Results: I

A preliminary head squash/IFA of the mosquitoes used for MID_{50} titration of Day 3 Tonga 14616 and Tahiti 10000 were completely negative and showed **no signs of infection**, i.e., no fluorescence was exhibited in any of the head squashes. A subsequent qPCR test of half the homogenates showed lack of infection in all Tahiti 10000 and New Cal and Day 3 Tonga 14616.

Discussion

A number of causes for lack of infection are possible:

- Our 100x dilution of the Tahiti and New Caledonia samples in order to normalize MOI may have lowered MOI too much or somehow interfered with infection.
- Virus samples may have degraded from excessive freeze/thaw (samples were stored in freezers at Duke-NUS that were shared with others and thus there is always the risk that samples could be moved by others and accidentally left out to thaw).
- There might have been mistakes made in dilution and/or injection.

To test for virus degradation and the effect of diluting the virus for normalizing MOI, I used the same media that I had previously used for dilution and ran another qPCR.

- All samples were positive so the stock virus was **not** degraded and dilution had no significant effect. However, only infection would ensure samples were capable of **replication**.
- Time course experiment was therefore repeated but unfortunately, we lacked time to titrate inoculated mosquitoes with MID_{50}.

Time Course Experiment: II

Given the failure of the previous time course experiment (see above) to successfully infect *Ae. aegypti* with Tahiti and New Caledonia DENV-2 isolates (even though qPCR demonstrated that both isolate stocks and their dilutions had copy numbers equivalent to previous assays) it was decided to attempt again direct inoculation of *Ae. aegypti* females with normalized isolates diluted in DMEM-10, harvesting / freezing at -80°C at 3 time points, i.e, Days 3, 7, 10 with 10 mosquitoes per time point.

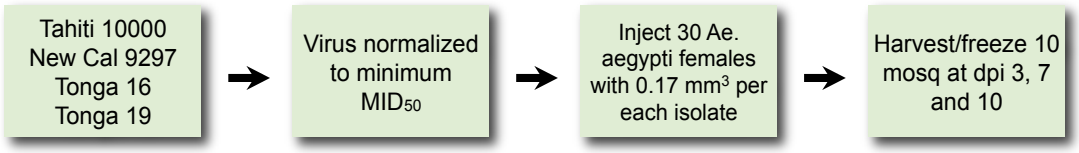


Table 8. MOI calculations for normalizing infectious titer for South Pac DENV-2 time course exp.

Virus	Stock titer (MID50)	To dilute by 10^{-2} divide by….	µL media to dilute 1 µL virus 10^{-2}	µL media *used* to dilute 5 µL virus	To dilute by 10^{-1} divide by….	µL media to dilute 1 µL virus 10^{-1}	µL media *used* to dilute 5 µL virus
Tahiti 10000	3.31E+07	2.58E+05	128	**640**	2.58E+06	13	**64**
New Cal 9297	2.34E+07	2.58E+05	90.70	**455**	2.58E+06	9	**45**
Tonga 14616	2.58E+05	2.58E+05	1.00	**0**	2.58E+06	0	**0**
Tonga 14619	2.61E+05	2.58E+05	1.01	**0**	2.58E+06	0	**0**

Results

Details on the method of SYBR Green qPCR used to measure these infected mosquitoes as well as the results are discussed in the following Chapter 9.

CHAPTER 9: VIRAL LOAD OF DENV-2 ISOLATES DETERMINED BY QPCR II

Introduction

Titration assays using qPCR carried out at Mahidol University, Bangkok School of Tropical Medicine, had the primary goal of titrating mosquitoes infected (by the intrathoracic method) at Duke-NUS as part of time course experiments (discussed in the previous Chapter 8.). As mentioned, these mosquitoes were originally planned by us to be titrated by means of MID_{50} but the time needed proved to be insufficient due to the need to move to Thailand. As methods used for carrying out qPCR at Mahidol were completely different from those used at University of Hawaii, a more full description is warranted. It also bears mentioning that in learning and carrying out this later qPCR, we are greatly indebted to the generous instruction and use of equipment by Dr. Pornsawan Leaungwutiwong and her laboratory staff, in particular Mr. Narin Thippornchai and Ms. Siriporn Cheattanadee, in the Department of Microbiology and Immunology at Mahidol University.

Table 9. Comparison of major differences between University of Hawaii and Mahidol qPCR methods:

	University of Hawaii at Manoa	Mahidol University
RNA Extraction	Qiagen QIAamp viral RNA mini kit	Biotechrabbit GenUP Total RNA kit
Chemistry	SYBR Green	TaqMan
Primer (Probe) gene target	3' Non-Coding Region (Chien et al. 2006) [39]	Envelope (Johnson et al. 2005) [117]
Reverse transcription	Two-step	One-step
Standard	Lab-adapted DENV-2 "DAKARA" tested by me using PFU and FFU	Lab-adapted "*D2 (16681), MK2-3*" independently tested using PFU and FFU

Standard

Standard curve of Mahidol University qPCR was based on lab-adapted DENV-2 strain acquired from Dr. Pornsawan and identified as "*D2 (16681), MK2-3, C6/36-1, Sm-2, C6/36-5, 20 Feb 13."* Previous plaque and focus-forming assays in her lab had established an average pfu of 2.3E+05 per mL.

RNA Extraction

Since Dr. Pornsawan's laboratory carried out qPCR of clinical samples for DENV detection and viremia measurement on a routine basis, protocols were completely worked out and required no optimization on my part. However, although Dr. Pornsawan's laboratory routinely uses the Qiagen QIAamp viral RNA mini kit for RNA extraction, as a means of saving funds, the use of a different and less expensive kit from another manufacturer was explored; the Biotechrabbit GenUP Total RNA kit. In using both kits to extract RNA from the same standard and then running all RNA under the same qPCR conditions, a threshold cycle of less than one ($Ct < 1$) was found in comparing results. Thus encouraged, a test run of the standard curve was conducted to ensure that methods were free of error.

Unfortunately, although amplification curves of the standards were evenly spaced, suggesting that the qPCR itself was successful, high Ct values lead me to believe that my RNA extraction was suboptimal. Dr. Pornsawan offered to have her staff attempt to repeat both the extraction (using the Qiagen kit) as well as the qPCR to check whether the problem was with either the virus standard or my lab technique. Unfortunately, her staff instead simply extracted RNA from the DENV-2 standard for me using the Qiagen kit (rather than the Biotechrabbit

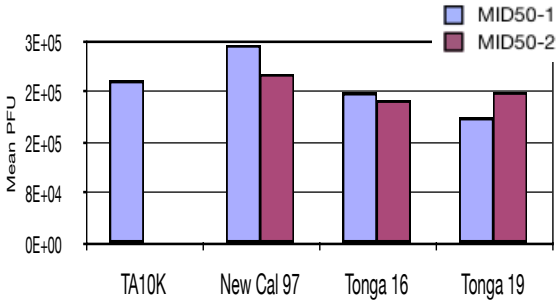
GenUP Total RNA kit) so that I could use this RNA as my standard for all subsequent qPCR assays. As we will see however, this may have led to subsequent problems since I used the Biotechrabbit GenUP Total RNA kit to extract RNA from samples while continuing to rely upon RNA extracted by the Qiagen kit for my standard curve.

Quantitative PCR assay of MID_{50} homogenate

My initial efforts at using qPCR at Mahidol were to test mosquitoes that had been inoculated in two separate MID_{50} assays at Duke-NUS. The first assay - named **MID50-1** – had come out lower than expected so had been followed up by another assay attempt - **MID50-2**. My goals in this qPCR then were: 1) confirm positive infection in mosquitoes from both previous assays, 2) test my new TaqMan protocols on at least the standard 3) test whether the later MID50 assay (MID50-2) was higher than the first as had been suggested by the respective MID50 results.

Table 10. Results of qPCR assay of MID_{50} homogenate

	Tahiti 10000	New Cal 9297	Tonga 14616	Tonga 14619
MID50-1	2.42E+05	2.95E+05	2.24E+05	1.87E+05
MID50-2	-	2.52E+05	2.13E+05	2.25E+05

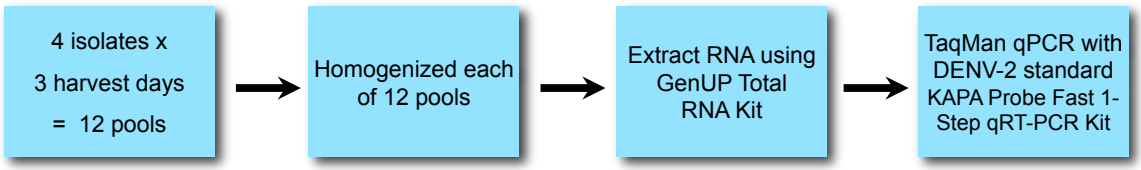


Results confirmed successful infection of mosquitoes with viable virus. And Ct values showed minimal variation *within* replicates suggesting that my qPCR methodology was improved. Mean PFU values did vary somewhat between the earlier attempt at MID50 (MID50-1) and the later (MID50-2). Note that measurements of the Tahiti and New Caledonia isolates from MID50-1 came out an average of 2 logs lower than from MID_{50} assays carried out a year

previously (although Tonga measurements were approximately the same). Thus MID50-2 was to see if the low titer of MID50-1 was a handling error or whether it suggested that my samples had deteriorated. Here it appears that if anything, G.E. PFU/mL values decreased in the later MID_{50} assay except with Tonga 19. (This exception might be due to shuffling of MID50 tubes when they were packed for shipment and accidental swapping of the Tonga 19 MID50-1 and MID-2 in inventory). In any case, all qPCR measurements are fairly similar which is even more odd.

Quantitative PCR assay of mosquitoes infected in Time-Course Experiment II

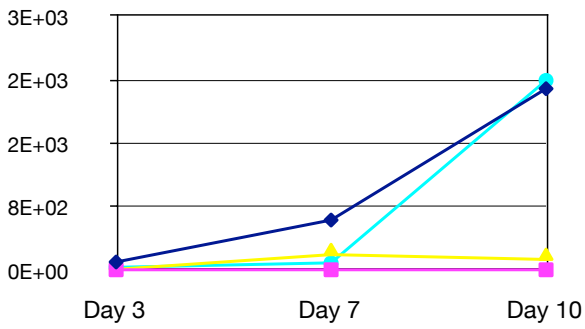
Treatment of mosquitoes inoculated as part of the second attempt at carrying out time-course experiments is illustrated in the following flow chart:



All mosquitoes for each of 4 isolates and 3 harvest days were homogenized as pools (n=12). However, my lab notes regarding the number of mosquitoes per pool turned out to be in error which meant that while most pools had 7 mosquitoes other pools had as few as 4, thus requiring normalization of subsequent copy number results.

Table 11. Results of qPCR of mosquitoes infected in Time Course Experiment II

	Day 3	Day 7	Day 10
Tahiti 10000	9.21E+01	5.88E+02	2.14E+03
New Cal 9297	3.41E-02	1.05E-01	8.64E-02
Tonga 14616	5.46E+00	1.79E+02	1.22E+02
Tonga 14619	2.91E+01	8.20E+01	2.24E+03



The results from the qPCR of pooled mosquitoes infected as part of the second time-course experiment were difficult to interpret. Unlike the first time course experiment, this titration did show evidence that mosquitoes were successfully infected at least with the Tahiti 10000 and Tonga 14619 (in contrast to the first time course in which infection failed in all days Tahiti 10000 and New Cal and Day 3 Tonga 14616).

However, this limited comparison of Tahiti versus Tonga shows no indication that the Tahiti 10000 isolate replicates faster than Tonga 14619, a result that would seem to contradict the results of previous replication experiment (Figure 22, upper left). In addition, viral loads determined by qPCR were still surprisingly low (considerably lower titers than those seen in the previous qPCR assay), which may have been an artifact of the assay (the dilutions for normalization) or possibly due to problems with the original inoculation.

Quantitative PCR of stock South Pacific DENV-2

Given the uncertain results from qPCR of mosquito samples infected with South Pacific DENV-2 isolates, I turned to original samples of the isolates that had been grown in C6/36 cells. While not the same passage as those samples previously tested by SYBR Green, they were only one passage later. The goal was to compare these qPCR results with those gained from the SYBR Green qPCR at University of Hawaii and the measurements for MID_{50} at Duke-NUS.

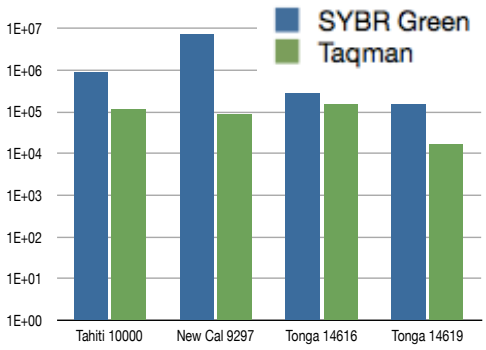
Table 12. Passage history of assayed South Pacific isolates

Isolate	Harvest day	Passage	Isolation	Volume	Harvest date
Tahiti 10000	10	3		0.5 mL	2/28/12
New Cal 9297	10	3	Mosq C6	1.0 mL	2/28/12
Tonga 14616	10	3	Mosq C6	0.5 mL	2/28/12
Tonga 14619	11	3	Mosq C6	0.5 mL	3/30/12

Results

Table 13. Results of qPCR of stock DENV-2 isolates

Isolate	Sample Ct-1	Sample Ct-2	Ct Mean	TaqMan Mean	SYBR Green
Tahiti 10000	19.79	19.72	19.75	1.18E+05	8.90E+05
New Cal 9297	20.13	20.22	20.18	8.84E+04	7.09E+06
Tonga 14616	19.36	19.35	19.35	1.55E+05	2.73E+05
Tonga 14619	22.30	22.99	22.64	1.68E+04	1.53E+05



As the table shows, while Ct values are consistent across replicates, the resulting pfu values do not appear to have any particular correlation with those from my SYBR Green qPCR assays. Also, unlike the SYBR Green qPCR, the TaqMan assay results show little difference in G.E. pfu values between what are ordinarily low-titer isolates such as the Tonga versus isolates those from the islands of Tahiti or New Caledonia.

Discussion

There were a number of problems with my qPCR titration of the mosquitoes infected in the time course experiments but the basic problem was that my TaqMan qPCR showed no apparent correlation with the results of other assays and in particular with my previous qPCR assays using SYBR Green. There are a number of possible reasons for this:

1. Using different primers may have contributed a significant source of variability in my results. The TaqMan primer/probe targeted the E protein – perhaps the most variable protein in DENV – whereas my SYBR Green probe was specific to the 3'NCR, which is very highly

conserved.

2. The choice of using TaqMan rather than SYBR Green seemed to promise more accurate results. However, apart from the inherent problems with comparing titration results when using different methods, the choice of TaqMan might have actually further decreased assay accuracy in using primer/probes of poor specificity.

   Of the two widely used real-time RT-PCR formats, SYBR Green I based assays are less expensive, more flexible, and less susceptible to false negatives due to single nucleotide polymorphisms in the probe sequence than assays that use TaqMan. Although a single point mutation in the probe region can reduce target detection by 47% in TaqMan assays, SYBR Green I is a nonspecific dsDNA binding dye that only requires design of oligonucleotide primers for PCR to measure fluorescence emission.[179]

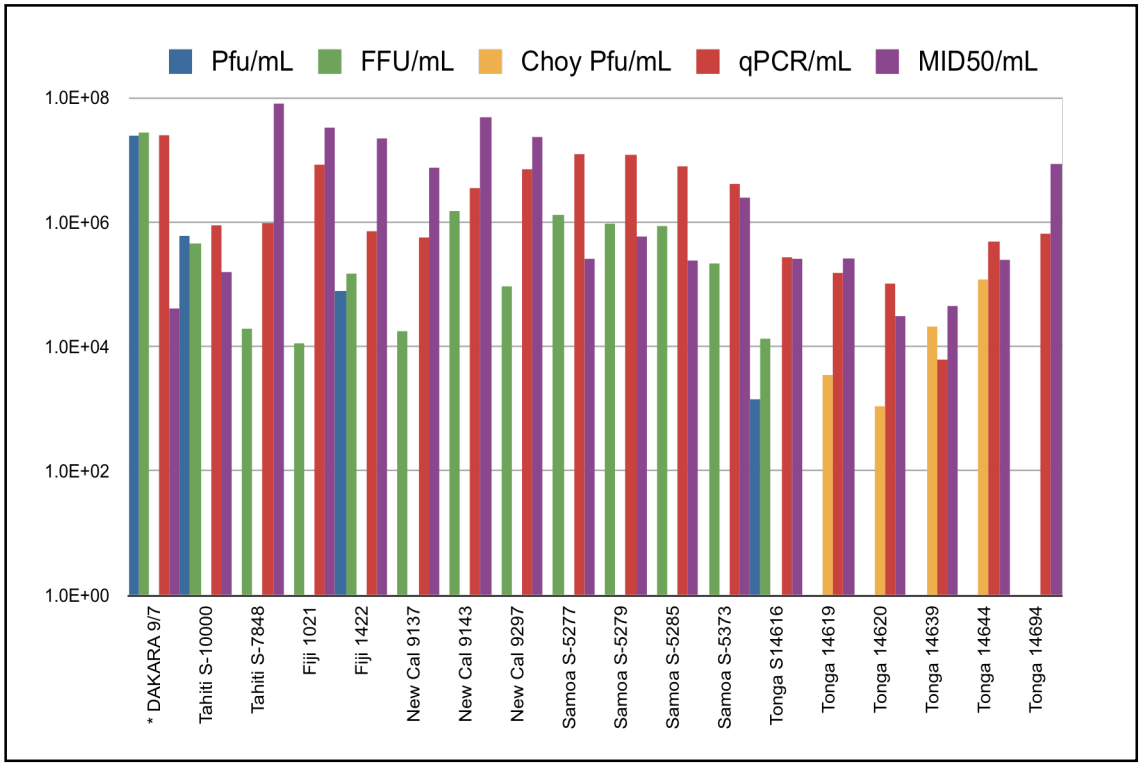
3. As previously mentioned, when I attempted to use the Biotechrabbit GenUP Total RNA kit to extract RNA from my standard and my qPCR results appeared less than optimal, I then opted to use RNA extracted using the Qiagen QIAamp viral RNA mini kit. However, I subsequently used the Biotechrabbit kit for extraction of the time course mosquitoes. This meant that I had never actually verified that my Biotechrabbit extractions were optimal and meant that Ct values of standards and unknowns were not really comparable.

CHAPTER 10: SUMMARY OF RESULTS AND DISCUSSION

Spearman's Correlation

The Spearman's Correlation Coefficient was applied to measure the statistical correlation between the various methods used over the course of this research to measure viral titer. Spearman's is a nonparametric measure of the strength of association that exists between two variables, in this case between any two methods of viral quantification.

For context, the chart (previously shown in Figure 23, Chapter 7) is included below to illustrate all titrations created during the course of this research.



A matrix showing the results of Spearman's Correlation test between paired data between methods (from Table 6; the standard DAKARA was not included) is shown in Table 14 below.

Table 14. Spearman's Correlation Test of titration data. N = no. of samples, Rs = Spearman's Correlation Coefficient (strength of association) and p = statistical significance. Colors indicate strength of association (*Rs*) where red = High, yellow = Moderate, blue = Low X = no comparison, and - = same method. Tests run in IBM SPSS Statistics for Mac v20.

	MID50	SYBR qPCR	Choy PFU (BHK-21)	FFA (Vero)	PFU (Vero)
MID50	-	N=17 *Rs*=0.288 p=0.262	N=4 *Rs*=0.400 p=0.600	N=12 *Rs*=0.025 p=0.94	N=3 *Rs*=0.500 p=0.333
	SYBR qPCR	-	N=4 *Rs*=0.400 p=0.600	N=12 *Rs*=0.455 p=0.138	N=3 *Rs*=1.000 p=0.000
		Choy PFU (BHK-21)	-	X	X
			FFA (Vero)	-	N=3 *Rs*=1.000 p=0.000
				PFU (Vero)	-

Given the variety of methods used over the course of this research to measure the viral titer of DENV-2 isolated from the South Pacific epidemic sweep, it is reasonable to speculate on how well these methods agree with one another. The Spearman's Correlation Test allows us to make some comparisons and the table above provides a graphic illustration of the strength of correlation between methods. Because there are a different number of samples associated with each method, *Rs* is not strictly comparable. Thus, note that those method comparisons with high *Rs* (red) also have few samples.

In general *Rs* values match expectations, e.g., since PFU (plaque assays) and FFA (focus

forming assays) utilize similar methods and both grow virus in Vero cells it is not surprising that they would show a high *Rs* value. The moderate correlation between SYBR Green qPCR and FFA is notable given that the majority of the isolates measured by these two methods could not be titrated by PFU but were able to be titrated by FFA, perhaps indicating a lack of plaque-forming phenotype. Perhaps most surprising it that the lowest *Rs* is found in comparing MID_{50} with other methods (except Choy perhaps). Possible explanations include: a) MID_{50} being the only method in which virus is grown in mosquitoes rather than a mosquito cell line b) MID_{50} used an later cell passage from all except for the Choy plaque assay (and latter was in BHK cells rather than Vero) c) the higher potential for "operator error" inherent in MID_{50} technique.

This analysis suggests that the assay methods have a modest degree of correlation but perhaps lend more support for settling upon a single assay method sensitive enough to measure all isolates and provide consistency across successive measurements.

Discussion

DENV pathogenesis has been a subject of serious scientific enquiry for more than 100 years. The progress made in the last ten years has been outstanding, exemplified by the successful trials and licensing of the first vaccine against DENV, Dengvaxia.[60,254] Yet the most basic of questions regarding DENV pathogenesis still await answers. We still lack a fundamental understanding of the mechanisms that determine why those infected with DENV have such a variety of clinical outcomes and indeed, still lack reliable markers for identifying individuals at risk for severe disease. The research described in this dissertation has been directed towards increasing our understanding of DENV pathogenicity through comparisons of phenotypic differences between DENV that were previously documented as differing in both their clinical and/or epidemic

outcomes as well as genetically.

Thus, this dissertation has described research carried out to explain the phenomena of a DENV-2 outbreak of dramatic attenuation on the island of Tonga as compared to outbreaks on other South Pacific islands surrounding it and which we were subsequently able to show correlated with specific amino acid substitutions. The aim with this current research was to identify specific phenotypic traits that correlate with virulence and epidemic potential, and using both *in vitro* and *in vivo* models, to explore their relationship with genetic variation in the DENV-2 epidemic strains. Among the variety of phenotypic differences that this research hoped to examine, viral productivity, i.e., the number of virus produced per unit volume, that is, *titer*, received the most attention.

As noted previously, evidence for molecular markers for pathogenesis in DENV is generally lacking. Given a lack of an effective animal model for DENV studies and the current poor understanding of the functional aspects of DENV structure, it is difficult to attribute specific correlates between viral phenotypes and pathogenesis. Nevertheless, titer has the strongest claim to being a candidate for a viral phenotypic marker given that a number of papers have identified a relationship between peak viremia titer and disease severity as well as increased rates of *Aedes* vector infection. For example, in a prospective study of dengue-infected children in Thailand, Vaughn et al. (2000) noted that disease severity was most strongly associated with DENV-2 serotype infections, secondary infections, and higher peak titers; indeed "as peak viremia titer increased so did the pleural effusion" with little to no pleural effusion detected until reaching higher peak levels of viremia.[237] Other studies have also found a strong association between disease severity and viremia levels; some with high *early* viremia determined to be

more significant than peak viremia (although peak viremia was often missed because it occurred prior to enrolment).[138,221] while Wang et al. (2003) found high *late* viremia a more accurate marker for severe dengue.[242] In terms of the role of human viremia in mosquito transmission, a study in Vietnam by Nguyen et al. (2013) found that high early DENV plasma viremia was associated with longer human infectiousness leading to increased prevalence of infection among mosquitoes allowed to blood feed.[167]

In spite of these many studies, the relationship between DENV titer, human viremia and disease severity is still unclear. Data on viremia levels in naturally occurring DENV infections is still sparse because, as mentioned above, clinical observation usually takes place long after the onset of infection, and thus the level of viremia already decreasing. In addition, it is not clear that studies have been done to isolate virus from infected patients and compare isolate titer with patient disease severity. Nonetheless, a correlation between high viral load and disease severity has long been suggested and indeed is assumed to be important in both antibody dependent enhancement and viral pathogenicity. [87,100]

In this context, the unusually low titer of the DENV-2 isolated from Tonga patients was a distinctive characteristic that drew attention from the beginning. As Dr. Gubler wrote his description of the Tonga outbreak,[85]

> Despite ideal timing, collection, and storage of serum specimens from the 1974 outbreak the virus isolation rate was considerably lower than that obtained in other dengue type 2 outbreaks in Tahiti and New Caledonia and that of the 1975 outbreak in Tonga – where timing, collection, and storage were less satisfactory. *It is logical to attribute the abortive nature of the 1974 outbreak and undetected presence on the island of dengue type 2 prior*

to August 1973 to the relatively low viremias observed. It also is natural to wonder if relatively low viremia is not associated with relatively benign disease. (Emphasis added).

In this context, what is most striking is that, regardless of the method used to measure titer, isolates from the attenuated outbreak on Tonga consistently demonstrated the lowest titer compared to those DENV-2 isolated from any of the other South Pacific outbreaks. Figure 26 below (also shown as Figure 25 on page 140) illustrates clearly how the majority of low titer isolates, as measured by the MID_{50} assay, fall within the Tonga clade and as a group demonstrate generally lower titer than those isolates from the other South Pacific outbreaks.

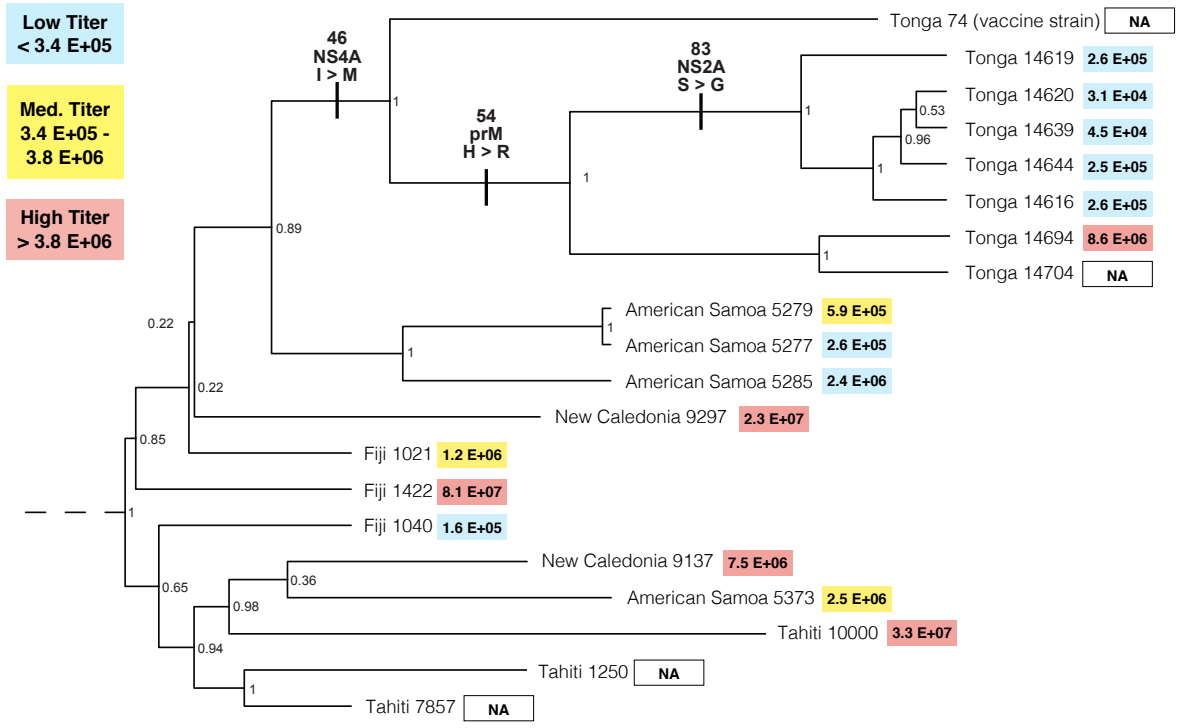


Figure 26. ***MID_{50}*** *titers of each isolate mapped onto phylogenetic tree for purposes of comparison.*

The concentration of low titer isolates within the Tonga clade is even more pronounced when titers from the SYBR Green qPCR assay are mapped upon the phylogenetic tree of the

South Pacific sweep. (Figure 27.)

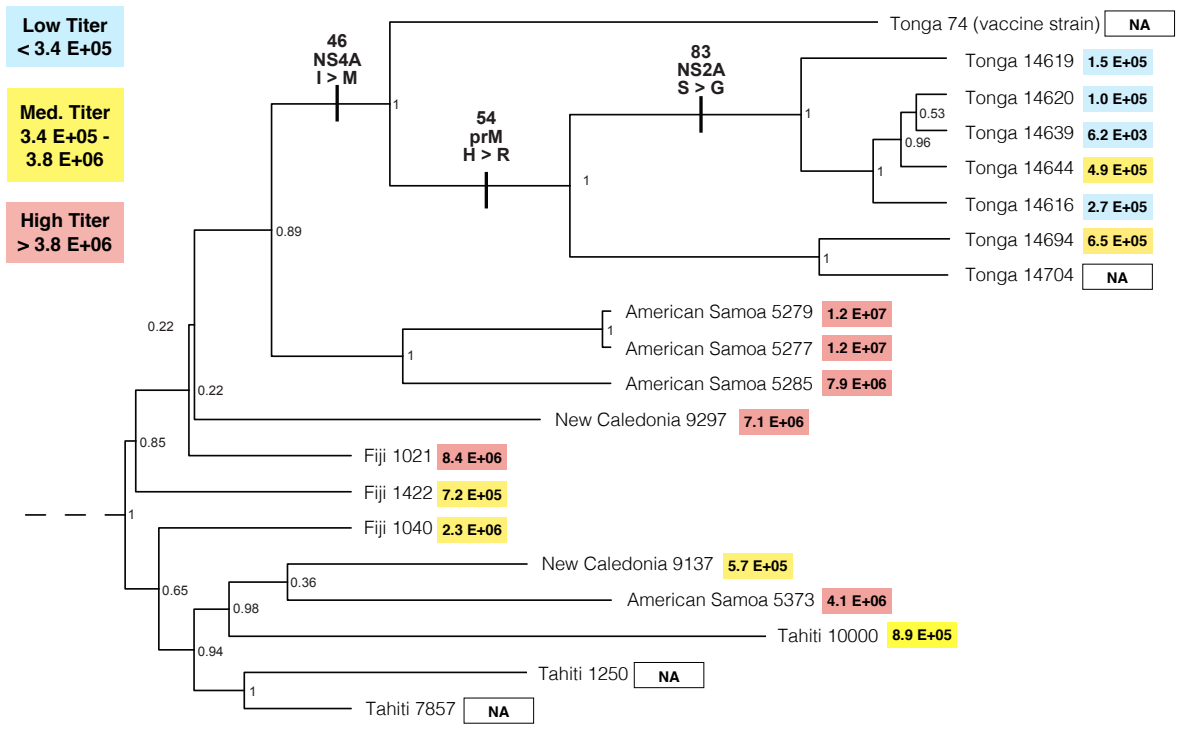


Figure 27. ***Quantitative PCR*** *titers of each isolate mapped onto phylogenetic tree for purposes of comparison.*

Such agreement between these two titer assay methods is striking given the preceding analysis using the Spearman's Correlation Test of titration data. That test showed the MID_{50} and qPCR assays as having a relatively poor level of correlation. This is unsurprising for the reasons previously given: a) DENV was replicated in C6/36 cells in the qPCR assay and adult mosquitoes in MID_{50}, b) MID_{50} used an later cell passage from the SYBR Green qPCR, c) MID_{50} only detected infectious virus while the SYBR Green qPCR measured the quantity of nucleic acid. Yet in spite of their differences both assays clearly demonstrate that, as a group, the Tonga clade is distinctively lower in titer than DENV isolated from the other outbreaks in the South Pacific DENV-2 epidemic sweep.

Also worth noting is the phylogenetic clustering of the Tonga clade with the Tonga 74 vaccine strain; this strain shared an amino acid substitution in the NS4A gene region and also exhibited an attenuated phenotype both in terms of its failure to infect the midgut or head of *Ae. aegypti* and in its relatively low replication rate in SCID mice transplanted with HuH-7 human hepatoma cells (a mean titer of 7.9 E+05 as measured by plaque assay with Vero cells).[23] Blaney et al. (2004) point out that as a member of the DENV-2 American genotype, the Tonga isolates (along with the rest of the South Pacific sweep) share point mutations that, when in other research were introduced into DENV-2 of the more virulent Asian genotype, were found to result in reduced replication within dendritic cells and monocytes.[45] Blaney et al. (2004) go on to suggest that the introduction of further mutations might lead to a "small, incremental increase in attenuation," a possible explanation for the lower viral titer exhibited by those DENV-2 isolated from Tonga compared with other American genotype DENV-2 isolated from the South Pacific sweep.

A final observation, albeit anecdotal, refers back to remarks first made in Chapter 6 (*Time-course experiments for replication dynamics* in vitro). As written there in reference to passaging the DENV-2 isolates from the South Pacific sweep "we have been struck by the difficulty in growing the Tonga isolates, their consistently poor fluorescence using the Direct Fluorescent Assay to assess DENV-2 infections in C6/36 cells (data not shown) and their consistently low qPCR titers even after passage."

Thus, the results from the variety of titration assays carried out over the course of this dissertation research corroborate our previous phylogenetic analysis demonstrating genetic clustering correlating with the attenuation of the Tonga isolates. Together, their conclusions

appear to support the original hypothesis: *evolutionary changes in the DENV genome can give rise to significant variations in disease severity and epidemic potential between different populations of DENV*.

Given the lack of an animal model with which to compare DENV variation directly, measurements of specific virus phenotypes, such as virus quantity, both *in vitro* and *in vivo* as have been carried out here, offer possible insights into the role of DENV evolution on disease severity. In addition, this work is strengthened by its use of low passage DENV from outbreaks whose epidemiology and clinical nature has been relatively well documented.

Nonetheless, this study has its weaknesses which can be summarized as two-fold: first, as the Spearman's Correlation analysis (Table 14) points out, the variety of titration methods used only showed a modest degree of correlation. For the purposes of this study and with time permitting, it would have been optimal to eventually settle upon a single assay method sensitive enough to measure all isolates and provide consistency across successive measurements. However, further investigation into the causes of such variation in titer would be interesting to pursue. As pointed out in the Discussion in Chapter 7, to have higher titers from MID_{50} than from qPCR for some of the South Pacific isolates was unexpected given that MID_{50} measures only infective DENV, which given the poor rates of DENV maturation, is usually a small subset of the larger virion population. Such a result may only be a result of experimental error but it is intriguing that as a group, Tonga showed the greatest variation in high titer between methods (3 Tonga isolates showed the highest titer using MID_{50} and 2 isolates were highest using qPCR; see Figure 24, page 138) while also showing the least variation in log difference between titers (Table 6, page 137).

A second weakness of these research results was the lack of further experimental evidence in addition to the titrations. Aim 1 of this dissertation research proposed to measure and compare a variety of phenotypes among the samples isolated from the South Pacific DENV-2 sweep. While viral productivity, i.e., viral titer, was examined, further assays were proposed but were never carried out or were unsuccessful in achieving results. They will be discussed in the following section "Future Directions."

Future Directions

Focus-forming assay with BHK-21

As mentioned above, establishing a single, reliable, sensitive titration method proved to be a particular obstacle in the research. Among other titration methods, both the plaque assay and focus-forming assay (FFA) were attempted but in using Vero cells neither method was capable of detecting samples isolated from the Tonga outbreaks. However, subsequent work by a colleague, Dr. Milly Choy, using BHK-21 cells proved more successful revealing that a change in cell type might be a more viable approach. Another recommendation for reattempting FFA using BHK-21 cells is that this assay measures infective infectious virions rather than the RNA measured by qPCR. Since viral RNA can be incompletely transcribed or not yet assembled into a virion within the cell, or that which is associated with immature or defective virus, FFA would seem to have the advantage of being a more direct measure of phenotype.

Time-course experiments

Successful titrations measured stock virus whose starting concentrations were never normalized. Time-course measurements would start with identical MOI and thus allow for a more accurate

comparison of rates of viral replication; that is, the number of virus produced per cell.

Comparison of South Pacific DENV-2 among various cell types

Preliminary work with the DENV-2 isolated from the South Pacific sweep was conducted primarily in the *Ae. albopictus* cell line C6/36 and in Vero cells (kidney epithelial cells from the African green monkey). However, given the marked variation in the susceptibility to infection among cell types by different DENV strains, particularly those strains which are of low passage and the tropism of DENV to different cell types, it is important to compare infectivity in a range of cells. Moreover, immune dysfunctions specific to cell lines, such as the lack of anti-viral RNA interference (RNAi) response by C6/36 cells and the defective interferon (IFN) response by Vero cells need to be taken into account. This is particularly significant given that two of the three amino acid substitutions defining the Tonga clade are in the NS2A and NS4A gene regions and both NS2A and NS4A have been shown to act as IFN antagonists by inhibiting IFN antiviral immune response both alone and in coordination with one another. Accordingly, it would still be useful to compare the infectivity of selected DENV-2 South Pacific isolates in Vero (and possibly C6/36) cells against similar cell types.

Measuring the ratio of mature to immature virions

An alternative explanation for poor rates of viral isolation and generally lower viremia seen during the Tonga outbreak compared with isolation rates and patient viremia on other islands might be that the DENV-2 isolated from the attenuated 1974 outbreak on Tonga produce a higher ratio of immature (noninfectious) to mature (infectious) virions compared with the other DENV-2 from the South Pacific sweep. This might be a consequence of the substitution in the

prM gene region shared by members of the Tonga clade (histidine to arginine substitution at residue 54). Most studies carry out Western blots utilizing a specific anti-M antibody capable of distinguishing between M and prM based on the greater molecular weight (thus lower band) of immature (pr + M) vs. mature (M only).

LITERATURE CITED

1. Aaskov J, Buzacott K, Field E, Lowry K, Berlioz-Arthaud A, Holmes EC: Multiple recombinant dengue type 1 viruses in an isolate from a dengue patient. **J Gen Virol 88**:3334–3340, 2007

2. Achee NL, Gould F, Perkins TA, Reiner RC, Morrison AC, Ritchie SA, et al: A critical assessment of vector control for dengue prevention. **PLoS Negl Trop Dis 9**:e0003655, 2015

3. Adams B, Holmes EC, Zhang C, Mammen MP, Nimmannitya S, Kalayanarooj SM, et al: Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. **Proceedings of the National Academy of Sciences 103**:14234–14239, 2006

4. Alphey L, Alphey N: Five things to know about genetically modified (GM) insects for vector control. **PLoS Pathog 10**:e1003909, 2014

5. Amarasinghe A, Kuritsk JN, Letson GW, Margolis HS: Dengue virus infection in Africa. **Emerging Infect Dis 17**:1349–1354, 2011

6. Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, et al: Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. **PLoS Negl Trop Dis 5**:e1311, 2011

7. Armstrong P, Rico-Hesse R: Differential susceptibility of Aedes aegypti to infection by the American and Southeast Asian genotypes of dengue type 2 virus. **Vector Borne Zoonotic Dis 1**:159–168, 2001

8. Armstrong P, Rico-Hesse R: Efficiency of dengue serotype 2 virus strains to infect and disseminate in Aedes aegypti. **Am J Trop Med Hyg 68**:539–544, 2003

9. Ashburn P, Craig C: Experimental investigations regarding the etiology of dengue. **J Infect Dis 4**:440–475, 1907

10. Bae H-G, Nitsche A, Teichmann A, Biel SS, Niedrig M: Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. **J Virol Methods 110**:185–191, 2003

11. Baer A, Kehn-Hall K: Viral concentration determination through plaque assays: using traditional and novel overlay systems. **J Vis Exp**:e52065, 2014

12. Balsitis SJ, Harris E: Animal Models of Dengue Virus Infection and Disease: Applications, Insights and Frontiers. **Frontiers in Dengue Virus Research**:103–120, 2010

13. Bancroft T: On the etiology of dengue fever. **Australian Medical Gazette 25**:17–18, 1906

14. Bancroft WH, Top FH, Eckels KH, Anderson JH, McCown JM, Russell PK: Dengue-2 vaccine: virological, immunological, and clinical responses of six yellow fever-immune recipients. **Infect Immun 31**:698–703, 1981

15. Barnes W, Rosen L: Fatal hemorrhagic disease and shock associated with primary dengue infection on a Pacific island. **Am J Trop Med Hyg 23**:495–506, 1974

16. Barrick JE, Lenski RE: Genome dynamics during experimental evolution. **Nat Rev Genet 14**:827–839, 2013

17. Basu A, Chaturvedi UC: Vascular endothelium: the battlefield of dengue viruses. **FEMS Immunology & Medical …**:2008

18. Bennett SN, Drummond AJ, Kapan DD, Suchard MA, Munoz-Jordan JL, Pybus OG, et al: Epidemic dynamics revealed in dengue evolution. **Mol Biol Evol 27**:811–818, 2010

19. Bennett SN: Evolutionary Dynamics of Dengue Virus. **Frontiers in Dengue Virus Research**:157–172, 2010

20. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, Vorndam V, et al: Molecular evolution of dengue 2 virus in Puerto Rico: positive selection in the viral envelope accompanies clade reintroduction. **J Gen Virol 87**:885–893, 2006

21. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, Vorndam V, et al: Selection-driven evolution of emergent dengue virus. **Mol Biol Evol 20**:1650–1658, 2003

22. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al: The global distribution and burden of dengue. **Nature 496**:504–507, 2013

23. Blaney JE, Hanson CT, Hanley KA, Murphy BR, Whitehead SS: Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2. **BMC Infect Dis 4**:39, 2004

24. Brathwaite Dick O, San Martín JL, Montoya RH, del Diego J, Zambrano B, Dayan GH: The history of dengue outbreaks in the Americas. **Am J Trop Med Hyg 87**:584–593, 2012

25. Bravo JR, Guzman MG, Kouri GP: Why dengue haemorrhagic fever in Cuba? 1. Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). **Trans R Soc Trop Med Hyg 81**:816–820, 1987

26. Bray M, Lai CJ: Dengue virus premembrane and membrane proteins elicit a protective immune response. **Virology 185**:505–508, 1991

27. Burke DS, Nisalak A, Johnson DE, Scott RM: A prospective study of dengue infections in Bangkok. **Am J Trop Med Hyg 38**:172–180, 1988

28. Cahour A, Pletnev A, Vazielle-Falcoz M, Rosen L, Lai CJ: Growth-restricted dengue virus mutants containing deletions in the 5' noncoding region of the RNA genome. **Virology 207**:68–76, 1995

29. Capeding MR, Tran PNH, Hadinegoro PSRS, Ismail HIHM, Chotpitayasunondh T, Chua MN, et al: Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. **Lancet 384**:1358–1365, 2014

30. Carey DE: Chikungunya and dengue: a case of mistaken identity? **Journal of the history of medicine and allied sciences 26**:243–262, 1971

31. Carrington LB, Simmons CP: Human to mosquito transmission of dengue viruses. **Front Immunol 5**:290, 2014

32. Castanha PM, Nascimento EJM, Cynthia B, Cordeiro MT, de Carvalho OV, de Mendonça LR, et al: Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection. **J Infect Dis**:2016

33. Chamberlain RW, Sudia WD: Mechanism of transmission of viruses by mosquitoes. **Annu Rev Entomol 6**:371–390, 1961

34. Chan KWK, Watanabe S, Kavishna R, Alonso S, Vasudevan SG: Animal models for studying dengue pathogenesis and therapy. **123**:5–14, 2015

35. Chan M, Johansson MA: The Incubation Periods of Dengue Viruses. **PLoS ONE 7**:e50972, 2012

36. Chang GJ: Molecular biology of dengue viruses. **In: Gubler, D & Kuno, G; Dengue and Dengue Hemorrhagic Fever**:1997

37. Chastel C: Eventual role of asymptomatic cases of dengue for the introduction and spread of dengue viruses in non-endemic regions. **Front Physiol 3**:70, 2012

38. Chen H-L, Lin S-R, Liu H-F, King C-C, Hsieh S-C, Wang W-K: Evolution of dengue virus type 2 during two consecutive outbreaks with an increase in severity in southern Taiwan in 2001-2002. **Am J Trop Med Hyg 79**:495–505, 2008

39. Chien L-J, Liao T-L, Shu P-Y, Huang J-H, Gubler DJ, Chang G-JJ: Development of real-time reverse transcriptase PCR assays to detect and serotype dengue viruses. **J Clin Microbiol 44**:1295–1304, 2006

40. Choy MM, Ellis BR, Ellis EM, Gubler DJ: Comparison of the mosquito inoculation technique and quantitative real time polymerase chain reaction to measure dengue virus concentration. **Am J Trop Med Hyg 89**:1001–1005, 2013

41. Chungue E, Laudon, Glaziou P: Dengue and Dengue Haemorrhagic Fever in French Polynesia - Current Situation. **Tropical Medicine 35**:209–215, 1993

42. Cleland J, Bradley B, Macdonald W: Dengue fever in Australia: its history and clinical course, its experimental transmission by Stegomyia fasciata, and the results of inoculation and other experiments. **Journal of hygiene 16**:317–419, 1918

43. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet F, Weaver SC: Arbovirus evolution in vivo is constrained by host alternation. **Proceedings of the National Academy of Sciences 105**:6970–6975, 2008

44. Cologna R, Armstrong P, Rico-Hesse R: Selection for virulent dengue viruses occurs in humans and mosquitoes. **J Virol 79**:853–859, 2005

45. Cologna R, Rico-Hesse R: American genotype structures decrease dengue virus output from human monocytes and dendritic cells. **J Virol 77**:3929–3938, 2003

46. Colton L, Biggerstaff BJ, Johnson A, Nasci RS: Quantification of West Nile virus in vector mosquito saliva. **J Am Mosq Control Assoc 21**:49–53, 2005

47. Commission SP: Recent historical perspectives of dengue in the South Pacific. **The South Pacific Commission Dengue Newsletter 1**:1–18, 1974 Available: message:%3CB8D04C08-E3E9-41EE-9076-2E58375C4E1D@hawaii.edu%3E.

48. Cummings DAT, Iamsirithaworn S, Lessler JT, McDermott A, Prasanthong R, Nisalak A, et al: The impact of the demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical modeling. **PLoS Med 6**:e1000139, 2009

49. de Lamballerie X, Crochu S, Billoir F, Neyts J, de Micco P, Holmes EC, et al: Genome sequence analysis of Tamana bat virus and its relationship with the genus Flavivirus. **Journal of General Virology 83**:2443–2454, 2002

50. Dejnirattisai W, Supasa P, Wongwiwat W, Rouvinski A, Barba-Spaeth G, Duangchinda T, et al: Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. **Nat Immunol 17**:1102–1108, 2016

51. Diallo M, Sall AA, Moncayo AC, Ba Y, Fernandez Z, Ortiz D, et al: Potential role of sylvatic and domestic African mosquito species in dengue emergence. **Am J Trop Med Hyg 73**:445–449, 2005

52. Diamond MS: Evasion of innate and adaptive immunity by flaviviruses. **Immunol Cell Biol 81**:196–206, 2003

53. Diamond MS, Pierson TC: Molecular Insight into Dengue Virus Pathogenesis and Its Implications for Disease Control. **Cell 162**:488–492, 2015

54. Dokland T, Walsh M, Mackenzie JM, Khromykh AA, Ee K-H, Wang S: West Nile virus core protein; tetramer structure and ribbon formation. **Structure 12**:1157–1163, 2004

55. Domingo E: Virus evolution. **Fields Virology, 5th ed**:2007

56. Drake JW, Charlesworth B, Charlesworth D, Crow JF: Rates of spontaneous mutation. **Genetics 148**:1667–1686, 1998

57. Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. **BMC Evol Biol 7**:214, 2007

58. Duarte E, Clarke D, Moya A, Domingo E, Holland J: Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. **Proceedings of the National Academy of Sciences 89**:6015–6019, 1992

59. Dupont-Rouzeyrol M, O'Connor O, Calvez E, Daurès M, John M, Grangeon J-P, et al: Co-infection with Zika and Dengue Viruses in 2 Patients, New Caledonia, 2014. **Emerging Infect Dis 21**:381–382, 2015

60. East S: World's first dengue fever vaccine launched in the Philippines. **editioncnncom**:2016 Available: http://edition.cnn.com/2016/04/06/health/dengue-fever-vaccine-philippines/. Accessed 21 February 2017

61. Estallo E, Lamfri M, Scavuzzo C, Almeida F: Models for predicting Aedes aegypti larval indices based on satellite images and climatic variables. **J Am Mosq Control Assoc 24**:368–376, 2008

62. Esteva L, Vargas C: Coexistence of different serotypes of dengue virus. **J Math Biol 46**:31–47, 2003

63. Farrar JJ, Hien TT, Horstick O, Hung NT, Jaenisch T, Junghanns T, et al: Dogma in Classifying Dengue Disease. **Am J Trop Med Hyg 89**:198–201, 2013

64. Ferguson N, Anderson R, Gupta S: The effect of antibody-dependent enhancement on the transmission dynamics and persistence of multiple-strain pathogens. **Proceedings of the National Academy of Sciences 96**:790–794, 1999 Available: http://www.pnas.org/cgi/doi/10.1073/pnas.96.2.790.

65. Flasche S, Jit M, Rodriguez-Barraquer I: Comparative modelling of dengue vaccine public health impact (CMDVI). **World Health …**:2016

66. Franz AWE, Kantor AM, Passarelli AL, Clem RJ: Tissue Barriers to Arbovirus Infection in Mosquitoes. **Viruses 7**:3741–3767, 2015

67. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van den Hurk A, et al: Limited dengue virus replication in field-collected Aedes aegypti mosquitoes infected with Wolbachia. **PLoS Negl Trop Dis 8**:e2688, 2014

68. Fulmali PV, Walimbe A, Mahadev PVM: Spread, establishment & prevalence of dengue vector Aedes aegypti (L.) in Konkan region, Maharashtra, India. **127**:589–601, 2008

69. G P Kouri MGGJRBCT: Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. **Bull World Health Organ 67**:375, 1989

70. Gaunt M, Sall A, de Lamballerie X, Falconar A, Dzhivanian T, Gould EA: Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. **J Gen Virol 82**:1867–1876, 2001

71. Getis A, Morrison A, GRAY K, Scott TW: Characteristics of the spatial pattern of the dengue vector, Aedes aegypti, in Iquitos, Peru. **Am J Trop Med Hyg 69**:494–505, 2003

72. Goldman N, Yang Z: A codon-based model of nucleotide substitution for protein-coding DNA sequences. **Mol Biol Evol 11**:725–736, 1994

73. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai C-J: Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. **Proceedings of the National Academy of Sciences 104**:9422–9427, 2007

74. Graur D, Li W-H: Fundamentals of Molecular Evolution, 2nd Ed. **Sinauer Associates**:2000

75. Green S, Rothman AL: Immunopathological mechanisms in dengue and dengue hemorrhagic fever. **Curr Opin Infect Dis 19**:429–436, 2006

76. Gubler DJ: Cities spawn epidemic dengue viruses. **Nat Med 10**:129–130, 2004

77. Gubler DJ: Dengue and dengue hemorrhagic fever: its history and resurgence as a public health problem, in Gubler DJ, Kuno G(eds): **Dengue and Dengue Hemorrhagic Fever, ed 2.** CAB international, 1997

78. Gubler DJ: Dengue and dengue hemorrhagic fever. **Clin Microbiol Rev 11**:480–496, 1998

79. Gubler DJ: Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. **Trends Microbiol 10**:100–103, 2002

80. Gubler DJ: The global emergence/resurgence of arboviral diseases as public health problems. **Arch Med Res 33**:330–342, 2002

81. Gubler DJ, Clark GG: Dengue/dengue hemorrhagic fever: the emergence of a global health problem. **Emerging Infect Dis 1**:55–57, 1995

82. Gubler DJ, Kuberski T, Rosen L: 30 dengue cases in American Samoa in 1972. 2009

83. Gubler DJ, Kuno G, Markoff L: **Flaviviruses**. 2007, pp 1153–1252

84. Gubler DJ, Ooi EE, Vasudevan S, Farrar J: **Dengue and Dengue Hemorrhagic Fever, 2nd Edition**. ed 1, CABI, 1997

85. Gubler DJ, Reed D, Rosen L, Hitchcock JR: Epidemiologic, clinical, and virologic observations on dengue in the Kingdom of Tonga. **Am J Trop Med Hyg 27**:581–589, 1978

86. Gubler DJ, Rosen L: A simple technique for demonstrating transmission of dengue virus by mosquitoes without the use of vertebrate hosts. **Am J Trop Med Hyg 25**:146–150, 1976

87. Gubler DJ, Suharyono W, Lubis I, Eram S, Gunarso S: Epidemic dengue 3 in central Java, associated with low viremia in man. **Am J Trop Med Hyg 30**:1094–1099, 1981

88. Guha-Sapir D, Schimmer B: Dengue fever: new paradigms for a changing epidemiology. **Emerg Themes Epidemiol 2**:1, 2005

89. Guy B, (null), Barrere B, Malinowski C, Saville M, Teyssou R, et al: From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. **Vaccine 29**:7229–7241, 2011

90. Guzman MG, Harris E: Dengue. **Lancet 385**:453–465, 2015

91. Guzman MG, Kourí G: Dengue haemorrhagic fever integral hypothesis: confirming observations, 1987-2007. **Trans R Soc Trop Med Hyg 102**:522–523, 2008

92. Halstead SB: Dengue haemorrhagic fever--a public health problem and a field for research. **Bull World Health Organ 58**:1–21, 1980

93. Halstead SB: Immunological Parameters of Togavirus Disease Syndromes, in Schlesinger RW(ed): **The Togaviruses: Biology, Structure, Replication, ed 1.** New York: Academic Press, 1980

94. Halstead SB, Nimmannitya S, Cohen SN: Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. **Yale J Biol Med 42**:311–328, 1970

95. Halstead SB, Simasthien P: Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. **Yale J Biol Med 42**:276–292, 1970

96. Halstead S: Epidemiology of dengue and dengue hemorrhagic fever, in Gubler DJ, Kuno G(eds): **Dengue and Dengue Hemorrhagic Fever, ed 2.** CAB international, 1997

97. Halstead SB: Dengue: the syndromic basis to pathogenesis research. Inutility of the 2009 WHO case definition. **Am J Trop Med Hyg 88**:212–215, 2013

98. Halstead SB: Dengue. **Lancet 370**:1644–1652, 2007

99. Halstead SB: Neutralization and antibody-dependent enhancement of dengue viruses. **Adv Virus Res 60**:421–467, 2003

100. Halstead SB: Pathogenesis of dengue: challenges to molecular biology. **Science 239**:476–481, 1988

101. Halstead SB, Diwan AR, Marchette NJ, Palumbo NE, Srisukonth L: Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. I. Attributes of uncloned virus at different passage levels. **Am J Trop Med Hyg 33**:654–665, 1984

102. Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM: Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. **Lancet Infect Dis 10**:712–722, 2010

103. Halstead SB, Udomsakdi S, Singharaj P, Nisalak A: Dengue and Chikungunya Virus Infection in Man in Thailand, 1962-1964: III. Clinical, epidemiologic, and virologic observations on disease in non-indigenous white persons. **Am J Trop Med Hyg**:1969

104. Hammon WM, Rudnick A, Sather GE: Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. **Science 131**:1102–1103, 1960

105. Heinz FX, Allison SL: Structures and mechanisms in flavivirus fusion. **Adv Virus Res 55**:231–269, 2000

106. Holmes EC: Patterns of intra- and interhost nonsynonymous variation reveal strong purifying selection in dengue virus. **J Virol 77**:11296–11298, 2003

107. Holmes EC, Burch SS: The causes and consequences of genetic variation in dengue virus. **Trends Microbiol 8**:74–77, 2000

108. Holmes EC, Twiddy S: The origin, emergence and evolutionary genetics of dengue virus. **Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 3**:19–28, 2003

109. Horstick O, Jaenisch T, Martínez E, Kroeger A, See LLC, Farrar J, et al: Comparing the Usefulness of the 1997 and 2009 WHO Dengue Case Classification: A Systematic Literature Review. **Am J Trop Med Hyg 91**:621–634, 2014

110. Hotta: Twenty Years of Laboratory Experience with Dengue Virus. **Applied Virology**:228–265, 1965

111. Houng HH, Hritz D, Kanesa-thasan N: Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3'-noncoding sequence. **J Virol Methods 86**:1–11, 2000

112. Houng HS, Chung-Ming Chen R, Vaughn DW, Kanesa-thasan N: Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1-4 using conserved and serotype-specific 3' noncoding sequences. **J Virol Methods 95**:19–32, 2001

113. Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP: Bayesian inference of phylogeny and its impact on evolutionary biology. **Science 294**:2310–2314, 2001

114. Jarman RG, Holmes EC, Rodpradit P, Klungthong C, Gibbons RV, Nisalak A, et al: Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. **J Virol 82**:5494–5500, 2008

115. Jenkins GM, Pagel M, Gould EA, de A Zanotto PM, Holmes EC: Evolution of base composition and codon usage bias in the genus Flavivirus. **J Mol Evol 52**:383–390, 2001

116. Jetten T, Focks D: Potential changes in the distribution of dengue transmission under climate warming. **Am J Trop Med Hyg 57**:285–297, 1997

117. Johnson BW, Russell BJ, Lanciotti RS: Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. **J Clin Microbiol 43**:4977–4983, 2005

118. Junjhon J, Edwards TJ, Utaipat U, Bowman VD, Holdaway HA, Zhang W, et al: Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. **J Virol 84**:8353–8358, 2010

119. Kalayanarooj S, Vaughn DW, Nimmannitya S, Green S, Innis BL, Rothman AL, et al: Early clinical and laboratory indicators of acute dengue illness. **J Infect Dis 176**:313–321, 1997

120. Katzelnick LC, Fonville JM, Gromowski GD, Bustos Arriaga J, Green A, James SL, et al: Dengue viruses cluster antigenically but not as discrete serotypes. **Science 349**:1338–1343, 2015

121. Kaushik A, Pineda C, Kest H: Diagnosis and management of dengue fever in children. **Pediatr Rev 31**:e28–35, 2010

122. Kawiecki AB, Christofferson RC: Zika Virus-Induced Antibody Response Enhances Dengue Virus Serotype 2 Replication In Vitro. **J Infect Dis 214**:1357–1360, 2016

123. Khromykh AA, Meka H, Guyatt KJ, Westaway EG: Essential role of cyclization sequences in flavivirus RNA replication. **J Virol 75**:6719–6728, 2001

124. Khromykh AA, Varnavski AN, Sedlak PL, Westaway EG: Coupling between replication and packaging of flavivirus RNA: evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. **J Virol 75**:4633–4640, 2001

125. Klasse PJ: Molecular determinants of the ratio of inert to infectious virus particles. **Prog Mol Biol Transl Sci 129**:285–326, 2015

126. Konishi E, Kuno G: **In Memoriam: Susumu Hotta (1918-2011).** 2013, pp 843–844

127. Kouri GP, Guzmán MG, Bravo JR: Why dengue haemorrhagic fever in Cuba? 2. An integral analysis. **Trans R Soc Trop Med Hyg**:1987

128. Kuberski TT, Rosen L: A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. **Am J Trop Med Hyg 26**:533–537, 1977

129. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB: Phylogeny of the genus Flavivirus. **J Virol 72**:73–83, 1998

130. Kuno G: Research on dengue and dengue-like illness in East Asia and the Western Pacific during the First Half of the 20th century. **Rev Med Virol 17**:327–341, 2007

131. Kümmerer BM, Rice CM: Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. **J Virol 76**:4773–4784, 2002

132. Lambrechts L, Scott TW, Gubler DJ: Consequences of the Expanding Global Distribution of Aedes albopictus for Dengue Virus Transmission. **PLoS Negl Trop Dis 4**:1–9, 2010

133. Lanciotti RS, Lewis JG, Gubler DJ, Trent DW: Molecular evolution and epidemiology of dengue-3 viruses. **J Gen Virol 75 (Pt 1)**:65–75, 1994

134. Laoprasopwattana K, Libraty DH, Endy TP, Nisalak A, Chunsuttiwat S, Vaughn DW, et al: Dengue Virus (DV) enhancing antibody activity in preillness plasma does not predict subsequent disease severity or viremia in secondary DV infection. **J Infect Dis 192**:510–519, 2005

135. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, de Chacon, et al: Dengue virus structural differences that correlate with pathogenesis. **J Virol 73**:4738–4747, 1999

136. Lequime S, Fontaine A, Ar Gouilh M, Moltini-Conclois I, Lambrechts L: Genetic Drift, Purifying Selection and Vector Genotype Shape Dengue Virus Intra-host Genetic Diversity in Mosquitoes. **PLoS Genet 12**:e1006111, 2016

137. Libraty DH, Acosta L, Tallo V, Segubre-Mercado E, Bautista A, Potts J, et al: A Prospective Nested Case-Control Study of Dengue in Infants: Rethinking and Refining the Antibody-Dependent Enhancement Dengue Hemorrhagic Fever Model. **PLoS Med 6**:e1000171, 2009

138. Libraty DH, Endy TP, Houng H-SH, Green S, Kalayanarooj S, Suntayakorn S, et al: Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. **J Infect Dis 185**:1213–1221, 2002

139. Lindenbach BD, Rice CM: Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. **J Virol 73**:4611–4621, 1999

140. Lindenbach BD, Thiel H-J, Rice CM: **Flaviviridae: the Viruses and Their Replication**. 2007, pp 1101–1152

141. Liu WJ, Chen HB, Wang XJ, Huang H, Khromykh AA: Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. **J Virol 78**:12225–12235, 2004

142. Liu WJ, Wang XJ, Clark DC, Lobigs M, Hall RA, Khromykh AA: A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. **J Virol 80**:2396–2404, 2006

143. Loison G, Rosen L, Papillaud J, Tomasini J, Vaujany J, Chanalet G: La dengue en Nouvelle-Caledonie (1971-1972). **Bulletin de la Societe de Pathologie Exotique 66**:511–519, 1973

144. Lorenz IC, Allison SL, Heinz FX, Helenius A: Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. **J Virol 76**:5480–5491, 2002

145. Low JG, Sung C, Wijaya L, Wei Y, Rathore APS, Watanabe S, et al: Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. **Lancet Infect Dis 14**:706–715, 2014

146. Maciel-de-Freitas R, Peres R, Souza-Santos R, Lourenco-de-Oliveira R: Occurrence, productivity and spatial distribution of key-premises in two dengue-endemic areas of Rio de Janeiro and their role in adult Aedes aegypti spatial infestation pattern. **Trop Med Int Health 13**:1–7, 2008

147. Mackenzie JM, Khromykh AA, Jones MK, Westaway EG: Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. **Virology 245**:203–215, 1998

148. MacKenzie JS, Gubler DJ, Petersen LR: Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. **Nat Med 10**:S98–109, 2004

149. Maddison DR, Maddison WP: MacClade 4: Analysis of phylogeny and character evolution.

Version 4.06. **Sinauer Associates**:2003

150. Maguire T, Miles JA, Macnamara FN, Wilkinson PJ, Austin FJ, Mataika JU: Mosquito-borne infections in Fiji. V. The 1971-73 dengue epidemic. **Journal of hygiene 73**:263–270, 1974

151. Mangada MM, Igarashi A: Molecular and in vitro analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities. **Virology 244**:458–466, 1998

152. Markoff L: 5“- and 3-”noncoding regions in flavivirus RNA. **Adv Virus Res 59**:177–228, 2003

153. Martina BEE, Koraka P, Osterhaus ADME: Dengue virus pathogenesis: an integrated view. **Clin Microbiol Rev 22**:564–581, 2009

154. McArthur MA, Sztein MB, Edelman R: Dengue vaccines: recent developments, ongoing challenges and current candidates. **Expert Rev Vaccines 12**:933–953, 2013

155. Medina F, Medina JF, Colón C, Vergne E, Santiago GA, Munoz-Jordan JL: Dengue virus: isolation, propagation, quantification, and storage. **Curr Protoc Microbiol Chapter 15**:15D.2.1–15D.2.24, 2012

156. Miller S, Kastner S, Krijnse-Locker J, Bühler S, Bartenschlager R: The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner. **J Biol Chem 282**:8873–8882, 2007

157. Monath TP: Dengue: the risk to developed and developing countries. **Proceedings of the National Academy of Sciences 91**:2395–2400, 1994

158. Moreau J, Rosen L, Saugrain J, Lagraulet J: An epidemic of dengue on Tahiti associated with hemorrhagic manifestations. **Am J Trop Med Hyg 22**:237–241, 1973

159. Morens DM: Antibody-dependent enhancement of infection and the pathogenesis of viral disease. **Clin Infect Dis 19**:500–512, 1994

160. Morens DM, Halstead SB: Disease severity‐related antigenic differences in dengue 2 strains detected by dengue 4 monoclonal antibodies. **J Med Virol 22**:169–174, 1987

161. Mukhopadhyay S, Kuhn RJ, Rossmann MG: A structural perspective of the flavivirus life cycle. **Nat Rev Micro 3**:13–22, 2005

162. Munoz-Jordan JL, Sánchez-Burgos GG, Laurent-Rolle M, García-Sastre A: Inhibition of interferon signaling by dengue virus. **Proceedings of the National Academy of Sciences 100**:14333–14338, 2003

163. Murgue B, Roche C, Chungue E, Deparis X: Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996-1997 dengue-2 outbreak in French Polynesia. **J Med Virol 60**:432–438, 2000

164. Murray NEA, Quam MB, Wilder-Smith A: Epidemiology of dengue: past, present and future prospects. **CLEP 5**:299–309, 2013

165. Mustafa MS, Rasotgi V, Jain S, Gupta V: Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. **Med J Armed Forces India 71**:67–70, 2015

166. Nelson DL, Cox MM: Lehninger Principles of Biochemistry, 3rd Ed. **Worth Publishers**:2000

167. Nguyen NM, Duong THK, Trung VT, Nguyen THQ, Tran CNB, Long VT, et al: Host and viral features of human dengue cases shape the population of infected and infectious Aedes aegypti mosquitoes. **Proceedings of the National Academy of Sciences 110**:9072–9077, 2013

168. Nisalak A, Halstead SB, Singharaj P, Udomsakdi S, Nye SW, Vinijchaikul K: Observations related to pathogenesis of dengue hemorrhagic fever. 3. Virologic studies of fatal disease. **Yale J Biol Med 42**:293–310, 1970

169. Normile D: Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. **Science 342**:415, 2013

170. OhAinle M, Balmaseda A, Macalalad AR, Tellez Y, Zody MC, Saborio S, et al: Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. **Sci Transl Med 3**:114ra128, 2011

171. Oishi K, Saito M, Mapua CA, Natividad FF: Dengue illness: clinical features and pathogenesis. **J Infect Chemother 13**:125–133, 2007

172. Ooi EE, Goh K-T, Gubler DJ: Dengue prevention and 35 years of vector control in Singapore. **Emerging Infect Dis 12**:887–893, 2006

173. Papa A, Karabaxoglou D, Kansouzidou A: Acute West Nile virus neuroinvasive infections: cross-reactivity with dengue virus and tick-borne encephalitis virus. **J Med Virol 83**:1861–1865, 2011

174. Paul LM, Carlin ER, Jenkins MM, Tan AL, Barcellona CM, Nicholson CO, et al: Dengue virus antibodies enhance Zika virus infection. **Clin Transl Immunology 5**:e117, 2016

175. Ponlawat A, Harrington L: Blood Feeding Patterns of Aedes aegypti and Aedes albopictus in Thailand. **J Med Entomol 42**:844–849, 2005

176. Pybus OG, Rambaut A, Holmes EC, Harvey PH: New inferences from tree shape: numbers of missing taxa and population growth rates. **Systematic Biology 51**:881–888, 2002

177. Reed L, Muench H: A simple method of estimating fifty per cent endpoints. **Am J Epidemiol 27**:493–497, 1938

178. Reiter P, Gubler DJ: Surveillance and control of urban dengue vectors, in Gubler DJ, Kuno G(eds): **Dengue and Dengue Hemorrhagic Fever.** CAB international, 1997

179. Richardson J, Molina-Cruz A, Salazar MI, Black WC: Quantitative analysis of dengue-2 virus RNA during the extrinsic incubation period in individual Aedes aegypti. **Am J Trop Med Hyg 74**:132–141, 2006

180. Rico-Hesse R: Microevolution and virulence of dengue viruses. **Adv Virus Res 59**:315–341, 2003

181. Rico-Hesse R: Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. **Virology 174**:479–493, 1990

182. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, et al: Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. **Virology 230**:244–251, 1997

183. Rigau-Pérez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV: Dengue and dengue haemorrhagic fever. **Lancet 352**:971–977, 1998

184. Rodenhuis-Zybert IA, van der Schaar HM, da Silva Voorham JM, van der Ende-Metselaar H, Lei H-Y, Wilschut J, et al: Immature Dengue Virus: A Veiled Pathogen? **PLoS Pathog 6**:e1000718, 2010 Available: http://www.plospathogens.org/article/info%3Adoi%2F10.1371%2Fjournal.ppat.1000718.

185. Rodhain F: The role of monkeys in the biology of dengue and yellow fever. **Comp Immunol Microb 14**:9–19, 1991

186. Rodhain F, Rosen L: Mosquito vectors and dengue virus-vector relationships. **In: Gubler, D & Kuno, G; Dengue and Dengue Hemorrhagic Fever**:1997

187. Ronquist F, Huelsenbeck JP: MrBayes 3: Bayesian phylogenetic inference under mixed models. **Bioinformatics 19**:1572–1574, 2003

188. Rosen L: The Emperor's New Clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. **Am J Trop Med Hyg 26**:337–343, 1977

189. Rosen L, Gubler DJ: The use of mosquitoes to detect and propagate dengue viruses. **Am J Trop Med Hyg 23**:1153–1160, 1974

190. Rothman AL: Viral pathogenesis of dengue infections, in Gubler DJ, Kuno G(eds): **Dengue and Dengue Hemorrhagic Fever.** CAB international, 1997

191. Rothman AL: Dengue: defining protective versus pathologic immunity. **J Clin Invest 113**:946–951, 2004

192. Rothman AL, Ennis FA: Dengue Vaccine: The Need, the Challenges, and Progress. **J Infect Dis 214**:825–827, 2016

193. Rudnick A: Studies of the ecology of dengue in Malaysia: a preliminary report. **J Med Entomol 2**:203–208, 1965

194. Rudnick A: Dengue fever epidemiology in Malaysia 1901–1980. 1986

195. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, et al: Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. **The Lancet 380**:1559–1567, 2012

196. Sabin A: Research on dengue during World War II. **Am J Trop Med Hyg 1**:30–50, 1952

197. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, et al: Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. **Am J Epidemiol 120**:653–669, 1984

198. Schliessmann DJ, Calheiros LB: **Review of the Status of Yellow Fever and Aedes Aegypti Eradication Programs in the Americas**. Mosquito News, 1974

199. Scott RM, Eckels KH, Bancroft WH, Summers PL, McCown JM, Anderson JH, et al: Dengue 2 vaccine: dose response in volunteers in relation to yellow fever immune status. **J Infect Dis 148**:1055–1060, 1983

200. Scott RM, Nimmannitya S, Bancroft WH, Mansuwan P: Shock syndrome in primary dengue infections. **Am J Trop Med Hyg 25**:866–874, 1976

201. Sharp TM, Hunsperger E, Munoz-Jordan JL, Margolis HS, Tomashek KM: Sequential episodes of dengue--Puerto Rico, 2005-2010. **Am J Trop Med Hyg 91**:235–239, 2014

202. Shepard DS, Undurraga EA, Halasa YA: Economic and disease burden of dengue in Southeast Asia. **PLoS Negl Trop Dis 7**:e2055, 2013

203. Sierra B de LC, (null), Kourí G, Guzman MG: Race: a risk factor for dengue hemorrhagic fever. **Arch Virol 152**:533–542, 2007

204. Sierra B, Alegre R, Pérez AB, García G, Sturn-Ramirez K, Obasanjo O, et al: HLA-A, -B, -C, and -DRB1 allele frequencies in Cuban individuals with antecedents of dengue 2 disease: advantages of the Cuban population for HLA studies of dengue virus infection. **Hum Immunol 68**:531–540, 2007

205. Siler JF, Hall MW, Hitchens A: Dengue, its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity and prevention. **Philippine Journal of Science 29**:1–304, 1926

206. Sim S, Aw PPK, Wilm A, Teoh G, Hue KDT, Nguyen NM, et al: Tracking Dengue Virus Intra-host Genetic Diversity during Human-to-Mosquito Transmission. **PLoS Negl Trop Dis 9**:e0004052, 2015

207. Simmons CP, Farrar JJ, Nguyen VVC, Wills B: Dengue. **N Engl J Med 366**:1423–1432, 2012

208. Simmons JS, John JHS, Reynolds FHK: Experimental studies of dengue. **Philippine Journal of Science 44**:1–251, 1931

209. Sinha G: Sanofi's dengue vaccine first to complete phase 3. **Nature Biotechnology 32**:605–606, 2014

210. Smith C: The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. **The Journal of tropical medicine and hygiene 59**:243–251, 1956

211. Srikiatkhachorn A, Rothman AL, Gibbons RV, Sittisombut N, Malasit P, Ennis FA, et al: Dengue--How Best to Classify It. **Clin Infect Dis 53**:563–567, 2011

212. Stamatakis A, Hoover P, Rougemont J: A rapid bootstrap algorithm for the RAxML Web servers. **Systematic Biology 57**:758–771, 2008

213. Steel A, Gubler DJ, Bennett SN: Natural attenuation of dengue virus type-2 after a series of island outbreaks: a retrospective phylogenetic study of events in the South Pacific three decades ago. **Virology 405**:505–512, 2010

214. Suwonkerd W, Mongkalangoon P, Parbaripai A, Grieco J, Achee N, Roberts D, et al: The effect of host type on movement patterns of Aedes aegypti (Diptera: Culicidae) into and out of experimental huts in Thailand. **J Vector Ecol 31**:311–318, 2006

215. Swofford DL, Sullivan J: Phylogeny inference based on parsimony and other methods using PAUP*. **The Phylogenetic Handbook: A Practical Approach to DNA and …**:2003

216. Syenina A, Jagaraj CJ, Aman SA, Sridharan A, John ALS, Medzhitov R: Dengue vascular leakage is augmented by mast cell degranulation mediated by immunoglobulin Fcγ receptors. **eLife Sciences 4**:e05291, 2015

217. Teixeira MDG, (null), Barreto ML, Costa MDCN, Ferreira LDA, Vasconcelos PFC, et al: Dynamics of dengue virus circulation: a silent epidemic in a complex urban area. **Trop Med Int Health 7**:757–762, 2002

218. Thai KTD, Phuong HL, Thanh Nga TT, Giao PT, Hung LQ, Van Nam N, et al: Clinical, epidemiological and virological features of Dengue virus infections in Vietnamese patients presenting to primary care facilities with acute undifferentiated fever. **J Infect 60**:229–237, 2010

219. Tolou HJ, Couissinier-Paris P, Durand JP, Mercier V, de Pina JJ, de Micco P, et al: Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences. **J Gen Virol 82**:1283–1290, 2001

220. Trent DW, Grant JA, Rosen L, Monath TP: Genetic variation among dengue 2 viruses of different geographic origin. **Virology 128**:271–284, 1983

221. Tricou V, Minh NN, Farrar J, Tran HT, Simmons CP: Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with dengue. **PLoS Negl Trop Dis 5**:e1309, 2011

222. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S: A single mutation in chikungunya virus affects vector specificity and epidemic potential. **PLoS Pathog 3**:e201, 2007

223. Tun-Lin W, Kay BH, Barnes A: Understanding productivity, a key to Aedes aegypti surveillance. **Am J Trop Med Hyg 53**:595–601, 1995

224. Turtle L, Bali T, Buxton G, Chib S, Chan S, Soni M, et al: Human T cell responses to Japanese encephalitis virus in health and disease. **J Exp Med 213**:1331–1352, 2016

225. Twiddy SS, Farrar JJ, Vinh Chau N, Wills B, Gould EA, Gritsun T, et al: Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. **Virology 298**:63–72, 2002

226. Twiddy SS, Holmes EC: The extent of homologous recombination in members of the genus Flavivirus. **J Gen Virol 84**:429–440, 2003

227. Twiddy SS, Holmes EC, Rambaut A: Inferring the rate and time-scale of dengue virus evolution. **Mol Biol Evol 20**:122–129, 2003

228. Twiddy SS, Woelk CH, Holmes EC: Phylogenetic evidence for adaptive evolution of dengue viruses in nature. **J Gen Virol 83**:1679–1689, 2002

229. Umareddy I, Tang KF, (null), (null), Vasudevan SG, (null), et al: Dengue virus regulates type I interferon signalling in a strain-dependent manner in human cell lines. **J Gen Virol 89**:3052–3062, 2008

230. Uzcategui NY, Camacho D, Comach G, Cuello de Uzcategui R, Holmes EC, Gould EA: Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. **J Gen Virol 82**:2945–2953, 2001

231. van der Schaar HM, Rust MJ, Waarts B-L, van der Ende-Metselaar H, Kuhn RJ, Wilschut J, et al: Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. **J Virol 81**:12019–12028, 2007

232. Vasilakis N, Cardosa J, Hanley KA, Holmes EC, Weaver SC: Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. **Nat Rev Micro 9**:532–541, 2011

233. Vasilakis N, Deardorff ER, Kenney JL, Rossi SL, Hanley KA, Weaver SC: Mosquitoes put the brake on arbovirus evolution: experimental evolution reveals slower mutation accumulation in mosquito than vertebrate cells. **PLoS Pathog 5**:e1000467, 2009

234. Vasilakis N, Shell EJ, Fokam EB, Mason PW, Hanley KA, Estes DM, et al: Potential of ancestral sylvatic dengue-2 viruses to re-emerge. **Virology 358**:402–412, 2007

235. Vasilakis N, Weaver SC: The history and evolution of human dengue emergence. **Adv Virus Res 72**:1–76, 2008

236. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al: Dengue in the early febrile phase: viremia and antibody responses. **J Infect Dis 176**:322–330, 1997

237. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al: Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. **J Infect Dis 181**:2–9, 2000

238. Waggoner JJ, Balmaseda A, Gresh L, Sahoo MK, Montoya M, Wang C, et al: Homotypic Dengue Virus Reinfections in Nicaraguan Children. **J Infect Dis 214**:986–993, 2016

239. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al: The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. **Nature 476**:450–453, 2011

240. Wang E, Ni H, Xu R, Barrett ADT, Watowich SJ, Gubler DJ, et al: Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. **J Virol 74**:3227–3234, 2000

241. Wang S, He R, Anderson R: PrM- and cell-binding domains of the dengue virus E protein. **J Virol 73**:2547–2551, 1999

242. Wang W-K, Chao D-Y, Kao C-L, Wu H-C, Liu Y-C, Li C-M, et al: High levels of plasma dengue viral load during defervescence in patients with dengue hemorrhagic fever: implications for pathogenesis. **Virology 305**:330–338, 2003

243. Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A: Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. **Am J Trop Med Hyg 36**:143–152, 1987

244. Watts DM, Porter KR, Putvatana P, Vasquez B, Calampa C, Hayes CG, et al: Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. **Lancet 354**:1431–1434, 1999

245. Weaver SC, Barrett ADT: Transmission cycles, host range, evolution and emergence of arboviral disease. **Nat Rev Micro 2**:789–801, 2004

246. WHO: **Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control.** ed 2, Geneva, Switzerland: WHO, 2007

247. WHO: Dengue vaccine: WHO position paper – July 2016. **Weekly epidemiological record 91**:349–364, 2016

248. WHO: Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. 2009

249. WHO: **Global Strategy for Dengue Prevention and Control 2012-2020**. 2012

250. Wilder-Smith A, Gubler DJ: Geographic expansion of dengue: the impact of international travel. **Med Clin North Am 92**:1377–90– x, 2008

251. Wiwanitkit V: The importance of accurate diagnosis of dengue fever. **Future Virol 7**:53–62, 2012

252. Worobey M, Holmes EC: Evolutionary aspects of recombination in RNA viruses. **J Gen Virol 80 (Pt 10)**:2535–2543, 1999

253. Yauch LE, Shresta S: Mouse models of dengue virus infection and disease. **Antiviral Res 80**:87–93, 2008

254. Zachri E, Planasari S: Dengue Fever Vaccine Available in Indonesia. **entempoco**:2016 Available: https://en.tempo.co/read/news/2016/10/17/310812742/Dengue-Fever-Vaccine-Available-in-Indonesia. Accessed 21 February 2017

255. Zanotto PM, Gould EA, Gao GF, Harvey PH, Holmes EC: Population dynamics of flaviviruses revealed by molecular phylogenies. **Proceedings of the National Academy of Sciences 93**:548–553, 1996

256. Zellweger RM, Prestwood TR, Shresta S: Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. **Cell Host Microbe 7**:128–139, 2010

257. Zhang C, Mammen MP, Chinnawirotpisan P, Klungthong C, Rodpradit P, Nisalak A, et al: Structure and age of genetic diversity of dengue virus type 2 in Thailand. **J Gen Virol 87**:873–883, 2006

258. Zhang Y, Zhang W, Ogata S, Clements D, Strauss JH, Baker TS, et al: Conformational changes of the flavivirus E glycoprotein. **Structure 12**:1607–1618, 2004

259. Zompi S, Harris E: Animal models of dengue virus infection. **Viruses 4**:62–82, 2012