Dengue Virus Evolution and Vertical Transmission in *Wolbachia*-infected *Aedes aegypti* mosquitoes



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Abstract

Aedes aegypti is the primary vector of dengue, which causes an estimated 100 million infections annually. Biocontrol methods, specifically using the endosymbiotic bacterium *Wolbachia* for population replacement of *Wolbachia*-infected mosquitoes and population suppression, have recently emerged as novel strategies for dengue vector control. However, the evolutionary adaptation of DENV under *Wolbachia* pressure within *Ae. aegypti* could potentially compromise the antiviral properties of this bacterium, thereby affecting the long-term efficacy of this approach in dengue prevention. In addition, the influence of *Wolbachia* on the vertical transmission of DENV in wild-type *Aedes aegypti* mosquitoes remains unexplored.

In Chapter 3, I developed a robust whole-genome sequencing assay designed to recover DENV genomes. This assay laid the foundation for the evolutionary exploration detailed in Chapter 4, where I detected and investigated the adaptive variant E203K of DENV-*w*Mel within *Ae. aegypti*. I have shown the fitness trade-off of this variant in the absence of *w*Mel infection in *Ae. aegypti* and in human cells. In Chapter 5, I demonstrated that vertical DENV transmission in field-reared *Ae. aegypti* does occur but is rare, and this vertical transmission rate is reduced significantly in the presence of *w*Mel.

Overall, this thesis contributes to understanding the molecular evolution of DENV in *Wolbachia*-infected *Ae.aegypti,* and the significant inhibitory effect of *Wolbachia* on vertical transmission of DENV. These results have addressed some vital questions in the field of dengue biocontrol and provides insights regarding the long-term benefits of *Wolbachia*-introgression field releases.

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Declaration

I take full responsibility for the content of this thesis, having written every section with the guidance and support of my supervisors, Professor Cameron Simmons and Associate Professor Sophie Yacoub.

I conducted the majority of the laboratory experiments, including homogenization, RT-PCR of mosquitoes, and genome sequencing (with assistance from Vi). However, it is important to acknowledge the significant contributions of the Insectary Team at OUCRU, and Dr. Daniela da Silva Goncalves (for Chapters 5). The insectary team played a crucial role in mosquito preparation, blood feeding, intrathoracic inoculation, and mosquito collection. Dr. Kathryn Edenborough provided valuable support for the evolutionary analysis. Enrolment of patients was carried out by the medical professionals and nurses at the Hospital for Tropical Diseases and the OUCRU.

I want to clarify that this thesis has not been submitted for a degree or any other qualification at this or any other university.

Publications arising from this thesis

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Abbreviations

Ae. aegypti	*Ades aegypti*
Ae. albopictus	*Aedes albopictus*
C	Capsid
CDS	CoDing Sequence
CHIKV	Chikungunya virus
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CPE	Cytopathic Effects
CS	Cyclization sequences
Ct	Cycle threshold
D. melanogaster	*Drosophila melanogaster*
DALYs	Disability-adjusted life years
DAPC	Discriminant Analysis of Principal Components
DB	Dumbbell Structures
DCs	Dendritic cells
DENV	Dengue virus
DENV-1(-2, -3, -4)	Dengue virus serotype 1 (2, 3, 4)
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DI	Domain I
DII	Domain II
DIII	Domain III
DNA	Deoxyribonucleic Acid
DSS	Dengue shock syndrome
E gene	Envelope gene
E protein	Envelope protein
EDTA	Ethylenediaminetetraacetic Acid
EIP	Extrinsic Incubation Period
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine serum

Fw	Forward
GM	Growth Media
HT	Horizontal Transmission
ID	Identifier
IFBS	Inactivate Fetal Bovine serum
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIP	Intrinsic incubation period
IMD	Immune Deficiency Pathway
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription Pathway
kDa	Kilo Dalton
M	Membrane protein
MD	Mosquito Diluent
ML tree	Maximum Likelihood Tree
ML	Maximum Likelihood
ML	Maximum Likelihood
MM	Maintain Medium
MOI	Multiplicity of Infection
NGS	Next Generation Sequencing
NPA	Negative Percent Agreement
NS	Non-structural protein
NS1	Nonstructural protein 1
NS2A	Nonstructural protein 2A
NS2B	Nonstructural protein 2B
NS3	Nonstructural protein 3
NS4A	Nonstructural protein 4A
NS4B	Nonstructural protein 4B
NS5	Nonstructural protein 5
ORF	Open Reading Frame
OxTREC	Oxford Tropical Research Ethics Committee
PAHO	Pan American Health Organization
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline

PCR	Polymerase chain reaction
PP	Pulse pressure
PPA	Positive Percent Agreement
PPA	Positive Percent Agreement
prM	Precursor membrane
PRS	Population Replacement Strategy
RC	Replication Complex
RdRp	RNA-dependent RNA polymerase
rH	relative humidity
ROS	Reactive Oxygen Species
Rps17	Ribosomal Protein S17
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
Rv	Reverse
SLA	Stem-loop A
SNVs	Single Nucleotide Variants
T-25	Plug seal cap flask
TCID50	Median Tissue Culture Infectious Dose
TE	Tris-EDTA buffer
TGN	Trans-Golgi network
UAR	Upstream AUG Regions
UTR	Untranslated regions
Ver	Version
ViPR	Virus Pathogen Database and Analysis Resource
VR	Variable Region
vRNA	virion RNA
VT	Vertical Transmission
WGS	Whole Genome Sequencing
WHO	World Health Organization
wsp	*Wolbachia* surface protein
Wt	Wild type
YF	Yellow fever
YLDs	Years lived with disability
ZIKV	Zika virus

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CHAPTER 1

INTRODUCTION

1.1 Introduction to dengue

In this section, I describe the historical origins, transmission cycle, and global impact of dengue, which has elevated it to a significant public health concern.

1.1.1 History of dengue

Dengue has a long and diverse history, with early reports of illnesses resembling dengue dating back centuries. The earliest recorded evidence of a dengue-like illness can be traced to a Chinese encyclopedia of disease symptoms and remedies, first published during the Chin Dynasty (265 to 420 A.D.) and subsequently edited in 610 A.D. (Tang Dynasty) and 992 A.D. (Northern Sung Dynasty) [1]. In this encyclopedia, the disease was referred to as "water poison" and was associated with flying insects near water sources [2].

Even before the 18th century, outbreaks of illnesses clinically compatible with dengue occurred in various regions. There were reports of potential dengue outbreaks in the French West Indies in 1635 and in Panama in 1966 [3,4]. These early cases demonstrated the wide geographic distribution of dengue or similar illnesses before the first known endemic of a dengue-like illness in the 18th century. In 1779 and 1780, major epidemics of an illness resembling dengue occurred on three continents, namely Asia, Africa, and North America [3,5–7]. The specific locations affected by these epidemics included Batavia (Jakarta), Indonesia, Cairo, Egypt, and Philadelphia in North America. Although it remains uncertain whether the epidemics in Batavia and Cairo were dengue, the outbreak in Philadelphia in 1780 was believed to have been dengue [8].

The limited endemic presence of dengue infection in Africa, relative to the higher

burdens observed in Asia and the Americas, can be elucidated through a multifaceted lens. Firstly, the pervasive focus on combating prevalent health issues, notably malaria and neglected tropical diseases, has directed attention away from *Aedes*-borne arboviruses in the African continent. The magnitude of these diseases has often overshadowed the emergence and impact of dengue. Secondly, challenges in accurate identification of arboviral infections in resource-poor settings in Africa contribute to underreporting, as fevers of unknown origin are frequently recorded as malaria. The lack of precise diagnostic tools and robust surveillance systems further hampers the recognition of major arboviral infections in the region. Thirdly, the intricate dynamics of *Aedes* species in Africa, including *Aedes aegypti (Ae. aegypti)* and *Aedes albopictus*, pose challenges in understanding their distribution and vectorial capacity. While *Aedes aegypti* almost certainly originated in Africa, studies indicate variations in vector competence across populations [9–12]. The presence of sylvatic *Aedes* species [13,14], although less likely to present an immediate threat in urban environments, adds complexity to the transmission dynamics. Fourthly, Africa *Ae. aegypti* in was traditionally considered less competent for flaviviruses than global counterparts [15,16]. However, recent findings reveal highly variable vector competence in African *Ae. aegypti*, dependent on specific mosquito-virus pairings [17–19] and exhibiting genotype-by-genotype specificity, aligning with patterns seen in other host-pathogen systems [20].

Key milestones in understanding dengue occurred during the early 20th century. In 1906, Thomas Lane Bancroft proposed the role of *Aedes* mosquitoes as vectors for dengue virus (DENV) transmission, which was subsequently confirmed in 1907 by Ashburn and Craig, who identified the viral etiology of the disease [21,22]. In 1943, Japanese scientists Ren Kimura and Susumu Hotta identified and isolated the first serotype of the dengue virus (DENV), known as DENV-1 [23], during their investigation of blood samples collected from patients during the 1943 dengue epidemic in Nagasaki, Japan. One year later, Albert B. Sabin and Walter Schlesinger isolated two additional DENV serotypes, named DENV1 and

DENV2, while conducting research on the leading causes of fever among soldiers in Asian and Pacific countries [24,25]. In 1953, two more serotypes, DENV-3 and DENV-4, were discovered in Manila, isolated from pediatric patients suffering from haemorrhagic disease, which exhibited a fatality rate of 10% [26].

1.1.2 Epidemiology of dengue and its impact on affected regions

Dengue poses a significant global public health challenge, characterized by a high burden of infections and considerable morbidity and mortality. However, accurately determining the true incidence of dengue is a complex task due to variations in reporting systems and under-diagnosis. For instance, a systematic analysis unveiled an additional 19.7 million cases excluded from the formal healthcare system, as reported by the Institute for Health Metrics and Evaluation's Global Burden of Disease Study 2013 [27].

Estimates indicate an annual range of 284 to 528 million DENV infections, with 96 million cases being symptomatic and detectable through reporting systems [28]. Southeast Asia exhibited the highest age-standardized incidence rates, with an average of 34.3 cases per 1,000 people, underscoring the large burden of dengue in the region [28]. Notably, reported dengue cases to the World Health Organization (WHO) have risen over eight-fold from 505,430 cases in 2000 to 4.2 million cases in 2019 [29]. According to one modelling estimate, an additional 2.25 (1.27–2.80) billion people will be at risk of dengue by 2080 [30]. Another study indicates 390 million DENV infections per year of which 96 million manifest clinically [31]. The factors associated with the high incidence of dengue in specific regions were investigated, revealing that dengue incidence or outbreaks are associated with several demographic, environmental, social, and ecological factors [32–38].

Dengue-related mortality is also a significant concern. According to The Global Burden of Dengue: an analysis from the Global Burden of Disease Study 2013, an average of

9,221 dengue-related deaths were estimated annually between 1990 and 2013, peaking at 11,302 deaths in 2010 [28]. Southeast Asia experienced the highest mortality rates, highlighting regional disparities in dengue's impact. Additionally, another study based on data from 32 countries estimated a global total of 13,586 dengue deaths in 2013, with 5,838 deaths among children and 7,748 among adults [15]. Findings from a systematic analysis based on the Global Burden of Disease Study 2017 [39] revealed that compared to 1990, dengue-related deaths increased from around 16,957 to 40,467 in 2017. Consistently, the highest death rates were observed in the Southeast Asia in both the 2013 and 2017 studies. In the Americas region, the Pan American Health Organization (PAHO) reported a total of 2,811,433 dengue cases in 2022, with 1,290 deaths (0.046%) [28].

To assess the impact on public health and economies worldwide, disability-adjusted life years (DALYs) and years lived with disability (YLDs) have been employed as fundamental metrics. DALYs provides a comprehensive measure of disease burden by integrating the years of life lost due to premature mortality and the years lived with disability [40,41]. In 2013, dengue resulted in an estimated 1.14 million DALYs globally, with 576,900 years lost to premature mortality and 566,000 years lived with disability [28].

The cost per dengue case varies depending on the disease severity and healthcare setting [27]. In 2013, the total annual global aggregate cost of dengue was estimated at USD 8.9 billion, equivalent to USD 1.56 per capita [27]. This comprehensive estimate encompasses the expenses related to non-fatal cases requiring hospitalization, ambulatory non-fatal cases, non-medical cases, and fatal cases. Dengue imposes a significant economic burden on both American and Southeast Asian countries. Among American countries, the annual economic burden of dengue was estimated at USD 2.1 billion between 2000 and 2007 [42]. For Southeast Asian countries, it amounted to 950 million USD from 2001 to 2010.

Notably, around 52–60% of these expenses resulted from productivity loss, making it a significant proportion of the overall economic burden [43].

In 2012, the WHO reported that Asia, particularly Southeast Asia, carried most of the global dengue burden, with an estimated annual cost of USD 1 billion in Southeast Asia alone [44]. These figures emphasize the urgent requirement for effective prevention, control, and management measures to alleviate the health and economic repercussions associated with dengue.

1.1.3 Overview of dengue virus (DENV) transmission cycles

DENV exhibits two distinct transmission cycles: sylvatic cycle and human cycle. While dengue is primarily known as a human disease transmitted mainly by *Aedes aegypti (Ae. aegypti)* mosquitoes in urban and peri-urban areas, sylvatic transmission occurs in certain regions where non-human primates and sylvan mosquito species are present. The sylvatic cycle involves non-human primates and arboreal strains of *Aedes* such as *Aedes luteocephalus*, *Aedes furcifer*, and *Aedes taylori* [45]. Infected non-human primates act as reservoir hosts [46], sustaining the virus within their populations. Mosquitoes that feed on these primates can acquire and transmit the virus to other non-human primates or, in some cases, humans who venture into sylvan environments areas [47,48]. Understanding the sylvatic transmission cycle is crucial for monitoring and controlling dengue as it contributes to the overall epidemiology and potential spillover events from wildlife populations to humans [47–49].

Urban transmission of DENV is primarily facilitated by the principal vector *Ae. aegypti* mosquito, operating within densely populated areas. *Ae. aegypti*, in particular, possesses ecological and behavioral characteristics that augment its efficiency as a vector. This species exhibits ovipositional preference for water storage containers and refuse in close proximity to human habitation. It enters human dwellings easily and engages in multiple

blood-feeding episodes during each gonotrophic cycle to sustain egg production and overall nutritional needs [50–58]. These behavioral traits contribute to increased vector competence, allowing for the transmission of DENV to multiple human hosts. Recent studies indicate that epidemic and endemic forms of DENV-1, -2, and -4 have evolved independently from sylvatic progenitors over the past 2,000 years. This evolution involved a shift in reservoir hosts from non-human primates to humans and a change in vector species from arboreal *Aedes* mosquitoes to *Ae. aegypti* and *Ae. albopictus* [59,60]. The urban cycle of DENV is influenced by various factors, including high population density, urbanization, *Aedes sp.* mosquito breeding sites, climate and weather patterns, socioeconomic factors, and travel and migration [22,61–67].

1.2 Disease manifestations

1.2.1 Disease classification

Dengue encompasses a diverse spectrum of clinical presentations. While the majority of DENV infections are either asymptomatic or characterized by mild symptoms, approximately 25% of individuals experience a self-limited febrile illness [68]. Among this, a small proportion may progress to clinically significant complications, such as systemic vascular leakage, coagulation abnormalities with associated bleeding, and organ involvement, notably affecting the hepatic or neurological systems [68].

The WHO established case classification guidelines in 1974, drawing upon investigations into disease patterns observed in pediatric populations in Thailand during the 1960s, with the aim of enhancing clarity in dengue distinctions. Clinical dengue disease was classified into two distinct entities, dengue fever (DF) and dengue haemorrhagic fever (DHF) [69]. These guidelines were widely published and edited multiple times in various countries. However, these guidelines became evident that the classification system lacked universal applicability for optimal clinical management. Consequently, in 2006, the WHO Dengue

Scientific Working Group advocated for further research in dengue diagnostics and prioritization of patients to optimize clinical management practices [70].

In 2009, the WHO introduced a new classification of dengue [68] following a multicenter study called DENCO [70] . This revised system reclassified dengue into non-severe and severe cases. According to the 2009 WHO dengue case classification, individuals with symptoms are identified as having dengue if they do not exhibit major complications. Patients manifest with at least one warning sign such as persistent vomiting, abdominal pain or tenderness, mucosal bleeding are sub-classified as dengue with warning signs. The updated classification system aims to facilitate efficient triage, effective clinical management, and improved global data collection. Nevertheless, debates continue regarding its practical utility, making it a subject of ongoing scientific discourse and investigation [71–73].

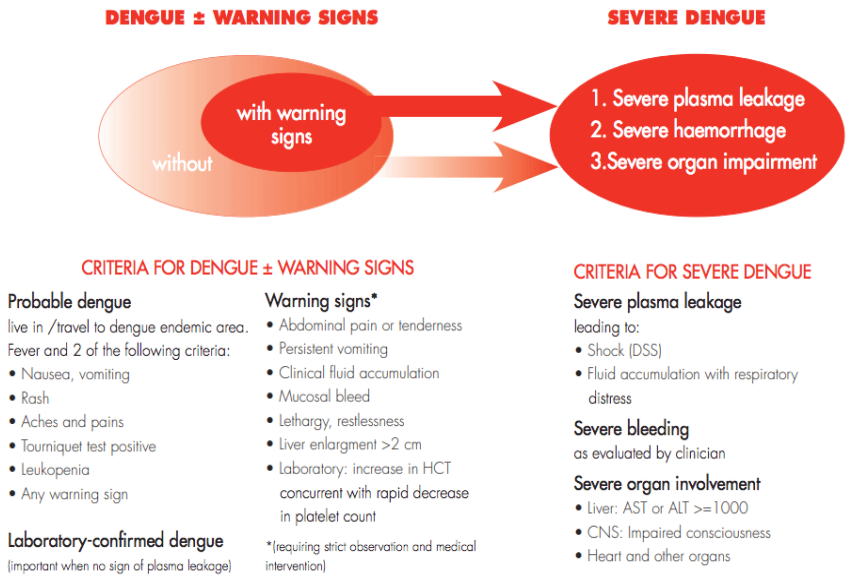


Figure 11: The 2009 WHO dengue case classification

1.2.2 Clinical phases

After an intrinsic incubation period (IIP) of 4-10 days, which represents the interval between initial infection and the onset of symptoms, typically fever, dengue patients progress through three distinct clinical phases (Figure 1-2) [74].

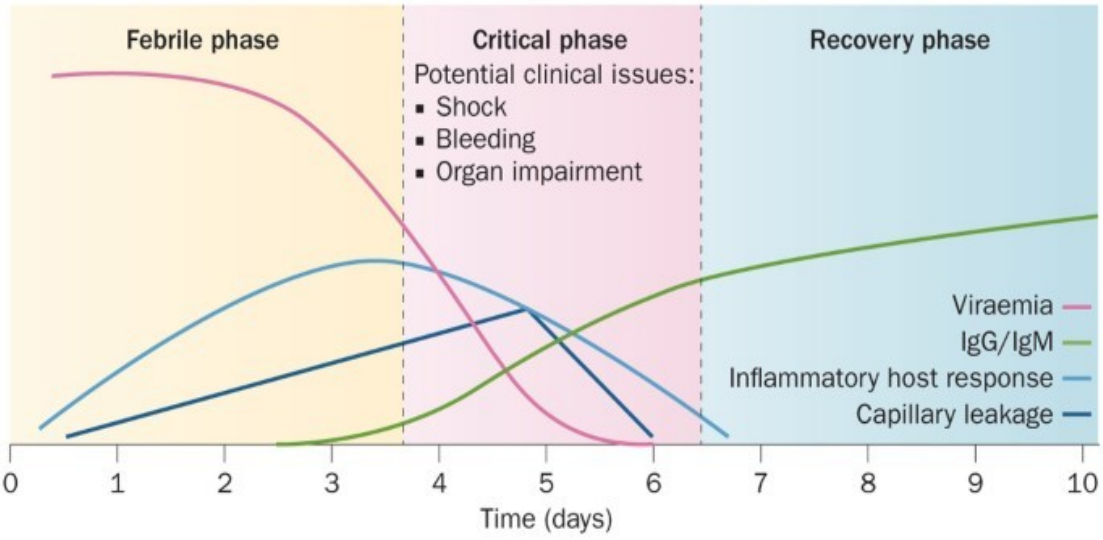


Figure 12: Dengue clinical phases and representation of the temporal relationship between the appearances of various biomarkers throughout the course of DENV infection.

Dengue virus infection undergoes through three distinct clinical phases. The febrile phase (lasting 3-7 days) is characterized by sudden onset of high fever. The critical phase occurs around defervescence (2-3 days), during which severe complications can manifest in a small proportion of patients. The recovery period (lasting 2-5 days) when the patient's symptoms resolve and clinical parameters normalize. From Yacoub S et.al, 2014

The febrile phase, the initial stage of DENV infection, presents as a short-lived acute febrile illness lasting approximately 2 to 7 days. Patients experience a sudden onset of high fever, accompanied by chills, profound malaise, vomiting, and constitutional symptoms [75–

77]. Some patients may also exhibit facial and truncal flushing around day 2 to 3, along with a transient macular rash [76,77]. While most patients improve as the fever subsides, it is crucial to be aware of potential complications that may arise during the defervescence period. Timely identification of these complications is essential for effective disease management.

During the critical phase, occurring around day 4 of illness, there is increased vascular permeability and plasma leakage, leading to potentially life-threatening DSS [78,79]. Haemoconcentration of 20% or more is a significant indicator, but its application for individual case management is challenging due to limited baseline hematocrit information [68]. Ultrasound studies reveal serosal effusions (pleural effusions, ascites, and gall bladder wall edema) as another indicator of leakage. However, clinically detecting them early is difficult until shock is established [78]. These studies have provided insights that minor signs of leakage may appear as early as day 2-3 of fever, indicating subtle vascular changes [80].

Dengue-induced shock usually occurs during defervescence, correlating with viral decline, suggesting an immune-mediated pathology [81]. Hypovolemic shock results from prolonged increased vascular permeability and plasma leakage [82]. Capillary leakage initially can be subclinical , then progress to compensated shock, and can potentially lead to hypotensive shock or cardiac arrest [74,83]. During the critical phase, a characteristic narrowing of pulse pressure (PP) is observed, with higher diastolic pressure and stable systolic pressure. A PP of 20 mm Hg or less is conventionally indicative of DSS, necessitating prompt fluid resuscitation [1]. Recurrent episodes of shock, known as "reshock," can occur 48-72 hours before the resolution of vasculopathy and are associated with increased mortality [78].

During the critical phase of DENV infection, patients commonly experience minor hemorrhagic manifestations, while organ impairment is less frequent, except in individuals

with underlying conditions or as a secondary infection [84]. Asymptomatic hepatomegaly and mild-to-moderate transaminases are common, while acute liver failure is rare. Additionally, neurological involvement, as well as ocular and cardiac manifestations such as myocardial dysfunction and rhythm disturbances, are recognized as part of the broader spectrum of dengue-associated complications [74,85,86]. Even in severe cases, patients usually achieve complete recovery within 1-2 weeks with appropriate supportive care. Transient vascular changes and abnormal hemostasis usually resolve within 48-72 hours. However, symptoms such as fatigue, lethargy, and rash may persist. In adults, the recovery period can be extended, lasting weeks to months [87,88]. In the recovery phase, plasma leakage and hemorrhage subside, and vital signs stabilize. A recovery rash may manifest, and this phase usually spans two to four days. However, adults might experience significant fatigue lasting for days to weeks following recovery.

1.2.3 Laboratory diagnosis

The laboratory diagnosis of dengue encompasses a diverse range of tests aimed at confirming the presence of DENV. These tests can be classified into three main categories: (1) those targeting the virus itself, including virus isolation and direct detection of viral genomic RNA; (2) those focused on detecting viral products, such as capturing and detecting the secreted NS1 protein; and (3) those measuring the host immune response to DENV infection, including the quantification of virus-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies. The selection and timing of specific laboratory tests for dengue diagnosis depend on various factors, including the clinical presentation, stage of illness, and resource availability. It is crucial to combine multiple diagnostic methods to enhance the accuracy and reliability of dengue diagnosis (Figure 1-3). In this section, I have listed the three main tests that have been widely used in many laboratories.

	Clinical Sample	Diagnostic Approach	Methodology	Time to Results
Virus and virus product detection	Acute serum (1–5 d of fever) and necropsy tissue	Virus isolation	Mosquito or mosquito cell culture inoculation	1 wk or more
		Nucleic acid detection	RT-PCR and real-time RT-PCR	1–2 d
		Antigen detection	NS1 Ag rapid test	Minutes
			NS1 Ag capture ELISA	1 d
			Immunohistochemistry	2–5 d
Serological response	Paired sera • S1: acute serum from 1–5 d • S2: convalescent serum 15–21 d	IgM or IgG seroconversion (S1 to S2)	ELISA HI	1–2 d
			Plaque reduction neutralization test	>7 d
	Serum after day 5 of fever	IgM detection	MAC-ELISA	1–2 d
			IgM rapid tests (lateral flow)	Minutes
		IgG detection	IgG ELISA	1–2 d

Figure 13: Laboratory diagnostic methods for the detection of Dengue infection.

Figure from Muller DA et.al, 2017

1.2.3.1 Nucleic acid testing

Reverse transcription polymerase chain reaction (RT-PCR) is a highly sensitive and specific method utilized in dengue diagnosis for the detection of viral RNA [89]. Real-time RT-PCR techniques further enhance the assay's sensitivity, specificity, and efficiency, enabling improved detection of DENV even in the later stages of illness, beyond five days after fever onset [90–93]. These techniques exhibit varying sensitivity levels, ranging from 50% to 99%, while maintaining a high level of specificity (99-100%) [90–93], making them valuable tools for accurate and reliable dengue diagnosis. In addition to detecting the presence of DENV, RT-PCR techniques also provide valuable information regarding dengue serotypes, and quantification of viral genome copies.

Despite these benefits, RT-PCR encounters challenges such as contamination risks and the requirement for expensive equipment. To address these limitations and improve accessibility, efforts are underway to develop alternative techniques suitable for low-resource settings. For instance, the POCKIT nucleic acid analyser enables point-of-care testing and field deployment [94,95].

1.2.3.2 Nonstructural protein 1 (NS1)

NS1, a glycoprotein secreted during the acute viraemic phase of DENV infection [96], is valuable for early diagnosis due to detectable levels early in infection , specificity to dengue, and abundance in sera [97,98]. The detection of secreted NS1 can be performed using

two formats: antibody-capture enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic tests (RDTs) [99–102]. NS1 assays offer the advantage of requiring only a single sample, allowing for swift detection and clinical management [103]. This is particularly beneficial in endemic areas where factors such as vaccination or previous flavivirus exposure can complicate diagnosis by antibody detection in serology samples [68,99]. The sensitivity and specificity of NS1 tests depend on dengue serotypes and immune status, significantly more sensitive for primary than secondary dengue [99–101,104]. Furthermore, NS1 sensitivity was higher in DENV-1 infected patients with primary dengue compared to secondary dengue at all stages of acute illness [100].

1.2.3.3 Serology testing

The detection and quantification of anti-dengue IgM and IgG antibodies are commonly employed for confirming DENV infection, utilizing ELISA format [105,106]. Serology assays present distinct advantages over real-time RT-PCR, including simplified execution, cost-effectiveness, decreased reliance on specialized equipment, and reduced personnel training requirements [97,107]. However, the sensitivity of IgM ELISAs is limited in the early phase of primary infection, with less than 50% sensitivity for at least four days after symptom onset, making them less useful for acute clinical management [108]. In contrast, secondary infections typically demonstrate lower IgM titres, which can even be undetectable, while IgG titres are elevated [109]. To distinguish between primary and secondary DENV infections, the IgM:IgG ratio has been used, with cut-off values ranging between 1.2 and 1.4 [68]. The specific cut-off value may vary depending on factors such as the duration of infection [110].

In dengue-endemic regions, the persistence of antibodies from prior infections presents a challenge in result interpretation [68,97,99]. To address this, the use of paired samples (acute and convalescent) is necessary to detect seroconversion and confirm acute

DENV infection. Additionally, cross-reactions with related flaviviruses can occur in antibody serology assays, especially in areas where these viruses co-circulate [68,99]. This cross-reactivity has been observed, including in individuals who have received flavivirus vaccinations [111,112].

1.3 Dengue virology

Understanding DENV structure and replication serves as a crucial bridge to improving laboratory diagnosis and facilitating vaccine/therapeutic targets. Section 1.3 provides fundamental knowledge of DENV structure and its replication features in human cells.

1.3.1 Molecular structure of Dengue virus (DENV)

DENV is a member of the *Flaviviridae* family and the *Flavivirus* genus [113]. DENV comprises four well-described serotypes, namely DENV-1 to DENV-4, which share approximately 65% amino acid sequence similarity.

Dengue virions, visualized through electron micrographs, are spherical with a smooth surface and a diameter of around 50 nm. They consist of an enveloped structure with a lipid bilayer enclosing an inner nucleocapsid core [114]. The virus is non-segmented and possesses a single-stranded positive-sense RNA genome, approximately 11 kb in length. The polyprotein undergoes both co- and post-translational processing, resulting in the generation of three structural proteins, namely Capsid (C), Envelope (E), and pre-Membrane (prM), as well as seven non-structural proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [114–116], Figure 1-4. The structural proteins play a role in the encapsidation of the viral RNA, nucleocapsid formation, membrane formation, maturation, and envelopment of the virion. The non-structural proteins have diverse enzymatic activities and contribute to various stages of the viral replication cycle [117].

The C protein, weighing 12 kilodalton (kDa), is a protein consisting of 100 amino acid residues. The C protein plays a vital role in the initial stages of DENV virion assembly by facilitating nucleocapsid formation [118] while M protein is involved in the organization and maturation of the DENV particle [119]. The E protein has three domains: domain I (DI) to III (DIII). DIII is responsible for receptor-binding activity, while domain II (DII) is the fusion domain with a hydrophobic fusion peptide [119].

The E protein is critical for the attachment of the virus to the host cell membrane and subsequent fusion [119]. NS5 protein functions as the viral RNA-dependent RNA polymerase (RdRp) and possesses nuclear localization sequences as well as S-adenosyl methionine methyltransferase activity. NS3 exhibits RNA-triphosphatase, nucleoside triphosphatase, and helicase activities, which are essential for RNA replication. NS2B acts as a viral serine protease, while other NS proteins, such as NS1(45 kDa), NS2A (22 kDa), NS4A, and NS4B, have diverse roles in viral replication, assembly, and release [42].

The genome of DENV contains untranslated regions (UTRs) at both the 5' and 3' ends flanking the open reading frame (ORF). The 5' end of the genome has a type I 5' cap that serves as the initiation site for translation, while the 3' end lacks a poly-A tail and instead has a stem-loop structure. The UTRs are required for efficient translation and replication of DENV [120,121]. A stem-loop A (SLA) locating in 5’-UTR is proposed acting as the promoter for the viral NS5 RdRp. The 5’- and the 3’-UTRs contain complementary Upstream AUG Regions (UAR) and cyclization sequences which mediate cyclization and RNA synthesis [121,122]. The length of the 5' UTR varies among the four DENV serotypes (DENV-1 to DENV-4), ranging from 95 to 101 nucleotides. In contrast, the length of the 3' UTR remains consistent across the serotypes, approximately 450 nucleotides [42,120,121]. The ORF encodes a polyprotein that serves as the precursor to 10 mature proteins.

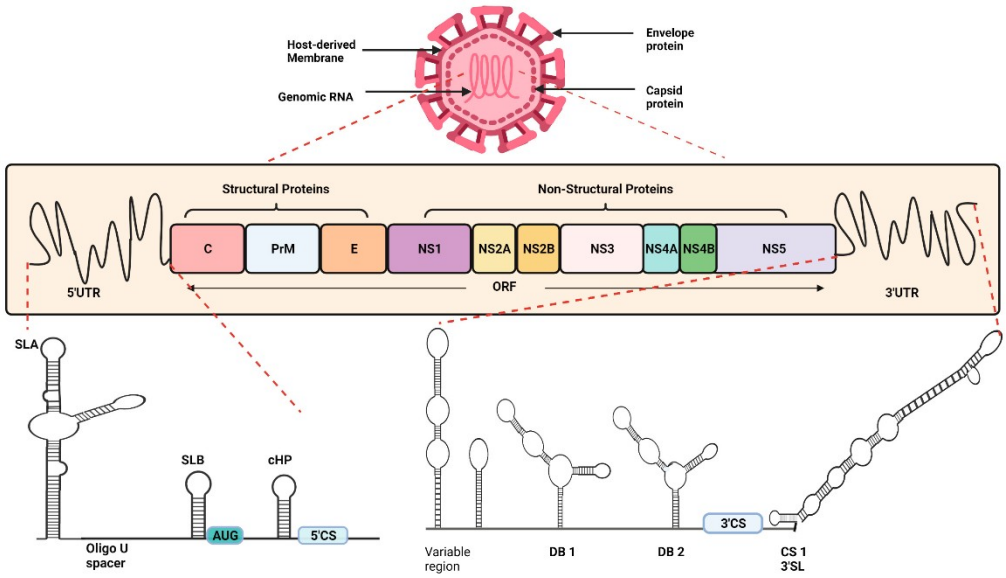


Figure 14: Dengue virus genome structure.

The viral genome consists of three segments: the 5' untranslated region (5'UTR), the open reading frame (ORF), and the 3' untranslated region (3'UTR). The ORF encodes a polyprotein that directs the synthesis of three structural proteins (Capsid - C, Pre-Membrane - PrM, and Envelope - E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins are components of the mature virus particle but they are not involved in viral replication while the NS proteins play important roles in replication. Figure from Nanaware et. al, 2021

1.3.2 Replication cycle of DENV in humans (Figure 1-5)

Following a mosquito bite, the delivery of DENV into the dermis allows for infection and replication in dendritic cells (DCs), specifically Langerhans cells, as well as keratinocytes residing in the basal and suprabasal layers of the epidermis [123–125]. The virus then

disseminates to the local lymph nodes either in association with infected migratory DCs or as free viruses present in the lymphatic fluid, leading to viremia [126]. The infection cycle of DENV in humans begins with the attachment of the virus to target cells. DENV demonstrates broad tropism, infecting various cell populations, including epithelial cells, fibroblasts, monocytes, macrophages, dendritic cells, B cells, T cells, endothelial cells, and hepatocytes [127–129].

The E protein interacts with host cell receptors and co-receptors, aiding viral entry. The M protein aids in viral binding to the host cell receptor. In mature virus particles, it forms the virus membrane. In immature particles, prM protein has a pr-domain to prevent premature virus-membrane interactions. DENV entry primarily occurs through clathrin-dependent receptor-mediated endocytosis, but alternative routes like fusion, diffusion, and micropinocytosis have been reported [128].

After receptor binding, virus is internalized into clathrin-coated endosomes and later matures or fuses with late endosomes. Acidic pH triggers E and PrM-E conformational changes [130]. In mature viruses, E protein re-arranges (triggering trimerization of the E protein in DIII), causing fusion loop to interact with endosomal membrane, releasing nucleocapsid into host cytosol [131]. Alternative virus trafficking pathways, such as direct penetration through the plasma membrane, the nucleocapsid is released into the host cytoplasm [128]. Capsid protein binds viral RNA, which un-coats via unknown mechanism, potentially involving ubiquitination [42,132]. Viral genome is transported to rough endoplasmic reticulum (ER) via cytoskeletal machinery [133].

Viral replication links to polyprotein translation, supported by ER membrane protein complex [134,135]. This replication process relies on a specialized replication complex located in a cytoplasmic compartment that extends into the ER [136]. The viral genome

structure enables distinct processes: the linear form is involved in translation and protein synthesis, while the circular form is dedicated to transcription [136]. Viral protein translation induces ER changes, forming convoluted membranes and vesicle packets. These structures connect to the cytoplasm through pore-like structures, supplying factors for viral replication [137]. Within vesicle packets, viral proteins such as NS5 (RdRp), NS3, and other viral NS proteins, along with host factors, form the replication complex [138].

Viral genome cyclization relocates NS5 to 3′ end for RNA synthesis. NS5 initiates RNA elongation by de novo synthesis of a CU dinucleotide [139]. The nascent positive strand, utilizing the negative strand as a template, displaces the pre-existing positive strand. This process leads to the amplification of positive strands of viral RNA. Additionally, the newly synthesized negative strand remains bound to the template positive strand, resulting in the formation of dsRNA [140]. Capping and methylation occur during transcription in the genomic viral RNA, while they are absent in dsRNA intermediates [141].

The positively charged capsid protein binds to the viral RNA, forming the nucleocapsid [142]. NS2 recruits polyproteins and proteases to the assembly site, leading to cleavage of prM protein and mature capsid protein [132,143,144]. The nucleocapsid buds from the ER membrane, assisted by E and prM proteins, and enters the ER lumen [118]. The prM protein shields the E protein's fusion loop to prevent premature fusion with the host membrane [118]. During viral egress, the E protein undergoes changes in the trans-Golgi network, transitioning from prM-E heterodimers to homodimers [145]. The prM protein is cleaved by furin [130,146,147], allowing viral egress. Mature virions are released into the extracellular medium, containing positive-sense single-stranded RNA encapsulated by C protein and surrounded by a lipid bilayer with viral envelope and membrane proteins forming a glycoprotein shell [148].

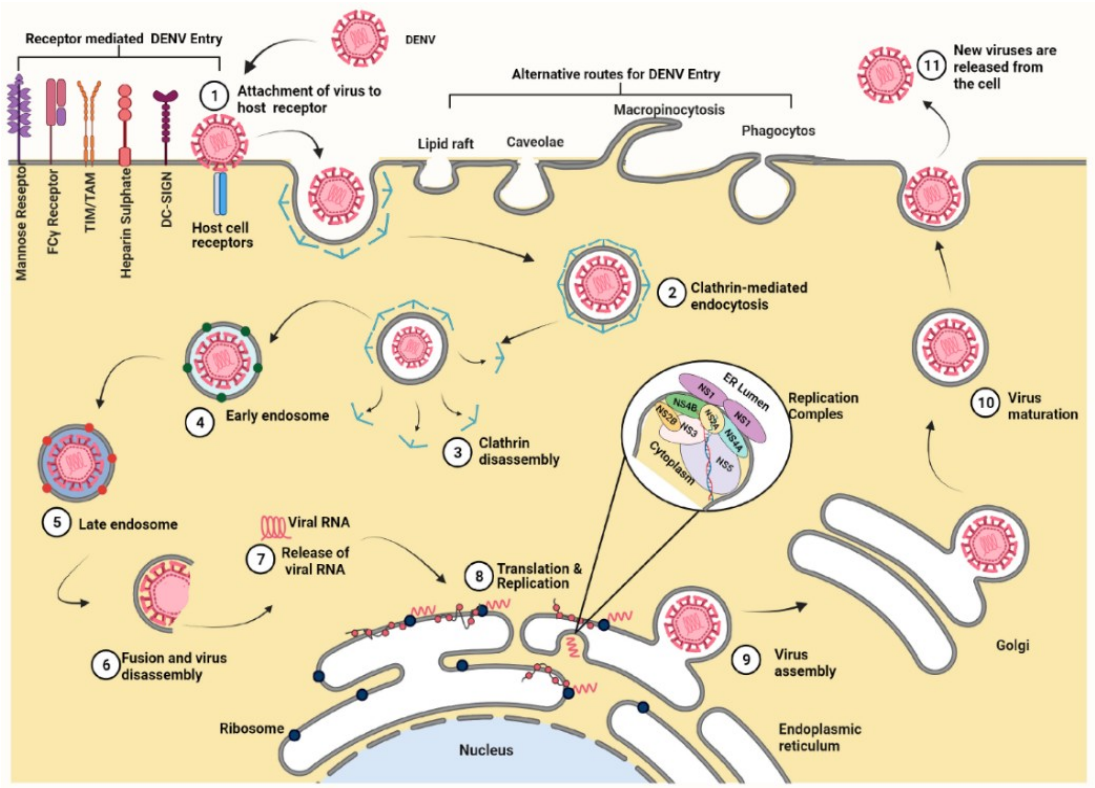


Figure 15: Replication cycle of DENV in human cells

The replication cycle of the virus begins with binding to host cell receptors for entry, preferably through receptor-mediated endocytosis, specifically clathrin-coated pits. The virus is internalized into a clathrin-coated bubble and undergoes endosomal processing as it moves through the early endosome with a slightly acidic pH. As the endosome matures, the pH decreases, triggering conformational changes that lead to fusion of the viral envelope with the host membrane, releasing the viral genome into the cytoplasm. The viral RNA then acts as a template for translation and replication in the endoplasmic reticulum (ER), facilitated by the replication complex formed by NS proteins. The replicated genome and viral proteins assemble into an immature virus particle in the ER, which undergoes furin-mediated maturation in the trans-Golgi network (TGN). Finally, the mature virus is exocytosed from the infected cell, completing the infection cycle. (Figure from Nanaware et al., 2021)

1.4 Molecular evolution of DENV

In this section, I describe the mechanisms driving the evolution of DENV. Understanding these drivers provides insights into virus adaptation and the spread of the disease.

1.4.1 Error-prone replication and intra-host variants

DENV, like other RNA viruses, relies on a low-fidelity RdRp encoded by the NS5 gene for replication. The estimated error-prone replication of DENV results in approximately one mutation per 11 kb DENV genome ($\sim 1 \times 10^{-4}$) [149]. This imperfect fidelity of RdRp leads to the accumulation of genetically distinct genomes, known as quasispecies [150]. Within individual human or mosquito hosts, it results in a population of genetic variants known as intra-host diversity [151]. It has been proposed that the inter-host diversity of DENV is shaped by a limited spectrum of within-host variants due to transmission bottlenecks that occur between vertebrate and vector hosts [151–153]. In mosquitoes, these bottlenecks including vector immune mechanisms such as RNA interference [151,154] and tissue barriers, along with natural selection, influence the genetic makeup of viral populations within individual mosquitoes, leading to intra-host variant DENV populations [155]. Despite these constraints, arboviruses manage to maintain genetic diversity through emergent viral variants resulting from random mutations and genetic variations within the limited number of surviving viruses within the arthropod vector population.

1.4.2 Recombination

Recombination events have been documented in non-segmented RNA viruses. In the case of DENV, recombination refers to the genetic exchange or recombination between different genotypes [60]. Being a positive-sense RNA virus, DENV has the ability to co-infect hosts with multiple genotypes, which suggests the potential occurrence of recombination events within hosts. Indeed, phylogenetic analyses have reported such recombination events in DENV [156–158]. Intra-serotypic recombination, involving genetic exchange within specific serotypes, has also been observed in DENV-1, DENV-2, and DENV-3, although these events are considered rare [157,159–164]. The inter-serotypic recombination, which involves genetic exchange between different DENV serotypes, has not been documented [165]. The detection of DENV recombination has been primarily based on the analysis of phylogenetic incongruence among wild-type viral sequences. DENV recombination has not been experimentally evidenced in-vitro, leaving the frequency and true occurrence of recombination in natural DENV populations under debate [166,167]. Recombinants in DENV do not show significant fitness advantages, and major expansions or persistent circulation of circulating recombinant forms are not prominent. In comparison to other viruses like human immunodeficiency virus, norovirus, and enterovirus, DENV recombination plays a limited role in shaping its diversity [168–170].

1.4.3 Purifying (Negative) selection

Phylogenetic studies examining the molecular evolution of DENV during natural transmission consistently demonstrated the prevalence of purifying selection [171–173]. This selective process was characterized by a predominance of synonymous mutations, indicating strong pressure against nonsynonymous mutations that result in amino acid changes. The presence of purifying selection has been observed in DENV datasets encompassing both continental-wide pandemics and localized epidemics [174]. These findings highlighted the role of negative selection as the virus alternates between its vector and vertebrate hosts [167].

While purifying selection has been proposed to contribute to lineage extinction and replacement, its precise role in lineage shifts and within-lineage variant extinction requires further investigation [175]. Purifying selection has also been observed to operate on within-human variant populations, albeit to a lesser extent in variant populations within vectors [151].

1.4.1 Adaptive evolution and positive (diversifying) selection pressure

The role of selection pressure in DENV evolution is a subject of ongoing research. While pervasive positive selection in human hosts has not been observed to define emergent clades and serotype replacement [176–178], positive selection has been associated with the introduction of epidemic clades in Puerto Rico [179]. Specific mutations in the Asian-American DENV 2 genotype during outbreaks in Peru were attributed to positive selection, although their correlation with enhanced in vitro replication was not observed [180].

Comparative analysis of DENV-2 strains revealed distinct selection pressures among genotypes, the Cosmopolitan and one of the two Asian genotypes [181] but no positive selection was observed during the emergence of the endemic DENV-2 lineage from ancestral strains [181]. Furthermore, selection pressure analyses on ancestral populations have not revealed pervasive adaptive evolution associated with the emergence of the four DENV serotypes [182]. However, episodic positive selection pressure has been observed during the divergence of major lineages within DENV-2, DENV-3, and DENV-4, as indicated by prior studies [161,183–185]. These findings highlight the role of positive selection in shaping the viral diversity of major DENV lineages. The majority of genes in DENV undergo purifying selection. However, in the NS2B and NS5 genes of DENV-2, several amino acid substitutions were found to be positively selected [167].

1.4.1 Constraints on DENV evolution imposed by mosquito-borne transmission

DENV serves as a fascinating example of the interplay between evolutionary processes and viral fitness. As arboviruses, they navigate diverse ecological niches, relying on both invertebrate mosquitoes and vertebrate hosts for replication and transmission. Despite lacking proofreading mechanisms [186], DENV exhibit remarkable genetic stability, possibly attributed to their unique demand for replication in distinct hosts, subjecting them to additional selective pressures compared to single-host viruses. This could lead to a compromise genome, where optimal replication in one host involves a fitness tradeoff in the alternate host [187,188]. An alternative explanation for the observed stability of arboviruses is the potential influence of persistent infections [189].

Recent studies have shed light on the impact of the human-to-mosquito transmission cycle on DENV evolution, revealing intriguing results. Alternating host passages have been found to enhance fitness within the retained host cell but come with a fitness trade off in the alternate host [190]. However, some experimental evidence contradicts the notion of negative fitness trade-offs from alternating host replication [191]. Additionally, DENV populations display extensive genetic diversity [151,192–194], which suggests the potential influence of different genome variants on viral fitness and adaptation to various environments. Furthermore, phylogenetic analyses underscore the significant impact of purifying selection on arboviruses [171–174], indicating substantial constraints on their evolution relative to single-host viruses. The intricate relationship between viruses, their vectors and human hosts involves co-evolution, resulting in long-term associations. A comprehensive understanding of these driving forces provides invaluable insights into virus adaptation and the dynamics of diseases like dengue, ultimately guiding the formulation of effective strategies for control and prevention.

In the following section, I will introduce *Ae. aegypti's* life cycle, historical spread, and virus-vector interactions, which has contributed to the expansion of dengue transmission around the world.

1.5 *Aedes aegypti,* the principal vector of dengue

1.5.1 History and spread of *Ae. aegypti*

Stegomyia is the most important subgenus of *Aedes sp.*, which includes *Ae. aegypti* vector [195,196]. The history of *Ae. aegypti* distribution was reviewed, suggesting that its ancestor, *Ae. aegypti formosus*, originated in sub-Saharan Africa where it still exists in forests [10,197]. This ancestral form laid eggs in tree holes and preferred non-human blood, exhibiting a darker morphology compared to the domestic form, *Ae. aegypti aegypti* (*Ae. aegypti)* [197,198].

The immigration of *Ae. aegypti* out of Africa and its introduction to the New World during the era of early European exploration and colonization was hypothesized, potentially facilitated by the slave trade [10,11]. Recent exome sequencing study suggest that domestication of *Ae. aegypti* occurred in Africa, resulting in the emergence of *Ae. aegypti* as a subspecies specialized in humans prior to its migration out of Africa [199]. Presently, *Ae. aegypti* is distributed throughout the tropics and subtropics, with variations in vector capacity among populations [9,10,13,200–202].

African populations tend to cluster genetically together, irrespective of their forest or domestic forms [14]. Within sub-Saharan Africa, *Ae. aegypti* populations exhibit significant variations in ecology, behavior, and appearance [14,202–205]. Some populations are less associated with humans, residing in forested areas and feeding on other mammals [10,13,200,201], while others have become domesticated, breeding in human habitats and feeding on humans. In East (Kenya) and West Africa (Senegal), both subspecies co-exist,

displaying distinct genetic identities in rural settings, while genetic introgression occurs in urban areas. Populations outside Africa show higher genetic structure, whereas ancestral populations in sub-Saharan Africa and those in Asia display lower levels of genetic differentiation [206].

Ae. aegypti exhibits a global distribution with variations in occurrence across different regions. More than 60% of all occurrence records for *Ae. aegypti* are from Asia and Oceania, while 35% are from the Americas. Notably, Africa and Europe have comparatively fewer unique occurrences, with only 575 records available [9]. The global expansion of *Aedes sp.*-borne arboviruses and the resulting public health threat can be attributed to urbanization, travel, inadequate vector control, and anthropogenic climate change [9,22,207–211].

1.5.2 Life cycle

The *Aedes sp.* mosquitoes have four life stages: egg, larva, pupa and adult (Figure 1-7). Female *Aedes sp.* mosquitoes exhibit oviposition behavior by depositing their eggs in diverse breeding sites, including both natural containers and artificial objects [212–215]. *Aedes* mosquito eggs enter dormancy to survive desiccation until water submersion, which initiates hatching and subsequent larval development within 2-7 days [216,217]. The larval stage comprises four instars (5-7 days), during which the larvae actively engage in feeding on microorganisms, organic matter, and algae present within the aquatic environment [218,219]. The duration of the larval stage is influenced by factors such as temperature, food availability, and interspecific competition, affecting the developmental timeline [220,221]. Following the larval stage, *Aedes sp.* mosquitoes progress to the pupal stage (2.0 - 3.6 days under optimal conditions), characterized by non-feeding and metamorphosis [222]. The pupal phase typically lasts several days before adult mosquitoes emerge. During this stage, the pupae exhibit mobility and exhibit responsive behavior to external stimuli, such as changes in light

intensity and vibrations [223]. Upon reaching adulthood, female *Aedes sp.* mosquitoes require a blood meal for egg development, while males primarily feed on nectar or plant juices [224].

Adult *Ae. aegypti* mosquitoes can fly shortly after emergence, but full maturity is reached 24-48 hours later [225]. They rest during the initial hours to allow for the hardening of their exoskeleton and wings [226]. Male mosquitoes undergo a 180° rotation of the terminalia post-emergence to enable mating, which takes around 18-24 hours [227,228]. Salivary glands in newly emerged females require time to mature [229], resulting in a delay in accepting blood meals for the first 18-24 hours or 20-40 hours [230,231]. The period between adult emergence and mating/blood feeding is considered an opportune time for dispersal from the larval habitat site. Along with the initial refractoriness of females to insemination, this has been proposed as a mechanism for preventing inbreeding [232]. Female *Ae. aegypti* mosquitoes are capable of flying long distances [233–235], but their dispersal is influenced by the availability of houses, hosts, and larval development sites [236].

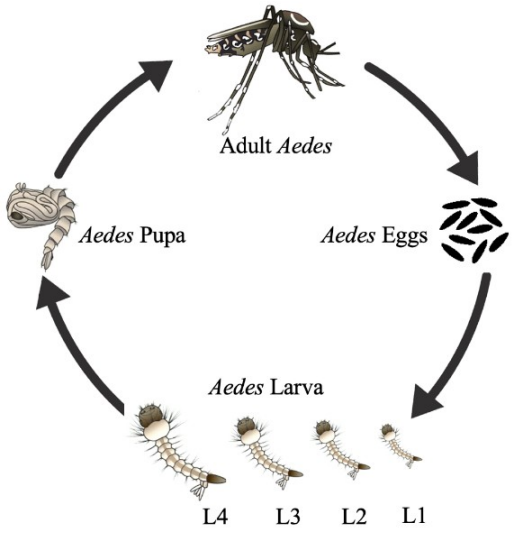


Figure 16: Life cycle of an *Aedes sp.* mosquito from eggs to adult

The cycle life includes four stages of development: egg, larva, pupa and adult. Source: Mahbubul Syeed et.al (2022)

1.5.3 Reproductive biology of *Ae. aegypti*

Ae. aegypti's reproductive cycle is driven by a series of intricate behaviors and physiological processes. This behavioral symphony culminates in the gonotrophic cycle (Figure 1-7), encapsulating host-seeking, blood ingestion, digestion, ovary maturation, and egg-laying, which underpins the mosquito's reproductive journey [237]. The timing of these processes, shaped by environmental cues, significantly influences population dynamics and vectorial capacity of *Ae. aegypti* [238]. This comprehensive understanding of multifaceted behaviors is pivotal for effective vector control and epidemiological studies.

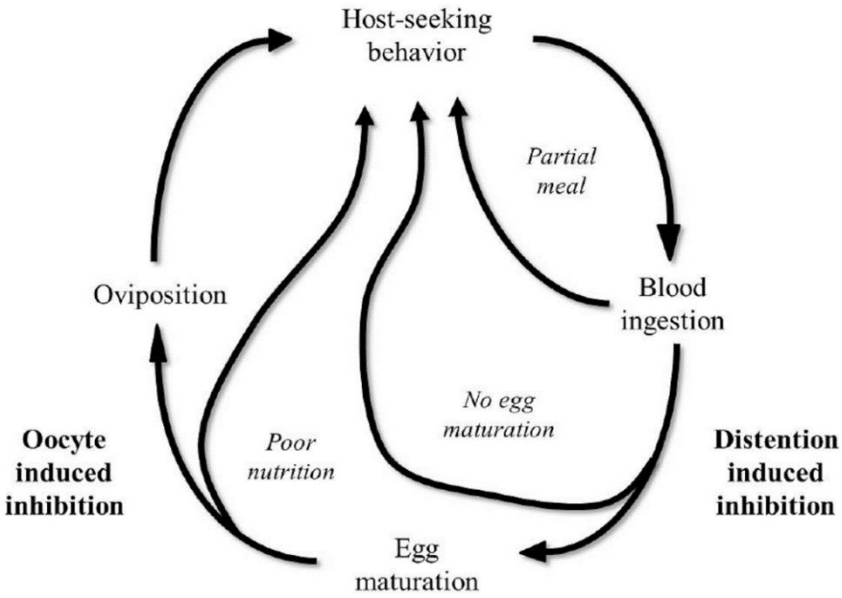


Figure 17: Gonotrophic cycle of *Ae. aegypti* . Figure from Klowden, M.J. et. al, 1994

Within 2-3 days after emergence, both male and female mosquitoes engage in mating, while females also take a blood meal necessary for egg development [239]. The simultaneous occurrence of mating and blood-feeding is facilitated by males being attracted to females. Male mosquitoes fly near the host to wait for female mosquitoes during their host-seeking flights [226]. In *Ae. aegypti*, females typically mate only once, as a single insemination event

provides sufficient sperm storage in the spermathecae to fertilize all the eggs produced during the female's lifetime. Additionally, the transfer of seminal fluid proteins during mating renders females unresponsive to further copulation [240–242].

Female *Ae. aegypti* mosquitoes exhibit persistent host-seeking behavior [243] throughout their life cycle, driven by their attraction to the exhaled carbon dioxide (CO_2) of the host [244]. Various factors, including vision, host body temperature, and humidity influence the approach of female *Ae. aegypti* mosquitoes toward their host [245]. While female *Ae. aegypti* primarily bite during the day, nocturnal activity indoors has also been reported [50]. Female *Ae. aegypti* mosquitoes, employing a sit-and-wait strategy [246], commonly engage in indoor biting [51], particularly in Asia and the Americas.

Ae. aegypti mosquitoes typically engage in multiple blood feedings within a single gonotrophic cycle [57,247,248], a phenomenon known as gonotrophic discordance [249,250]. The frequency of these feedings is regulated by many factors such as host defensive behavior, female body size, and local mosquito abundance [56,250] and has significant effects on their host-seeking behavior. Following large blood meals, the mosquitoes experience abdominal distension, which immediately leads to a short-term inhibition of host seeking [56], while a neuropeptide produced during oogenesis mediates a second long-term inhibition occurring 30 hours after a large blood meal [251,252]. Mechanisms overlap following blood repletion, suppressing host seeking until egg laying [251]. Nutritional status plays a role, with carbohydrate scarcity leading to energy-driven blood meal utilization, resulting in short-term inhibition and heightened likelihood of multiple feedings [253]. Partial blood meals, influenced by host movements or defensive behaviors, elevate the odds of multiple feedings in a single gonotrophic cycle, as they insufficiently inhibit long-term host seeking [254,255]. The digestion of the blood meal takes place in the midgut of *Ae. aegypti*, and the presence of symbiotic bacteria in the midgut contributes to digestion, nutrition, and development [256].

Oviposition behavior is a crucial aspect of *Ae. aegypti* reproduction. Once the eggs reach maturity, females actively seek optimal oviposition sites, frequently locating water-filled containers near human habitats [52,53]. These sites serve as both habitats and sustenance sources for mosquito larvae [257,258]. During the rainy season, the oviposition activity intensifies due to the increased availability of water-filled containers, leading to a subsequent rise in the abundance of the mosquito population [54]. Factors influencing oviposition site choice including the presence of conspecific larvae, container size, light-dark contrasts, specular reflectance from water surfaces, and environmental conditions. Gravid females are particularly attracted to specific physical attributes of oviposition sites, such as their size, contrast, and reflectance [53].

Understanding the life cycle of an *Ae. aegypti* mosquito, including its subsequent gonotrophic cycles, is pivotal for comprehending population dynamics and disease transmission potential. Typically, *Ae. aegypti* adults live for about 28 to 30 days under ideal conditions. However, research indicates that when provided with diets rich in honey, maltodextrin, or dextrose, their lifespan can extend significantly, up to 33, 35, and even 47 days, respectively [259]. Consequently, the gonotrophic cycles could span up to 5-6 cycles (Figure 1-8, adapted from [260]). While subsequent gonotrophic cycles generally follow a consistent pattern, the duration of each cycle can vary due to factors such as nutritional stress, population density, and temperature fluctuations, affecting the mosquito's fitness and longevity [261–263].

Understanding the gonotrophic cycle is critical not only for mosquito reproduction but also for disease transmission. Studies on vertical transmission of pathogens reveal an intriguing trend: later gonotrophic cycles show an increase in vertical transmission rates [264,265]. However, it's important to note that as mosquitoes age, both egg production and maternal survival decrease, potentially impacting the accuracy of vertical transmission rate

estimates [266]. Therefore, focusing on the second gonotrophic cycle might be the most appropriate strategy for observing and measuring vertical transmission rates. This approach could help minimize bias arising from factors such as initial virus dissemination to the ovaries or increased ovarian permeability during early oogenesis [267].

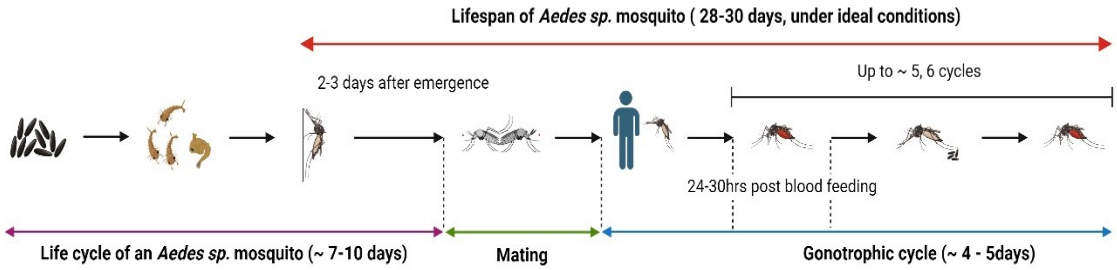


Figure 18: Time course of *Aedes aegypti* life cycle and gonotrophic cycle

1.6 DENV replication in *Ae. aegypti*

After having a blood meal from a viremic host containing DENV, *Ae. aegypti* initiates the replication process of the virus inside a mosquito. Successful DENV infection in the mosquito involves several key steps [268]. First, DENV establishes its infection in the mosquito's midgut. From there, it spreads within the midgut epithelium, allowing it to gain a foothold in mosquito. Subsequently, the virus disseminates from the midgut to other secondary tissues, further expanding its presence within the mosquito's body. In these secondary tissues outside the midgut, the virus undergoes secondary amplification, leading to higher viral loads. This amplification is crucial for the virus's ability to persist and thrive within the mosquito. Another critical stage involves the virus infecting the mosquito's salivary glands. In some cases, reproductive tissues may also be infected, possibly enabling vertical transmission (VT) of the virus to offspring, which is provided in detail in section 1.7. Finally, the virus is released into the mosquito's salivary, positioning it for horizontal transmission

(HT) to an uninfected vertebrate host during the mosquito's next blood meal. The period between the initial midgut infection and the subsequent transmission of DENV by *Ae. aegypti* is called the extrinsic incubation period (EIP), which typically lasts from 7 to 14 days at temperatures of 25-30°C [269].

1.7 Vertical transmission of DENV in *Ae. aegypti*

Here, we explore the phenomenon of vertical transmission (VT), revealing how DENV can be passed from infected female mosquitoes to their offspring, and describe challenges of determining these transmission rates in natural settings.

DENV is primarily transmitted between humans through the bite of infected female *Aedes sp*. mosquitoes, a process known as horizontal transmission (HT), where a mosquito becomes infected after ingesting blood from a viremic human host. Additionally, DENV can be transmitted vertically, from infected female mosquitoes to their offspring through the eggs [270–273]. The VT, specifically transovarial transmission, occurs during either follicle development or oviposition [274]. The detection of DENV in wild-caught male *Ae. aegypti* and *Ae. albopictus* mosquitoes provides strong evidence of VT occurring in nature [270,271,275,276]. Laboratory studies have also observed VT in both *Ae. aegypti* and *Ae. albopictus* mosquitoes [277,278]. Furthermore, VT has been observed in other mosquito-borne viruses such as Chikungunya virus (CHIKV) and Zika virus (ZIKV) [275,279–281]. This mode of transmission is considered a mechanism supporting DENV persistence in the absence of a host and/or under unfavorable conditions for mosquito activities [282–284].

It is challenging to determine whether a virus-infected female mosquito caught from the field acquired the virus horizontally or vertically. Nevertheless, the frequency of female infection in nature can vary widely, ranging from less than 1% to as high as 10% [285–287]. The presence of infected male mosquitoes and viruses in immature mosquitoes of any sex is a

clear indication of VT from mother to offspring [275]. In the field, estimates of VT in immatures and adult males differ among individuals, with some studies reporting 0% [286,288], while others show around 20% based on pooled samples or using the Ovitraps Index [289,290].

The EIP duration is influenced by mosquito species, individuals, virus species, and external factors such as temperature [291–293]. In natural settings, mosquitoes encounter daily temperature fluctuations and microclimatic variations, resulting in infected individuals being at in different infection development stages [294]. This variability contributes to varying perspectives on the frequency of vertical DENV transmission in the field, with some suggesting it is negligible, while others emphasize uncertainty [289,290]. Consequently, accurately estimating the VT rate in the wild requires collecting large samples of mosquitoes over consecutive days to obtain comparable infection rate estimates [275,295,296]. An ideal approach would involve using an unbiased trapping method that ensures all individuals have an equal chance of being collected. These captured mosquitoes would then undergo individual testing for the virus using an assay capable of consistently detecting the specific virus at any concentration. Individual testing of mosquitoes is often not feasible, leading to the need for a cost-effective alternative. In practice, achieving these ideal conditions can be challenging due to logistical and financial constraints. An alternative approach involves pooling mosquitoes and collectively testing them for virus presence. This pooled testing method improves sample processing efficiency and overcomes individual testing limitations [297–299].

1.8 Dengue prevention

Dengue prevention is a critical public health priority, and it involves a combination of measures to control the spread of the virus, currently this mainly includes vector control and vaccine development.

1.8.1 Vaccine advancements

Personal protective measures play a limited role in preventing DENV infection on an individual level. People living in dengue-prone areas are encouraged to take personal precautions to avoid mosquito bites. This includes wearing long-sleeved shirts, long pants, and socks to cover exposed skin [300]. Additionally, using mosquito repellents and sleeping under mosquito nets, especially during peak mosquito activity times (dusk and dawn), can provide some protection against mosquito bites and DENV infection [300].

An exciting new area of dengue prevention is the development and implementation of effective dengue vaccines. Dengvaxia (tetravalent dengue vaccine- CYD-TDV), the first commercially available dengue vaccine, is a live attenuated tetravalent chimeric vaccine that has been tested in a large number of children aged 2-14 years. While it has shown efficacy in reducing dengue incidence (56.5 % in the Asian population, 64.7% in American people) [301,302], its potential to cause antibody-dependent enhancement in dengue-seronegative individuals is an important consideration [303]. TAK-003, a promising recombinant chimeric vaccine, has demonstrated an overall efficacy of 72.7% (74.8% in the baseline seropositive population and 67% in the seronegative population) [304]. The efficacy against hospitalized dengue cases has shown 90% effectiveness in the baseline seropositive group and 87% in the baseline seronegative group [304]. It has been licensed in several countries worldwide, including Europe, the U.K., Brazil, Argentina, Indonesia, and Thailand [305,306]. Instituto Butantan, the U.S. NIH, and Merck have released the phase III trial results for their vaccine, Butantan-DV. This vaccine was developed using materials provided by the U.S. NIH and is similar in composition to the NIH's TV003 formulation. The overall efficacy was 79.6%, higher in dengue immune (89.2%) compared to non-immune (75.3%) [307]. The phase III trial is ongoing in Brazil until 2024 to provide more insights, particularly for DENV-3 and -4.

Dengue vaccine development faces several formidable challenges. One of the primary obstacles is the existence of four distinct four serotypes which makes it crucial to develop a vaccine that provides broad protection. There absence of validated immune correlate of protection, making it difficult to predict an individual's immunity against DENV infection. Moreover, animal models used in research do not comprehensively recapitulate the human DENV infection and disease experience. Immunologic assays also struggle to precisely define DENV type-specific (homotypic) immune responses. Large-scale efficacy trials across diverse populations and clinical endpoints are required to demonstrate vaccine benefits.

1.8.2 Therapeutics and Prophylactic drugs

One promising avenue involves the development of therapeutics and prophylactic drugs. In the realm of therapeutics, researchers are actively engaged in discovering antiviral drugs that can impede the replication of DENV. The aim is to develop drugs that target viral components or critical host mechanisms, offering both prophylactic and treatment options to effectively mitigate disease severity and reduce fatalities, thereby alleviating the overall burden of dengue. Since the early 2000s, several compounds have been tested in early proof-of-concept trials but, none was able to demonstrate clinical efficacy [308–312]. Numerous factors contribute to this disparity, including the nonspecific and wide spectrum of disease manifestations [2,68], limitations of existing model organisms [313],and the absence of a reliable biological marker correlating with clinical outcomes [314,315].

Turning to the prophylactic front, interventions could prove beneficial for individuals traveling to dengue-endemic regions (e.g., aid workers, tourists, business and military travelers, and expatriates), as well as vulnerable populations residing in such regions. Prophylactic measures can be applied across different phases of illness, including the incubation, febrile, and critical phases. In the incubation phase, prophylactic drugs aim to disrupt DENV replication [316,317]. Recently, JNJ-64281802, a novel anti-DENV small

molecule targeting DENV NS4B, has shown potent antiviral activity in nonclinical studies across all four DENV serotypes [317]. By disrupting the DENV viral replication, JNJ-64281802 could potentially reduce the viral load and/or prevent viremia from emerging and thereby could reduce or even prevent DENV infection-associated morbidity and mortality. A Phase 2 clinical trial is currently underway to assess the efficacy and safety of JNJ-64281802 for the prevention of dengue infection [318].

For the critical phase, supportive interventions such as platelet and plasma transfusion are considered [319–322]. However, there is a paucity of trial evidence regarding the role of platelet transfusion in patients with thrombocytopenia and moderate to severe bleeding. Further studies are crucial to determine the efficacy of platelet transfusion in such scenarios. Current guidelines recommend the administration of fresh packed cells or fresh whole blood for patients with moderate to severe bleeding in dengue [68].

1.8.3 Vector control strategies

One of the primary strategies in dengue prevention is mosquito control. Controlling the mosquito population is crucial to break the transmission cycle. This involves identifying and eliminating potential breeding sites, such as stagnant water in containers, discarded tires, and other water-holding objects [68,323]. Nevertheless, vector control encounters significant challenges due to urbanization, population growth, increased international travel, and the inadequate implementation of control programs in dengue-endemic countries [68,324].

Vector control strategies for the prevention of DENV transmitted by *Ae. aegypti* mosquitoes involve two main approaches: chemical control and biocontrol. These strategies aim to reduce the mosquito population and, consequently, the transmission of the virus.

1.8.3.1 Chemical control strategies

Chemical control methods utilize insecticides to target and eliminate adult mosquitoes or their larvae. It is essential to use these chemicals responsibly, following recommended guidelines and considering potential environmental impacts [300]. Indoor Residual Spraying (IRS) involves spraying insecticides on the interior walls of houses and buildings where mosquitoes rest, effectively killing adult mosquitoes on contact [325,326]. Space Spraying (Fogging) disperses insecticides as fine droplets in the air, eliminating adult mosquitoes in outdoor areas, commonly used during outbreaks or in high mosquito population areas [326]. Larvicides, on the other hand, are designed to target mosquito larvae in breeding sites such as stagnant water sources, reducing the mosquito population by eliminating them before they mature into adults [68,323].

Chemical approaches presents several challenges and limitations. These include the development of mosquito resistance [208–211] to chemicals due to their frequent use, potential harm to the environment and non-target organisms [327], and concerns about public health impacts [328].

1.8.3.2 Biological control

Biocontrol methods focus on using natural enemies or biological agents to control mosquito populations. These methods are considered more environmentally friendly and sustainable [329]. *Bacillus thuringiensis israelensis* (*Bti*), a naturally occurring soil bacterium, is commonly used as a larvicide in water bodies due to its toxin-producing abilities that specifically target mosquito larvae [329]. Another biocontrol method involves introducing *G. affinis, Poecilia reticulata, Tilapia mossambica*, and *Sarotherodon niloticus*, commonly known as mosquito fish, into household water containers as a method of control. These fish species feed on mosquito larvae and pupae, effectively helping to control mosquito breeding in domestic environments [330].

Currently, innovative biocontrol strategies involving *Wolbachia* introgression and genetic modification have been employed many areas. The sterile insect technique (SIT) is a species-specific and environmentally safe strategy for controlling insect populations. It relies on mass rearing, radiation-mediated sterility, and the release of numerous male insects into a designated area to compete for mates with wild males. The population decreases as wild females mate with the released sterile males [331,332]. However, challenges related to mosquito egg production and mass rearing have sometimes led to the failure of this control method [333].

The presented vector control approaches often encounter challenges in maintaining sustained disease reduction over extended periods, as evidenced by a lack of demonstrated disease reduction over longer timeframes [334,335]. The diminished long-term effectiveness of vector control methods can be attributed to various factors, including the emergence of insecticide resistance, globalization and climate change [9,22,207–211]. Thus, continuous evaluation of vector control strategies is crucial to identify interventions that may lose efficacy over time, refine existing methods, and explore innovative approaches, such as the *Wolbachia*-introgression method. Implementing rigorous evaluation methodologies focused on long-term sustainability and the broader impact on disease beyond immediate reductions is essential to ensure that vector control efforts yield lasting improvements in public health.

Wolbachia, a bacterium, interferes with viral replication inside the mosquito, reducing their ability to transmit viruses like DENV, ZIKV, CHIKV and *Plasmodium* parasites [336–340]. Releasing *Wolbachia*-infected mosquitoes into the wild has shown promise as an effective biocontrol strategy [341,342]. Subsequent sections delve into the *Wolbachia*-based approach for dengue control.

1.9 Introduction to *Wolbachia*-based biocontrol for dengue reduction

Wolbachia pipientis (*W. pipientis*) is a gram-negative, maternally inherited, endosymbiotic bacterium belonging to the order *Rickettsiales*, the class *Alphaproteobacteria* [343,344]. In 1924, Hertig and Wolbach first identified *Wolbachia* in the ovaries of *Culex pipens* mosquitoes and this particular species was officially named *Wolbachia pipientis* [345]. It occurs naturally in about 66% of all insect species, showcasing a wide array of ecological interactions with its eukaryotic host cells [346–348]. There is significant genomic diversity among strains, which is classified into seventeen supergroups (A to R, except G) based on genome differences, mainly through 16S ribosomal region phylogenetic analysis, with specific supergroups being predominant in certain hosts [349–351].

Since the development of the ability to generate novel *Wolbachia* symbiosis (transinfection) in mosquitoes via embryonic microinjection [352,353], numerous transinfected mosquito lines carrying diverse *Wolbachia* strains have been established and characterized. These transinfected lines are now being harnessed for disease control applications [337,342,353–359]. *Wolbachia*-carrying mosquitoes exhibit their antiviral ability against several arthropod-borne viruses, such as DENV [360], ZIKV [337,361], CHIKV [338,362], and Yellow fever (YF) [362]. This ability has provided an ideal opportunity to use this bacterium to limit the transmission of arboviruses including DENV by establishing *Wolbachia* to the wild mosquito population.

Ae. aegypti is not a natural host of *Wolbachia* [363]. *w*Mel is a strain of *Wolbachia* originally isolated from *D. melanogaster* [364]. Unlike *w*MelPop-CLA, which has more pronounced fitness costs [365], *w*Mel displays minimal impact on the fitness of *Ae. aegypti* mosquitoes [366]. While providing less viral protection compared to *w*MelPop-CLA [366], *w*Mel is considered a more favorable option for biocontrol strategies against mosquito-borne diseases. Recently, a significant advancement involved producing super-infected *Ae. aegypti*

mosquitoes carrying both *w*Mel and *w*AlbB strains [367]. These super-infected mosquitoes showed lower fitness costs and higher viral protection compared to mosquitoes infected with only *w*Mel [367]. The exact mechanism through which *Wolbachia* produces its antiviral effect remains not fully understood. There is evidence indicating that in some *Wolbachia*-host-virus associations, the primary effect is establishing tolerance to virus replication without significantly impacting virus titer [368].

In addition to its antiviral properties in *Ae. aegypti*, *Wolbachia's* potential to alter insect host physiology is investigated as a biotechnological approach for controlling insect populations. The subsequent sections will describe these topics.

1.10 *Wolbachia*-mediated inhibition of viral replication

1.10.1 *Wolbachia* alters the host's intracellular environment

Wolbachia, an intracellular bacterium, relies on several host factors to ensure its successful replication, transmission, and manipulation of the host. The process of *Wolbachia* infection is essential for maternal transmission and the induction of reproductive phenotypes [369,370]. However, *Wolbachia*'s influence extends beyond the developing oocytes; it infects various tissues and cell types within the host, resulting in significant effects on the host's physiology [371–374].

At the cellular level, the presence of *Wolbachia* leads to various differences when comparing infected and uninfected individuals (Figure 1-8). One of the key aspects of *Wolbachia*'s influence on the host is its close association with host-derived membranes. It resides within vesicles derived from the host's Golgi apparatus [375] and relies on the host's endoplasmic reticulum-associated protein degradation pathway to maintain its abundance within host cells [376]. Consequently, *Wolbachia*'s presence significantly alters the morphology of these intracellular membranes [376], affecting their normal functions.

Wolbachia also interacts closely with the host's cytoskeleton, utilizing host microtubules and actin [377] for its proper localization within the cell [371,378,379]. The bacterium employs various effector proteins secreted via the Type IV Secretion System to directly target host cellular processes. This ensures its successful maintenance [377,380,381] and reproduction within the host. Furthermore, the presence of *Wolbachia* has been observed to induce changes in host gene expression, affecting various biological processes. Notable differences in gene expression have been reported for antioxidant processes [373,382], metabolism [383], immune responses [373,383,384] and microRNAs [385,386]. *Wolbachia*'s surface proteins alone can elicit a transcriptional response from the host, contributing to the overall modification of the host's intracellular environment [428]. Additionally, studies suggest that *Wolbachia* infection may alter the epigenetic patterns of host genomes [388,389]. *Wolbachia*'s reliance on host nutrients suggests that there may be competition between the bacterium and the host for amino acids [390].

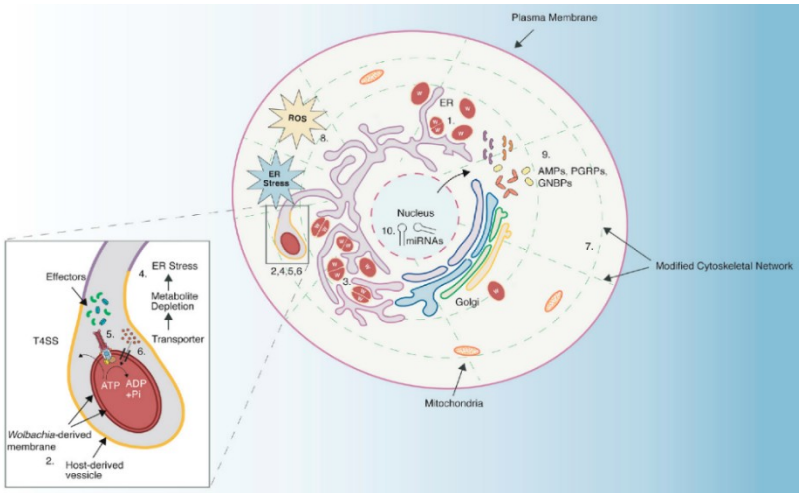


Figure 19: *Wolbachia* modifies the intracellular environment of the host

Wolbachia exhibits complex interactions within host cells, involving distinct localization around the nucleus and association with host membranes, particularly the endoplasmic reticulum (ER). It is enclosed by three membranes, including a host-derived vesicle and two *Wolbachia*-derived membranes, crucial for replication and division. During replication, daughter cells briefly share the host membrane before separating. A direct fusion with the ER and possession of a Type Four Secretion System (T4SS) allow *Wolbachia* to exchange proteins with the host cell and export effector proteins that influence cellular functions. The

bacterium's genome encodes transporters for nutrient uptake, sustaining its survival and replication. Additionally, *Wolbachia* can modify the host cell's cytoskeleton, impact cellular processes, and generate reactive oxygen species (ROS), causing cellular stress. *Wolbachia* infection triggers immune responses by upregulating immune-related genes and pathways, including antimicrobial peptides, Gram-negative binding proteins, peptidoglycan recognition proteins, and microRNAs, highlighting intricate host-bacterium interactions.

1.10.2 Competition for resources

Wolbachia, like other members of *Rickettsiales*, lacks the ability to synthesize its own cholesterol and depends solely on its host's cholesterol reserves for efficient growth and reproduction [371]. This characteristic is not unique to *Wolbachia*, as many mosquito-borne flaviviruses and alphaviruses also rely heavily on host cholesterol for successful infectivity, replication, virion assembly, and release from infected cells [391,392].

The antiviral protection provided by *Wolbachia* is thought to be density-dependent, wherein higher densities of *Wolbachia* might consume more host resources, creating an environment less conducive to viral proliferation [371]. DENV infection has also been associated with the accumulation of acyl-carnitines in the midgut of *Ae. aegypti*, suggesting that the virus may divert energy resources to support its replication [393]. Recent research on the *Aag2 Ae. aegypti*-derived cell line infected with *Wolbachia*, DENV, and ZIKV has shown that acyl-carnitines play a crucial role in this complex interaction [394]. During *Wolbachia* infection, acyl-carnitines are down-regulated. Moreover, inhibiting acyl-carnitines highlights their significant involvement in the replication of DENV and ZIKV. Surprisingly, depleting acyl-carnitines increases *Wolbachia* density, while adding acyl-carnitines impairs *Wolbachia*

production. Furthermore, the presence of acyl-carnitines leads to an increase in flavivirus infection in *Wolbachia*-infected cells. These results highlight the intricate interactions between acyl-carnitines, *Wolbachia,* and viral infections, shaping the intracellular environment and influencing pathogen replication [394,395].

A study analyzed the lipid profile of *Ae. aegypti* mosquitoes infected with DENV and the *Wolbachia*, *w*Mel strain [395]. The lipid profiles of wMel-DENV-infected and DENV-mono-infected mosquitoes are similar, suggesting that DENV induces lipids more prominently than *w*Mel does. The study suggests that *w*Mel-DENV may not directly compete for host lipids. Other studies have also demonstrated that manipulating host lipid profiles in mosquito cell lines or *Drosophila sp*. carrying various antiviral *Wolbachia* strains can diminish *Wolbachia*'s antiviral effectiveness. These studies indicate the importance of lipids in the antiviral state established by *Wolbachia* [396,397]. *Wolbachia*-induced changes in lipid levels could affect the cholesterol content of cellular membranes, potentially disrupting intracellular trafficking and the formation of replication complexes, thereby reducing energy availability for DENV replication [395].

1.10.3 *Wolbachia*-induced immune priming and its advantages in trans-infected mosquitoes

One fascinating aspect of *Wolbachia* is its ability to influence the host's immune system, leading to immune priming. When *Wolbachia* infects mosquitoes, it induces the upregulation of immune genes belonging to three major innate immune pathways: Toll, Immune Deficiency (IMD) pathway, and the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway [397–399]. Activation of these pathways triggers the expression of various antimicrobial peptides and immune effectors, contributing to the host's defense against pathogens.

This immune priming effect of *Wolbachia* has been observed in transinfected mosquito species like *Ae. aegypti*. Immune priming is advantageous, especially in transinfected mosquitoes, as it prepares their immune systems to mount a robust antiviral response. When these primed mosquitoes encounter viruses, they are more prepared to combat subsequent pathogen infections. This, in turn, can lead to reduced viral replication and transmission, potentially interfering with the spread of arboviruses like DENV, CHIKV, and ZIKV.

Furthermore, *Wolbachia* infection has been associated with the production of reactive oxygen species (ROS) in both flies and mosquitoes. In the case of *Ae. aegypti* mosquitoes infected with *Wolbachia*, ROS have been shown to modulate the Toll pathway, leading to restricted replication of DENV [396]. This effect is specific to *Ae. aegypti*, as *D. melanogaster* flies infected with *Wolbachia* did not show changes in immune gene expression. This suggests that the immune priming effects of *Wolbachia* may be specific to certain host species.

1.11 Utilizing *Wolbachia* as biocontrol tool for vector population control

This section explores the employment of two primary strategies: the population replacement strategy (PRS) and the incompatible insect technique (IIT) (Figure 1-9). Cytoplasmic incompatibility (CI) is a crucial element in the two *Wolbachia*-based vector control methods that are currently employed to reduce disease transmission.

1.11.1 Effect of *Wolbachia* on *Ae. aegypti*'s reproduction

Wolbachia infections have a profound impact on various aspects of host biology, encompassing physiology, immunity, ecology, evolution, and reproduction [400–403]. This bacterial group exhibits a wide distribution among insect species and is associated with diverse reproductive outcomes, including male killing [404], feminization [405],

parthenogenesis [406], and particularly, CI [407–409]. Among these manipulations, CI appears to be the most common in insects [402].

The utilization of *Wolbachia* and CI has emerged as an effective strategy for controlling agricultural pests and reducing the transmission of mosquito-borne human diseases [354,410]. *Wolbachia* has demonstrated success in managing insect populations [411–414]. However, despite its widespread use, the underlying molecular mechanisms of CI have long remained enigmatic. Recent breakthroughs have shed light on this phenomenon by identifying *Wolbachia* two-gene operons that encode CI factors (Cifs) responsible for CI and rescue (normal embryo viability results) processes [415,416]. The *Wolbachia* Cif proteins have become a central point of investigation. Studies have begun to unravel the biochemical activities and genetic effects of these proteins, providing valuable insights into the mechanisms of CI [415,416]. While this progress marks the early stages of biochemical analyses of CI, further research is essential to fully comprehend the intricate processes involved.

When *Wolbachia* is artificially introduced into *Ae. aegypti* [417], it strategically manipulates the host's reproductive system to enhance its transmission (Figure 1-10). CI allows the efficient spread of maternally transmitted *Wolbachia* within host populations, eliminating the need for horizontal transmission between individuals [418]. When uninfected females mate with *Wolbachia*-infected males, CI leads to reduced fertility or unviability of offspring [419–421]. In contrast, *Wolbachia*-infected females can produce viable offspring when mating with both infected and uninfected males [418,422,423].

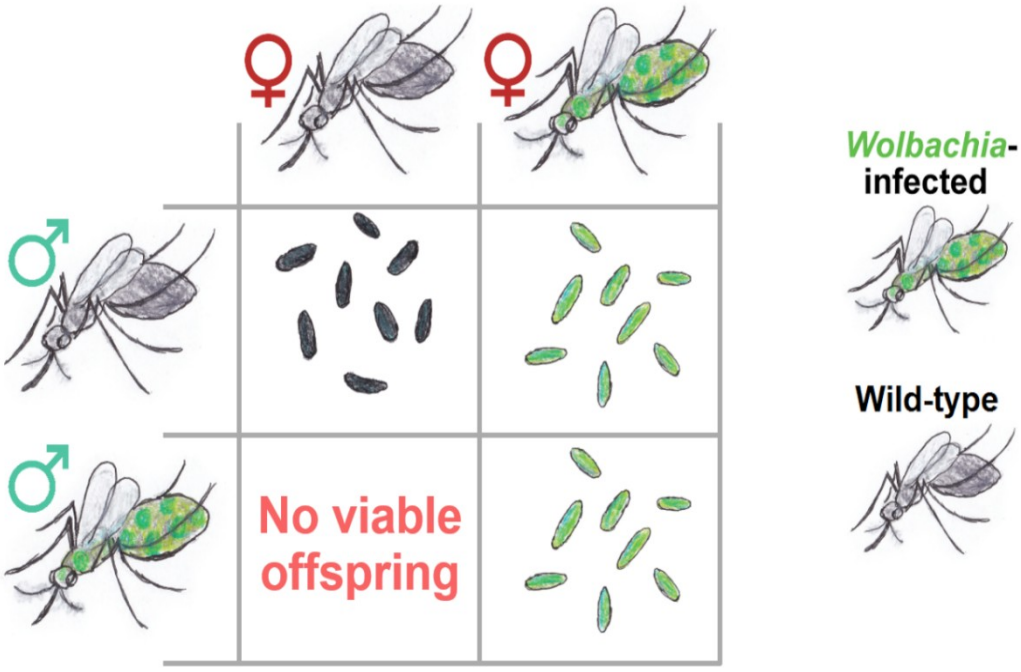


Figure 110: Cytoplasmic Incompatibility (CI) of *Wolbachia*.

When *Wolbachia* is introduced into mosquitoes, it induces a phenomenon known as cytoplasmic incompatibility. This condition causes a significant impact when infected males mate with females that are not carrying *Wolbachia*; their eggs fail to hatch. As a result, females infected with *Wolbachia* gain a competitive advantage, facilitating the widespread transmission of *Wolbachia* throughout wild mosquito populations.
Source:

1.11.2 Population Replacement Strategy (PRS)

In the PRS, symbiotic male and female mosquitoes are released to propagate and establish *Wolbachia* to the wild mosquito population, leading to a reduction in the mosquitoes' ability to transmit virus to humans. The PRS has seen success with the deployment of mosquitoes infected with *w*Mel strain of *Wolbachia*. For instance, in Northern Australia, the release of symbiotic mosquitoes resulted in the stable integration of *Wolbachia* into the wild *Ae. aegypti* population at frequencies above 80-90% [411]. Recently, multiple epidemiological studies, including a cluster randomized trial, have shown a significant decrease in dengue incidence in *w*Mel treated communities, demonstrating the remarkable effectiveness of *Wolbachia* introgression approach in dengue prevention [341,342,424,425]. The PRS has been implemented in dengue-endemic countries, such as Indonesia, Brazil, Colombia, Vietnam, Sri Lanka, Mexico, and some Pacific Islands.

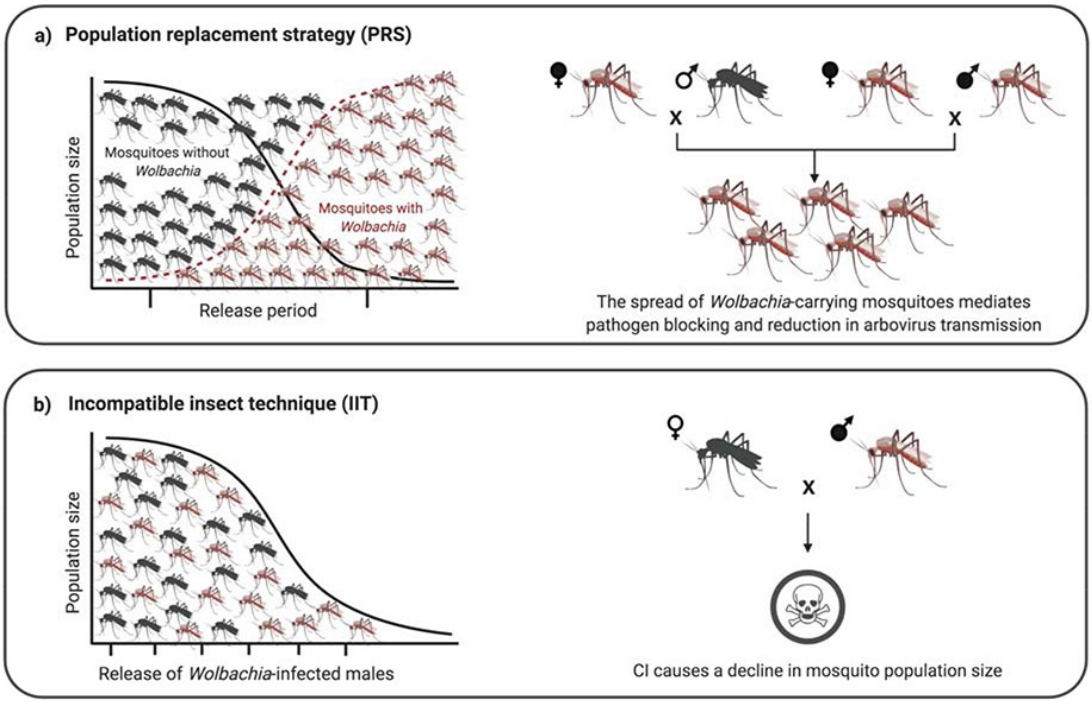


Figure 111: *Wolbachia*-mediated reduction in Vector competence and disease burden in humans.

a) Population Replacement Strategy: cytoplasmic incompatibility-inducing *Wolbachia* spreads in uninfected target populations, replacing native species with pathogen-blocking, *Wolbachia*-infected mosquitoes, reducing virus transmission. b) Incompatible Insect Technique (IIT): CI-causing male mosquitoes are released, leading to embryonic lethality when mating with

aposymbiotic females, reducing disease-transmitting mosquito populations. Source: Kaur R. et al, 2021

1.11.3 Incompatible Insect Technique (IIT)

The IIT [414,426,427], also known as Population Suppression, involves releasing symbiotic males that induce CI, leading to the inability to produce viable offspring when they mate with aposymbiotic females [407,411]. This disruption in the reproductive process results in the suppression of mosquito populations. Through continued release of infected males over time, the target mosquito population is reduced at the intended site. Multiple organizations are now deploying IIT including MosquitoMate (Kentucky, USA) [428], Verily (California, USA) [429], Commonwealth Scientific and Industrial Research Organization (Australia) [430], and Singapore's National Environment Agency (Singapore) [431]. Their efforts have demonstrated significant success, achieving a 95% population reduction in California, over 80% reduction in Australia, and a remarkable 90% reduction in Singapore.

1.12 Knowledge gaps and thesis objectives

Considering the benefits of the *Wolbachia*-introgression approach, evaluating factors that could undermine its efficacy in dengue prevention becomes a vital consideration [432,433]. Multiple studies have undertaken the task of dissecting the tripartite interplay *Wolbachia*, *Ae. aegypti*, and DENV. They have investigated into the stability of genomes within *Wolbachia*, *w*Mel strain inside *Ae. aegypti* in the regions where releases occurred. The findings of these investigations have been reassuring, indicating the maintenance of genome stability in both the *Wolbachia* and *Ae. aegypti* mosquito population [434,435]. Remarkably,

Wobachia- *w*Mel strain has retained its high prevalence within the *Ae. aegypti* population in these regions [436–438]. In addition, the greater *w*Mel-mediated virus blocking was observed in field- collected *w*Mel –carrying *Ae. aegypti* [261]. Distinct from the genetic stability of *Wolbachia* and *Ae. aegypti*, RNA viruses such as DENV possess high mutation rate. Given this characteristic, viruses are capable of accumulating genome mutations (variants) that facilitate replication within *Wolbachia*-carrying mosquitoes. These variants might experience rapid selection under *Wolbachia* pressure and are likely to persist in *Wolbachia-Ae. aegypti* population and then might exhibit robust replication within human hosts [439]. This raises a fundamental question: will DENV evolve to adapt to the antiviral property conferred by *Wolbachia* in *Ae. aegypti?* I will address this question in Chapters 3 and 4. In Chapter 3, I describe a robust whole-genome assay that enabled the recovery of complete viral genomes at various levels of viral abundance. This serves as the foundational tool to generate data for analysis in Chapter 4, where I investigate the evolutionary dynamics of DENV within *Wolbachia*-infected *Ae. aegypti*, employing a serial passage system.

The question regarding the role of vertically infected mosquitoes as potential reservoirs for subsequent horizontal virus transmission has been a matter of concern. While the existence of VT of DENV within *Ae. aegypti* is well-documented, the capacity of horizontal transmission of the virus acquired vertically to the human population remains unknown. By examining the potential for subsequent horizontal spread of vertically acquired virus, valuable insights could be gleaned for predicting and assessing transmission risk. Considering the regions where *Wolbachia*-infected *Ae. aegypti* has been established, a further fundamental question arises: Could *Wolbachia* influence this mode of transmission ? In Chapter 5, I will investigate the VT in wild-type and *Wolbachia*-infected *Ae. aegypti*, including the role of vertically infected mosquitoes as reservoirs for horizontal virus transmission.

The overarching aim of this thesis is to bridge these fundamental knowledge gaps in DENV transmission and provide evidence regarding the broader implications of *Wolbachia* introgression in preventing DENV transmission, encompassing the reduction of both vertical and horizontal transmission.

Chapter 2

MATERIALS AND METHODS

In this chapter, I describe the protocols applied in the three result chapters. The steps outlined in the Materials and Methods chapter provided the necessary input materials for conducting the research in Chapter 3, Chapter 4, and Chapter 5. Chapter 3 focused on developing the Whole Genome Sequencing method for DENV, which was then applied in Chapter 4 to investigate DENV evolution in *w*Mel-infected *Ae. aegypti*. Chapter 5 estimated the VT rate in field-reared *Ae. aegypti* and in *w*Mel-infected *Ae. aegypti*. More details on the specific methodology were described in the relevant chapters.

2.1 Mosquito rearing and maintenance

2.1.1 Floating eggs and rearing larvae

Filter papers containing mosquito eggs were floated into primary trays of fresh water. Mosquito eggs of each strain were floated in separate trays, labeled, and placed into an incubator. The trays were covered with a net to prevent mosquito from escaping and to prevent any loose mosquitoes in the incubator from laying fresh eggs in the trays. Water was changed daily, except for the first 2-3 days when dealing with small larvae. The larvae were maintained in the incubator at 28^{0}C, 65-85% relative humidity (rH) and equal length light: darkness (12hrs: 12 hrs). Water and fish food were changed or supplied depending on the developmental stage of the larvae (Table 2-1).

Table 2-1: The amount of food given to larvae at each development stage.

Development Stages	Condition	Food
Day 1: Floating Eggs	The filter paper containing mosquito eggs was placed in 1.5 L water (~10,000 eggs).	The eggs did not require any food at this stage, as they had not yet hatched, and providing food would have led to unclean water.
Day 2: Hatching Eggs		Each tray received a 100mg tablet of fish food.
Day 3 and 4: Controlling larval density	The larvae were split into 3 trays, with a density of approximately 3,300-3,500 larvae per 1.5L of water (equivalent to 1 larvae/0.45 ml of water).	Each tray received a 100mg tablet of fish food.
Day 5 and 6: Control the larval density	The larval density was continuously split and controlled. Each tray contained approximately 500 larvae in 1.5L of water (equivalent to 1 larvae/3 mL of water). Each day, they were provided with around 200mg of boiled shrimp and 200mg of boiled pork liver.	Boiled shrimp (200mg) and boiled pork liver (200mg) were provided each day

Day 7 to 10: Pupae collecting		A small amount of food (~50mg boiled shrimp + ~50mg boiled pork) was added to maintain the remaining larvae (pupae no longer feed.)

2.1.2 Pupae collection

After approximately 7-10 days, the larvae started to pupate. The collection of pupae varied based on the intended use of the mosquitoes (Table 2-2). After 6-7 days, 30-40 pupae were transferred to cups. Cups were coded as cup number, mosquito strains, and collecting date.

Table 2-2: Pupae collection methods based on future purpose of mosquitoes.

Purpose	Pupae Collection Method
Experimental work (i.e., blood feeding or inoculation)	The pupae were placed into the plastic cups with 20ml of water and covered by a net (with a limit of 45 females per cup).
For colony maintenance: Closed colonies	The required number of pupae of each sex in the same generation were transferred into a mosquito cage (400-500 pupae per gender). The mosquitoes were maintained for 4 weeks by providing both blood directly through feeding and a 10% sucrose solution.
For colony maintenance: Backcrossing	The females were 100% from the (hybrid) *Wolbachia*-infected population, while the males were 100% from the target population for back-crossing.

	These mosquitoes were allowed to emerge into the same cage, facilitating mating between them. As a result, the subsequent generations of progeny became increasingly similar to the target wild type population in terms of genetics. Direct blood feeding was provided to the mosquitoes during this process.
For colony maintenance: Outcrossing (To keep genetic diversity in the population, we performed outcrossing with 10% of male mosquitoes from the field)	All females were selected from the same population, while the number of available male pupae from that population was counted. To ensure sufficient mating opportunities, 10-20% more males were prepared from the F0/F1(*) wild type population. These two sets of males were put together for emergence. By including wild males, the females had the opportunity to mate with them, preserving genetic diversity. This process was repeated every two generations to prevent inbreeding or laboratory adaptation. Direct blood feeding was provided to the mosquitoes during this phase. (*) Mosquito generation: F1-the first generation, F2- the second generation

2.1.3 Emergence of adults

To collect the adult mosquitoes, an empty cup with a hole in the bottom was placed over the cups containing the pupae. The top of the empty cup was covered by tulle mesh. After two days, all the mosquitoes hatching from the pupae were collected in the upper cup, and the hole was covered by cotton wool. These cups containing the adult mosquitoes were

coded in the same way described above. Mosquitoes were maintained at 26-28°C, 65-85% rH, a length light: darkness (12hrs: 12 hrs), with access to 10% sucrose solution.

2.1.4 Maintaining adult mosquitoes

2.1.4.1 Mosquito maintenance for experiments

Mosquitoes were typically maintained for experiments by providing them with wet cotton wool soaked in a mixture of 10% sucrose solution and water. The mosquitoes aged 1-2 days were used for experiments. If the mosquitoes were scheduled for experiments the next day, they were only given water. However, if they were not yet required for experiments, they were provided with a combination of 10% sucrose and water. Removing sucrose from the mosquitoes 24 hours prior to experiments ensured their starvation and readiness to feed/receive inoculum during intrathoracic inoculations.

2.1.4.2 Blood feeding of mosquito colonies

The mosquitoes were given access to the arm or fingers of a volunteer for feeding. The temperature of the volunteers were checked each time to ensure they were less than 37.5°C prior to feeding the mosquitoes. Additionally, the mosquitoes had access to water and 10% sucrose solution, approximately three times per week. Each cage had a designated volunteer responsible for feeding, and their name was clearly labeled on the cage.

2.1.4.3 Collecting eggs for the next generation

The length of the gonotrophic cycle for *Ae. aegypti* mosquitoes was approximately 2-3 days. Typically, these mosquitoes were offered a blood meal every 2-3 days, around three times per week.

To collect mosquito eggs, strips of filter paper were placed inside the colony cages, within a cup containing approximately 7 mL of water. The water level was maintained at around halfway up the height of the paper, allowing the mosquitoes to lay their eggs just above the water line. Every two days, the eggs were collected by carefully removing the cup from the cage. The filter paper strip was replaced with a fresh one, and the water in the cup was replenished as needed. The cup was then returned to the cage to collect more eggs.

The collected filter paper strips, containing the eggs, were dried at room temperature for one day in individually labeled boxes to prevent contamination between different mosquito strains. The dried strips were then placed in small zip-lock bags, which were kept inside closed plastic boxes. The zip-lock bags remained open, while a small container with 25% KCl solution (34g KCl in 100ml H_2O) to maintain humidity. Each box was labeled with the mosquito strain, generation number, date of egg collection, and the name of the volunteer who fed the adult mosquitoes.

2.2 Establishment of DENV infection in *Ae. aegypti* mosquitoes

2.2.1 Preparation of Mosquito Diluent (MD)

Mosquito Diluent was used as a buffer for extracting mosquito saliva and preparing mosquito homogenates. To prepare 500ml of Mosquito Diluent (MD), RPMI-1640 (Sigma) and other components were used, with the corresponding volume listed in Table 2-3.

Table 2-3: Components of Mosquito Diluent (MD) in 500mL total of volume.

Components	Volume (mL)
Heat-inactivated FCS (Sigma)	10ml
Glutaminutese (Invitrogen)	5ml
Fungizone (Gibco)	5ml
Penicillin-Streptomycin (Invitrogen)	2.5ml

Gentamycin (Sigma)	0.5ml

2.2.2 Preparation of Squash buffer

To make the Squash buffer, the following components were mixed together in a bottle: 0.485g TRIS base, 0.149g EDTA, and 1.169g NaCl. The mixture was made up to a total volume of 400mL with distilled water, and the pH was adjusted to 8.2. Subsequently, the Squash buffer was autoclaved for sterilization. The final Extraction Buffer was prepared by adding Proteinase K to the Squash buffer at a ratio of 1:160.

2.2.3 Mosquito oral blood-feeding using DENV infected patient blood

2.2.3.1 Preparing the artificial blood meals

To prepare the artificial blood meals, the circulating water bath system was turned on until the water reached 37°C to maintain the warmth of the blood meals during mosquito feeding. Plastic cups covered with mesh netting contained approximately 30-50 naïve female mosquitoes.

To set up the mosquito exposures, nozzles were inserted on either side of glass feeders connected to rubber tubing. A prepared membrane feeder with tubing attached was gently placed on a mosquito cup and secured with a clamp to keep it in place. The membrane was positioned flush with the net, facilitating convenient access for mosquitoes from inside the cup. The second membrane feeder was carefully added to the top of a mosquito cup, secured with the clamp, and the chain of tubing and cups was extended. This process was repeated for as many feeders as there were mosquito cups. After connecting all membrane feeders and tubing, the circuit was connected back to the water bath, with one end of the series connected to the circulator of the water bath (the part that pushed water through the feeders), and the other end of the series consisted of rubber tubing submerged in the bath to

allow the circulated water to empty back into the bath. The water flow was activated to circulate through the feeders, maintaining the warmth of the blood.

The membranes, resembling skin, were prepared by tightly sealing small squares of stretched pig intestinal membrane to the bottom of glass feeders using rubber bands. Each cup was positioned correctly on stands, with one cup per stand. Inside the biosafety cabinet, 1mL of virus-blood meal was pipetted into the feeder's neck while holding it at an angle to allow air escape. The blood flowed through the neck into the reservoir, making contact with the membrane surface for mosquito access. After filling the feeders with blood, Parafilm was applied to cover their tops, minimizing evaporation.

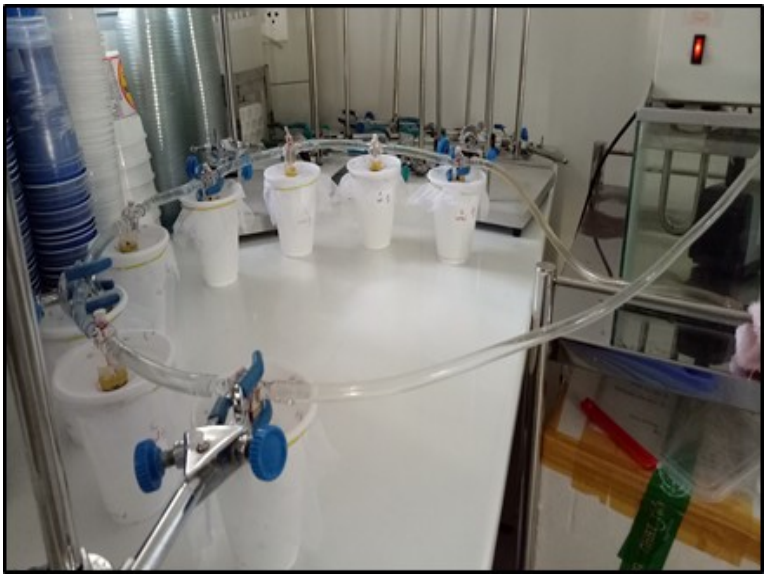


Figure 21: Preparing the artificial blood meals for mosquito feeding

2.2.3.2 Feeding and maintaining mosquitoes

Mosquitoes were allowed to feed for 30 minutes. After feeding, the mosquitoes were allowed to rest for a short time (~1-2 hours). Then, they were anesthetized by placing the cups in a refrigerator or cold room for about 1.5-2 minutes. Once fully anesthetized, the mosquitoes were transferred to a petri dish embedded in an ice tray. Only fully engorged females were collected and transferred to separate cups. The cups were placed inside a small cage covered with a cloth to create a dimly lit environment for the mosquitoes. They were then maintained under specific conditions, including a temperature of 28°C, relative humidity ranging from 65% to 85%, and a 12:12 hour light-dark cycle. Ad libitum sugar and water were provided. Mosquitoes were harvested around 7-14 days post-infection.

2.2.4 Collecting mosquito saliva [440]

After 24 hours of removing the sugar source, the cups holding the mosquitoes were immobilized. Subsequently, the mosquito cups were transferred to ice, ensuring complete immobility. The mosquitoes were then poured into a first petri dish, which was immediately covered.

Each mosquito was processed individually in a separate dish to prevent contamination and sample mixing. In the new petri dish, legs and wings of each mosquito were removed. Using sterile forceps, the proboscis of each mosquito was inserted into a filtered pipette tip containing a 1:1 solution of filtered inactivated fetal calf serum: sucrose 30%. The forceps were decontaminated with ethanol 70% between each sample. The volume of solution in the pipette tips did not exceed 5µL for collecting up to five mosquitoes with a single saliva sample.

The petri dish with the mosquito and pipette tip was placed in a tray filled with ice. The collection of the mosquito's saliva into the tip lasted 30 minutes. The collected saliva

were processed in two ways: stored in 2ml screw tubes at -80°C to preserve viral particles, or inoculated into naïve mosquitoes to test for virus presence.

2.2.5 Infecting mosquitoes by intrathoracic DENV inoculation [441]

To establish DENV infection in naive mosquitoes, various infectious sources were used, including filtered mosquito homogenate, laboratory strains of DENV, or mosquito saliva. The glass needle was inserted into the adapter of the injection system. The air valve was opened, and the plunger was compressed to expel any air from the syringe. Next, the sample intended for inoculation into the naïve mosquitoes was picked up and pipetted onto a clean glass slide. The needle was positioned at a 45° angle and inserted into the inoculum drop, while the plunger was pulled to draw the fluid into the needle, ensuring no air was trapped in the tip. The needle tip was gently inserted just below the exoskeleton at the injection site. The plunger was then compressed to inject 1µl of inoculum into the mosquitoes. The abdomen distention of the mosquito indicated successful inoculation.

The inoculated mosquitoes were anesthetized and placed in a new cup. The cup was then placed back on ice to keep the mosquitoes sedated until all were inoculated. The cup was labeled with information including patient code, mosquito strain, passage number, cohort number, and injection date. The mosquitoes were maintained with sucrose for 7 or 14 days.

2.2.6 Mosquito tissue collection and homogenization

2.2.6.1 Mosquito tissue collection

Mosquitoes derived from the same infectious sources (patient blood, infectious saliva, or mosquito homogenates) were anesthetized and placed in one petri dish. The petri dishes were placed into a big tray filled with ice. Mosquito whole bodies were collected and stored

individually in PCR-96 well plates or as pooled samples in tubes. The forceps were decontaminated with ethanol between each sample.

2.2.6.2 Mosquito homogenization [442]

Approximately 20-30 silica beads and the appropriate volume of buffers (MD or extraction buffer) were distributed into the sample containers. For pooled samples containing 10-20 mosquitoes, 1 mL of buffer was used, while for individual mosquitoes, 0.1 mL of buffer was added. The plate or tube was then homogenized at 30 Hz using the TissueLyser II for 2-5 minutes. After homogenization, the plate or tube was centrifuged at 1,400rpm for 1min. The mosquito homogenates were then processed in different ways, depending on the experimental objectives (Table 2-4).

Table 2-4: Buffers for Mosquito homogenization and processing of mosquito homogenates.

Using extraction buffer (Squash buffer)	Using MD
The homogenised sample plate was incubated at • 56°C for 10 minutes • 98°C for 15 minutes • Cooled down to 12°C. All of this was performed in a 96-well thermocycler. Then, stored at -20°C for up to 3 days for running the PCR.	The homogenates was be used for purified DNA/RNA extraction (store in -80^{0}C) or inoculation (keep on ice) procedures in later step.

2.3 Cell maintenance

2.3.1 Cell lines and cell media

The C6/36 cell, which was used in this study, is an insect cell line derived from Aedes albopictus, C6/36 (ATCC® CRL-1660™). The human cell lines used were hematopoietic malignant cells (K-562, ATCC® CCL-243™) and human monocytic cells (THP-1, ATCC® TIB-202™). The cell lines were propagated and maintained in the medium listed in Table 2-5.

Growth medium (GM) – Total 500mL	Maintain medium (MM) - Total 500mL
RPMI1640 (Sigma), 10% inactivated Fetal Bovine Serum (IFBS), 1% Glutaminutese, 1% Penicillin and Streptomycin (Sigma)	RPMI1640 (Sigma), 5% IFBS, 1% Glutaminutese, 1% Penicillin and Streptomycin (Sigma)

Table 2-5: Cell culture media components

2.3.2 Maintaining insect cell lines- C6/36

Cell confluence was checked before passaging. The spent cell culture media was removed and discarded from the T-25 flask. Cells were gently washed with sterile Phosphate Buffer Saline (PBS) 1x without disturbing the cell layer. The cell layer was detached by adding 0.5mL warmed Trypsin to the flask and incubated at room temperature for approximately 2 minutes. Once 80% of the cells had detached, 2mL of GM was added to the flask, and the medium was dispersed by pipetting over the cell layer surface several times. The cells were then transferred to a 15mL Falcon tube and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was re-suspended in 5mL of GM, and the resulting cell suspension was divided into 3-4 new T-25 flasks. The flasks were returned to the incubator set at 28°C

without CO_2. The sub-culture was performed when their confluence reached approximately 70-80%.

2.3.3 Maintaining suspension cells, THP-1 and K562

The cells were transferred to a 15mL Falcon tube and centrifuged at 1,500 rpm for 5 minutes. The resulting cell pellet was re-suspended in 1mL of GM. The total number of cells was determined using a hemocytometer. The required volume of media needed to dilute the culture down to a concentration of 10^4 cells/flask/5mL of pre-warmed GM was calculated. Subsequently, the cells were returned to the incubator set at 37°C with 5% CO_2. For both THP-1 and K562 cell lines, the subculture was performed when the cells were in the log-phase of growth, before they clumped together and the medium appeared turbid.

2.4 Cell infection and virus titration

2.4.1 Seed culture plate

Cells (C6/36, THP-1, and K562) were seeded in a well culture plate at a concentration of 10^6 cells/well. The plates were gently rocked back and forth and from side to side to ensure even distribution of the cells. The plate was then incubated at 28°C for 24 hours. For THP-1 and K-562 cells, 5% CO_2 was used during incubation, while C6/36 cells did not require CO_2. Subsequently, the cells were visualized under a light microscope to confirm that they were evenly distributed and had reached over 80% confluency.

2.4.2 Cell infection

The GM was aspirated, and the wells were subsequently rinsed 2-3 times with PBS, followed by two additional rinses with maintenance medium MM. Next, 0.2ml of each sample was introduced into the wells. For the mosquito homogenate samples, they were filtered before being introduced to the cells. The plate was then incubated for a period of two

hours, allowing for the adsorption of the inoculums at the appropriate temperature suitable for each specific cell line. After the adsorption period, MM was supplemented to each well, resulting in a final volume of 2ml per well. The plate was then incubated for 6-7 days, and daily observations were made for cytopathic effects (CPE), which typically manifested around 2-3 days of incubation. Following the observation period, the detection of DENV was performed using RT-PCR.

2.4.3 Harvesting supernatant

The cell supernatant was poured into a Falcon tube and centrifuged at 1,500 rpm for 5 minutes. Following centrifugation, the supernatant was transferred to a new Falcon tube. FBS was added to the supernatant, bringing the final volume to 2% of the supernatant volume for storage. The supernatant was aliquoted into sterile tubes, with each tube containing 100-500µl of the supernatant. Proper labeling was done on each tube, and they were subsequently stored at -80°C.

2.4.4 Titrating the virus concentration using Tissue Culture Infectious Dose (TCID50)

2.4.4.1 Seed culture plate with C6/36

C6/36 cells were seeded in a 96-wells plate at a concentration of 105 cells/well. The plates were gently rocked back and forth and from side to side to ensure even distribution of the cells. The plate was then incubated at 28°C for 24 hours, without CO_2. Subsequently, the cells were visualized under a light microscope to confirm that they were evenly distributed and had reached over 80% confluency.

2.4.4.2 Prepare serial dilutions of virus

A series of dilutions at a 1:10 ratio of the original virus sample was prepared. The first tube in the series was filled with 2.0 ml of MM, while the remaining six tubes were filled

with 1.8 ml of MM. The virus sample was vortexed, and then 20µl of the virus was transferred to the first tube. The original virus stock was briefly mixed and a 1:100 dilution was made by transferring 20µl of the virus stock to the first tubes of each column containing 1.8ml of MM. Subsequently, 200µl of the diluted virus was transferred to the second tubes in the series. This process was repeated to create a serial 1:10 dilution of the virus, ranging from 10^{-2} to 10^{-7}.

2.4.4.3 Infecting cell monolayer

The lid of the 96-well dish was labeled by drawing grid lines to define quadruplicates, and each grid was numbered to correspond to the virus sample. Additionally, four negative wells were included on each plate. The GM in each well was carefully discarded, and the plate was washed three times using 200µl of PBS and then two times with MM. Then, 50µl of the virus dilution was gently added to each well, infecting four wells per dilution. The plate was incubated at 28°C without CO_2 for 2 hours to allow the virus to adsorb to the cells. After the adsorption period, the virus dilution was discarded, and the wells were washed four to five times with MM. Next, 200µl of MM was added to each well, and the plates were returned to the incubator at 28°C without CO_2 for the observation of CPE over a period of five to six days.

2.4.4.4 Visualization and calculation of TCID50

All supernatants were collected and subjected to quantification of DENV genome copies using RT-PCR [4]. The titer was determined using the Muench and Reed method [443,444].

2.5 Virus quantification and *w*Mel detection in *Ae. aegypti* mosquitoes carrying *w*Mel

2.5.1 Quantitation of dengue viremia in patient plasma

Viral RNA was extracted using MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) on the MagNA Pure 96 System. The nucleic acid was stored at -200C. Viremia in patient plasma was quantified via RT-PCR as described previously [91].

2.5.2 Detecting the presence of DENV in *Ae. aegypti* and assessing density of *w*Mel in *Ae. aegypti*

The presence of DENV, and *w*Mel in mosquito homogenates was tested by one step qualitative multiplex real-time RT-PCR. The primers detecting *w*Mel located in WD0513 region of the *Wolbachia* genome [445]. The primers and probes of generic PCR for DENV detection were designed in 3'UTR region [355]. The *Ae. aegypti* rps17 gene was amplified and used as the internal control in the real-time RT-PCR reaction [446]. The RT-PCR was performed using LightCycler® Multiplex RNA Virus Master (Roche). The density of *w*Mel was presented as the ratio of wsp/rps17. The calculation used the delta CT method as described previously [447]. The components of real-time RT-PCR and the thermal cycling are described in Table 2-6.

Table 2-6: PCR reagents and standard cycling conditions for real-time RT-PCR detecting DENV and *Wolbachia, w*Mel.

Components	Volume (µL)	Final concentration
Molecular water	7.9	1x
qPCR reaction mix	4	1x
RT-Enzyme	0.1	0.1µM
Ae. aegypti Forward (10µM)	0.2	0.1µM
Ae. aegypti Reverse (10µM)	0.2	0.1µM

Ae. aegypti Probe-Cy5 (10µM)	0.2	
*w*Mel Forward (10µM)	0.4	0.2µM
*w*Mel Reverse (10µM)	0.4	0.2µM
*w*Mel Probe-HEX (10µM)	0.3	0.15µM
Den Group Forward (10µM)	0.5	0.25µM
Den Group Reverse (10µM)	0.5	0.25µM
Den Group Probe-FAM(10µM)	0.3	0.15µM
RNA/ DNA	5	
Thermal cycling name	**Temperature (^{0}C)**	**Number of cycles**
Reverse transcription	58^{0}C/ 30 minutes	1
Denaturation	95^{0}C/ 1 minute	1
Amplification	95^{0}C/ 15 sec	40
	60^{0}C/ 15 sec	
Cooling	37^{0}C/ 2 minutes	1

2.6 Biosecurity and biosafety measures

The specific recommendations on the design, construction, and use of a CL2 Insectary were based on guidelines provided by the American Committee of Medical Entomology (ACME) of the American Society of Tropical Medicine and Hygiene. Additional standards in facility design and work practices were followed in accordance with the Australian and New Zealand Standards (AS/NZS), with Oxford University Clinical Research Unit (OUCRU) implementing equivalent compliance measures.

Chapter 3

A SEROTYPE-SPECIFIC AND TILED AMPLICON MULTIPLEX PCR METHOD FOR WHOLE GENOME SEQUENCING OF DENGUE VIRUS

Abstract

Dengue is a major mosquito-borne disease problem in most of the tropical world. Dengue is caused by any of the four serotypes of dengue virus (DENV1-4). Cutting-edge technologies like Next Generation Sequencing (NGS) are revolutionizing virology, allowing in-depth exploration of DENV's genetic diversity. The sequencing methodology developed in this study utilized the multiplex tiling PCR approach for targeted DENV genome sequencing. The libraries were sequenced using Illumina's MiSeq platform. The assay demonstrated its ability to recover the full-length dengue genome across various viral abundances in clinical specimens with high-quality base coverage. The assay was employed to investigate the genome evolution of DENV in *w*Mel-infected *Ae. aegypti* in Chapter 4.

3.1 Introduction

DENV, belonging to the *Flavivirus* genus, comprises four serotypes (DENV1-4) and is responsible for a significant number of human infections worldwide [448]. DENV exhibits continuous evolution, although its rate of evolution is relatively slower compared to other single-stranded RNA viruses [449,450]. The lack of proofreading ability in the RNA-dependent RNA polymerase contributes to the diversification of genomic sequences within infected individuals [151] or viral quasispecies [150]. Multiple evolutionary forces, including genetic mechanisms, demographic processes, and natural selection, shape the viral population [60,151,159,171–173,179,180,193,194,451].

Next-Generation Sequencing technologies have revolutionized the study of DENV, enabling in-depth investigations into virulence, transmission, and host interactions [452–456]. Researchers employ metagenomic sequencing or target enrichment sequencing methods for viral sequencing. The target enrichment techniques falling into two categories: hybrid capture enrichment and amplicon-based enrichment [454,457–459]. Amplicon-based enrichment has proven indispensable in identifying, and screening genetic variants. Multiplex tiling PCR-based amplicon sequencing, offers high specificity and coverage while addressing technical challenges associated with sequencing emerging infectious diseases [460,461]. This approach is cost-effective, time-efficient, and enhances research, diagnostics, and viral disease surveillance. Nonetheless, it can introduce coverage gaps and targeted region dropouts, necessitating the reduction of coverage bias [462]. Moreover, the challenges of failing to achieve the full-length genome and accurately detecting low-abundance viruses with a cycle threshold (Ct) > 29 have been documented [462,463]. Additionally, the correlation between viral load and the proportion of viral reads can impact the accuracy of variant calling [464,465].

Here, we have developed a Whole Genome Sequencing (WGS) assay for DENV (DENV1-4) using an Amplicon Multiplex tiling PCR approach. This assay specifically targets the DENV genome, allowing for the sequencing of dengue genomes from human clinical specimens and mosquito samples. The primary objective of this study is to develop a reliable WGS assay for DENV (DENV1-4) that facilitates the sequencing of dengue genomes derived from clinical samples with moderate and low viral loads.

3.2 Material and Method

3.2.1 Virus stocks and clinical DENV specimens

To develop and validate the assay, we used the cultured strains of DENV serotypes 1 to 4 (DENV-1-4) isolated from Vietnamese patients. The corresponding genomic sequences of these strains were deposited in GenBank under the following accession numbers: DENV-1 (Genotype 1, FJ432734, 10,689bp, CDS = 10,179bp), DENV-2 (Asian I, EU482447, 10,678bp, CDS = 10,176bp), DENV-3 (Genotype II, EU660409, 10,663bp, CDS = 10,173bp), and DENV-4 (Genotype I, KY924607, 10,161bp, CDS = 10,161bp). These genomic sequences were generated by the Broad Institute Genome Sequencing Platform; Broad Institute. Microbial Sequencing Center; through the project Genome Resources in Dengue Consortium [466–468]. Viremic plasma from dengue patients were included in the evaluation. Additionally, we examined the genomes of DENV in thirteen homogenates of *Ae. aegypti* mosquitoes that were infected through oral-feeding and inoculation methods [190]. The DENV genome copy number were quantitated using a validated real-time RT-PCR [91].

We utilized low-passage isolates of DENV from virological-confirmed dengue cases from the phase III trials of the tetravalent dengue vaccine, CYD-TDV, which had previously undergone sequencing of the PrM-E gene using amplicon-based sequencing on 454 sequencers [469]. In this study, we performed the WGS protocol on these samples to assess

the capability of generating WGS on different genotypes for each DENV serotype. We accomplished this by comparing the Envelope (E) sequences from the new WGS method with the previously obtained E gene sequences. We selected viruses representing the prevalent genotypes in dengue endemic countries in Asia and the Americas.

3.2.2 Developing Whole Genome Sequencing (WGS) assay for DENV-1-4

3.2.2.1 Primer design

In the process of primer design, primer panels were designed using a tiling scheme adapted from the method described in reference [460]. Primer specificity was evaluated using Primer BLAST [470]. Through iterative rounds of computational mapping, screening, and manual refinement our primary objective was to target highly conserved regions, ensuring the efficacy of the primers.

By integrating degenerate bases strategically, we effectively managed mutant sites and addressed issues like self-dimers, cross-dimers, hairpins, and mismatches through manual modifications. To enhance functionality, we implemented ambiguity codes for nucleotide degeneracy, specifically for 3'-end mismatches, starting from the 3rd nucleotide mismatch position onward, with a maximum limit of 4 ambiguity codes within the primer. Additionally, primers were carefully designed with a similar annealing temperature, enabling their seamless use in a single reaction tube.

3.2.2.2 RNA extraction and cDNA synthesis

For RNA extraction, a volume of 140µL of the specimen was used, and viral RNA was eluted in 50µL of elution buffer. The MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, 06543588001) in the MagNA Pure 96 System were used to isolate viral RNA. The purified RNA was either immediately stored at -80°C or subjected to cDNA synthesis

using the SuperScript IV First-Strand Synthesis System (Cat # 18091200, Thermo Fisher Scientific).

3.2.2.3 Multiplex tiling PCR amplification

Full-length dengue genomes were generated through two multiplex PCR reactions utilizing two pools of primers. The concentration of the primers in the pools was adjusted to achieve complete coverage of coding sequences within the genomes. The two multiplex PCRs were performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, M0491L).

3.2.2.4 DENV WGS library construction using Nextera XT DNA Library preparation

The template DNA sample was used as input for library construction using the Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1024 or FC-131-1096). Library quantification was performed using the KAPA Library Quantification kit (Roche, KK-4824), and the quantity of each library was normalized to ensure equal representation in the pool sample. After the library pooling step, the library was ready for sequencing using the MiSeq kit (Illumina). The optimization procedure was initially conducted using virus stocks, and subsequently, the complete protocol was applied to clinical and mosquito samples.

3.2.3 Workflow for optimization and performance evaluation of DENV sequencing

Figure 3-1 illustrates the workflow for optimization and performance evaluation. Briefly, four virus stocks representative of the four DENV serotypes, along with viremic plasma from patients and DENV-infected mosquito samples, were used to generate DENV WGS libraries. We utilized isolates from all four DENV serotypes (DENV-1-4) for optimization. Different DENV serotypes 1 to 4 (DENV -1-4) inputs, low vs. high, were used to evaluate the capability to retrieve DENV genomes in virus supernatants at various levels of viral abundance. The plasma samples from the dengue patient and mosquito samples were

utilized to evaluate the ability to recover DENV genomes in clinical specimens. The DENV WGS libraries were sequenced in a pair-end on the Illumina MiSeq platform. We benchmarked the performance of the protocol in terms of mappability, viral genome coverage (%), uniformity, and reproducibility.

There were three different runs for the whole project. The first run included 29 libraries, with 14 samples being run as duplicates. The second run contained 25 libraries, with three different dilution levels for each serotype, and each sample was run in duplicate. The third run included 78 samples, consisting of plasma samples and DENV-infected mosquitoes. Negative controls, prepared from the amplicon step, were run in parallel with the samples to ensure procedural quality and were included in the sequence run.

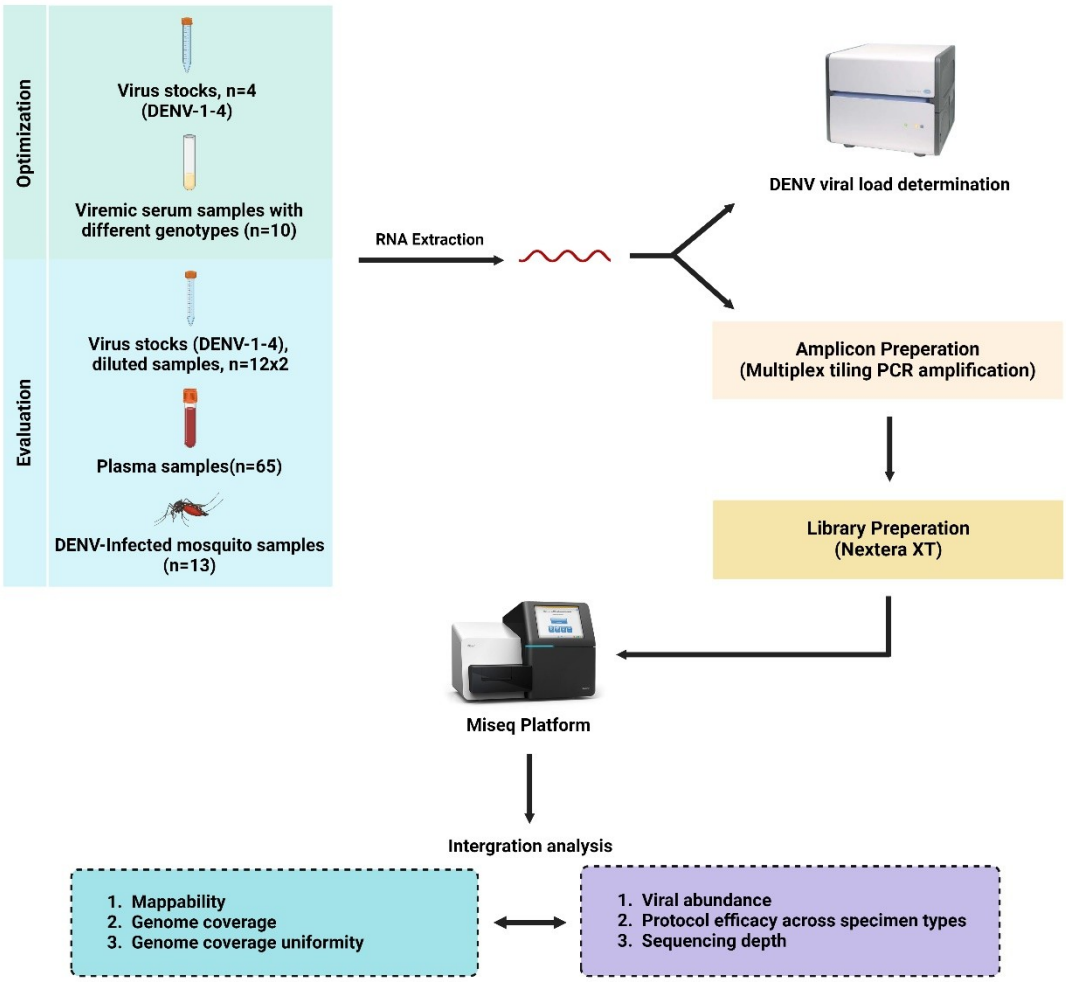


Figure 31: Schematic overview of the optimization and performance evaluation

DENV virus stocks of each serotype were employed in constructing DENV whole-genome sequencing (WGS) libraries through a multiplex tiling PCR approach. The level of viral abundance that is efficient for the protocol was evaluated. Seventy-eight samples (65 plasma samples and 13 mosquito samples) were used to evaluate the performance of the protocols. The capability of the specific serotype primer panels to capture genotypes was also evaluated using viremic serum samples. The performances of protocols were evaluated based on viral input, sequencing depth, mappability, viral genome coverage and coverage uniformity.

3.2.4 Downstream bioinformatics analysis (Figure 3-2)

3.2.4.1 Read trimming and filtering

Sequencing reads was pre-processed for subsequent analysis. This involved conducting quality control on raw reads and performing read trimming, which entailed removing adapter sequences, filtering out low-quality reads, and trimming off low-quality base pairs. Adapter sequences were removed from the 3' ends of the raw reads using the adapter trimming option available on the Miseq. CLC Genomics Workbench version 9.0 was used for quality control, trimming, de novo assembly, and read mapping [471].

Raw reads were trimmed based on a quality score limit of 0.05 and ambiguous (N) characters were removed with a limit of 2 allowed per read. Quality scores were calculated on a Phred scale and converted to base-calling error probability. A base trim operation removed 20 nucleotides at either the 3' or 5' end of the sequence reads, while a length trimming operation discarded reads shorter than 100 nucleotides.

3.2.4.2 Generating reference sequences for mapping

After de novo assembly of the trimmed reads, the longest contiguous sequence was blasted to the nucleotide collection in GenBank to identify the reference genomes with the highest similarity. This pre-selection step enhances the probability of choosing an appropriate reference for read mapping, thereby improving the accuracy of the analysis.

3.2.4.3 Reference mapping and extract consensus sequence

The trimmed reads were subsequently aligned to a selected reference homologous DENV sequence in order to generate a complete genome sequence suitable for reference-based mapping. The bioinformatics analysis followed the Quality Control Guidance for NGS Workflows [471] wherein the length fraction parameter was established at 0.5. This criterion specified that a read needed to correspond to at least half of the reference sequence to qualify for inclusion in the final mapping. A minimum fraction of identity, requiring a 50% match

between the read and the reference sequence, was also applied. Consensus sequences were extracted, employing a coverage threshold of 20X.

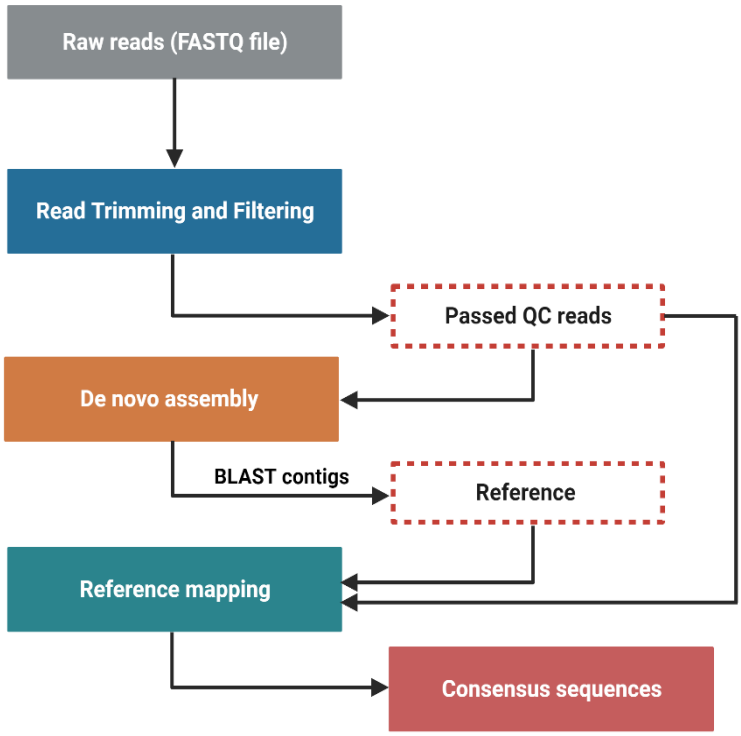


Figure 32: Downstream analysis

3.2.5 Assessment parameters

To assess the quality of sequencing runs, several parameters were evaluated, including the mapping rate, base coverage (maximum, median and minimum), the Phred quality score of Q30 (expressed as % Q30), viral genome coverage (presented as a percentage), and genome coverage uniformity (per position).

The mapping rate indicates the proportion of reads uniquely aligned to the DENV reference genome. Mapping rates above 80%, as indicated in [472], are considered excellent, while rates lower than 50% are concerning. The Q30 is considered a benchmark for quality in NGS, signifying that the probability of an incorrect base call is 1 in 1000. A minimum Q30

Phred quality score of 70% indicates that the sequencing data has a high level of accuracy and reliability [473]. Viral genome coverage indicates the length of genomes compared to the expected DENV genomes generated from the specific primer sets: DENV-1: 10,372bp, DENV-2: 10,433bp, DENV-3: 10,462bp, DENV-4: 10,365bp.

3.3 Results

3.3.1 Primer design for DENV-1 to DENV-4

Our goal was to maximize the assay's capacity to capture a wide range of DENV strains. To achieve this, we conducted primer alignments with a substantial dataset of representative DENV sequences, encompassing DENV-1 (1,310 sequences), DENV-2 (1,753 sequences), DENV-3 (805 sequences), and DENV-4 (250 sequences). This comprehensive alignment covered multiple regions, including Southeast Asia, with a specific focus on Vietnam, as well as other geographical areas such as Asia (India, China, Singapore, Thailand, Myanmar, Indonesia, Philippines, Cambodia) and the Americas (Brazil, Venezuela, Mexico, Nicaragua, USA).

The primer sets for DENV-1, DENV-2, DENV-3, and DENV-4 comprised 36, 35, 30, and 42 primer pairs, respectively, with an annealing temperature of 60°C (Appendix 1). The majority of primers in the primer pool were formulated with a working stock concentration of 10µM per primer. To achieve genome coverage uniformity, the concentration of several specific primers in the pool was increased to 20µM and 40µM. The primers were divided into two primer pools, Pool 1 and Pool 2, with equal volumes for each primer. Notably, the primers in Pool 1 and Pool 2 exhibit overlapping but not within reactions. Each primer pool was dedicated to a single PCR reaction, producing amplicons of 400-700bp with at least 100bp overlap between adjacent amplicons.

3.3.2 Amplicon preparation

3.3.2.1 cDNA synthesis

All cDNA synthesis reactions were made up on ice and were performed in 0.2µL 8-strip tubes in the Eppendorf Mastercycler Nexus. DENV RNA was subjected to cDNA synthesis using SuperScript IV First-Strand Synthesis System (Cat # 18091200, Thermo Fisher Scientific). The cDNA synthesis reactions were completed by two separate reactions and the final volume of reaction was 20µL. The components of cDNA mix and cycling program were described in the Table 3-1.

Table 3-7: Thermal cycling conditions and PCR reagents for cDNA synthesis

Components	**Volume (µL)**	**Final concentration**
Mix 1:		
Molecular water Random Hexamer (10ng/µL) dNTPs (10mM) RNA	3 1 1 8	 0.5ng 0.5mM of each
Pipette up and down to mix and heat the Mix 1 at 70^0C for 7 minutes. Set the Mix 1 reaction tubes on ice directly for 2 minutes.		
Mix 2:		
5x SSIV buffer DTT RNA inhibitor (40U/ µL) SSIV Reverse Transcriptase (200U/ µL)	4 1 1 1	1x 1x 2U/µL 10U/µL
Distribute 7µL of Mix 2 into the Mix 1 reaction tubes. Mixing up and place the cDNA tube in the thermal cycler.		
Temperature (^{0}C)	**Duration**	

23 50 55 80 4	10 minutes 45 minutes 15 minutes 10 minutes 2 minutes	
Add 1µL of RNAseH into cDNA reaction tubes and then incubate at 37^{0}C for 20 minutes. The RNAse H was added later to destroy remaining RNA in the RNA: cDNA hybrid. The cDNA tubes were stored at -20^{0}C.		

3.3.2.2 Amplicon amplification

The complete whole genome for each serotype was amplified in two multiplex PCR reactions using a high-fidelity DNA polymerase (Cat # M0491L, New England Biolabs). The same thermocycling conditions were applied to all four serotypes, Table 3-2. PCR amplification products were visualized by gel electrophoresis.

Table 3-8: PCR components and thermal program for DENV amplicon PCR

Components	**Volume (µL)**	**Final concentration**
Molecular water	14.25	
Pool 1-forward (10µM of each primer)	1	0.4µM
Pool 1-reverse (10µM of each primer)	1	0.4µM
5x Q5 reaction buffer	5	1x
dNTPs (10mM of each)	1	0.4mM
Q5® High-Fidelity DNA Polymerase	0.25	0.02U/µl
cDNA	2.5	
The reaction components of Pool 1 and 2 was similar and the total volume was 25µL.		
Thermal cycling name	**Temperature (^{0}C)**	**Number of cycles**
Pre-heat	98^{0}C/ 2 minutes	1

Amplification	95^0C/ 15 seconds 65^0C/ 15 minutes	35
Cooling	4^0C/ 2 minutes	1

3.3.2.3 Amplicon Quantification and Pooling

The amplicons were purified following the instructions provided by the Agencourt AMPure XP kit (A63882, Beckman Coulter). A volume of 30µL of TE buffer was added to recover the purified amplicons. The concentration of each amplicon was determined using the PicoGreen dsDNA quantitation assay kit (P7589, Invitrogen). To create the DNA standard for quantification, it was thawed from a concentration of 100ng/µL (100µg/mL). A dilution series was then prepared to establish the standard curve. For the standard curve, 100µL of each DNA standard dilution was transferred to eight wells, with two duplicated rows as shown in Table 3-3.

Table 3-9: The amounts of DNA per standard well

Tube #	**Well (100µL+100µL)**	**Dilution factor**	**DNA concentration**
Tube 1 (1ng/µL)	A1-2	100	100ng/well
Tube 2 (0.5ng/µL)	B1-2	2	50ng/well
Tube 3 (0.25ng/µL)	C1-2	2	25ng/well
Tube 4 (0.125ng/µL)	D1-2	2	12.5ng/well
Tube 5 (0.0625ng/µL)	E1-2	2	6.25ng/well
Tube 6 (0.0313ng/µL)	F1-2	2	3.13ng/well
Tube 7 (0.0156ng/µL)	G1-2	2	1.56ng/well
Tube 8 (blank)	H1-2		0.00ng/well

Next, 100µL of a 1:200 dilution of the Picogreen reagent in 1X TE buffer was added to each well containing the diluted amplicons (100x). Careful mixing was performed while ensuring protection from light at all times during the process. Subsequently, the fluorescence of each sample was measured using the FLUOstar OPTIMA instrument, with the emission wavelength set to 520 nm for FAM detection.

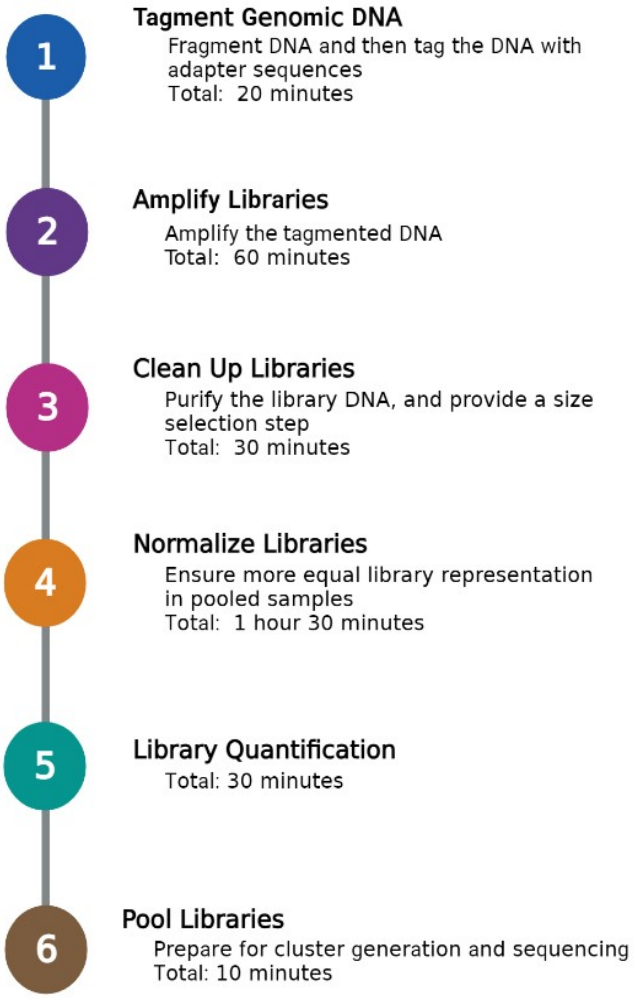
To standardize the DNA concentration, all amplicon samples were adjusted to a concentration of 4ng/µL. After the adjustment, an equal volume of each diluted amplicon derived from the same sample source, was combined. The resulting pooled sample, containing the two PCR products from a single sample, was stored at -20°C and used as the input for library construction.

3.3.3 Library preparation

After DNA quantification and dilution to 4ng/µL for each sample, the samples were used as input template for library construction, using Nextera XT DNA sample preparation kit (FC-131-1024 or FC-131-1096, Illumina). The library preparation process involved various steps, including tagmentation, adaptor ligation, library amplification, cleaning up, normalization, and library pooling (Figure 3-3). The library preparation involved six steps following the kit instructions.

MiSeq libraries were constructed using the Nextera transposome (1) and conducted at 55^0C/ 4 minutes. Ech amplicon pool was labbeled by dual-barcoded by combining one P5 barcode and one P7 barcode in each ready-to-use adapter (2). After library amplification, the reaction cleanup and removal of adapter-dimers were achieved by using Agencourt AMPure XP (Cat # A63881, Beckman Coulter) (3). Library quantification followed normalization step using KAPPA Library Quantification kit (Cat # KK4824, Roche) (4+5). After a library pooling step, the library then was ready to be sequenced using MiSeq v2 300 cycles (Illumina, MS-102-3002) or MiSeq v3 600 cycles (Illumina, MS-102-3003). The amount of input library

was within a range from 1.5 to 2nM. The optimization procedure was performed using virus cultures and the complete protocol was applied for clinical samples then.



3.3.4

Figure 33: Workflow for Whole Genome Sequencing (WGS) of DENV-1-4 using the Nextera XT library kit and MiSeq platform.

Generation of full-length sequences

The genomic sequences of four DENV serotypes were re-constructed using laboratory cell- adapted strains that were previously deposited in GenBank. The trimmed reads underwent de novo assembly and produced contigs with length ranging from 6,000 to 7,000bp. The longest contiguous sequences were compared to the nr/nt nucleotide collection in GenBank. The re-sequenced samples exhibited the highest similarity to four previously sequenced genomes derived from the same virus culture samples: DENV-1 (FJ432734), DENV-2 (EU482447), DENV-3 (EU660409), and DENV-4 (KY924607). These re-sequenced samples provided consensus sequences with a coverage depth of 20x, which were identical to their original sequences. The consensus lengths of DENV-1, DENV-2, DENV-3, and DENV-4 were 10,299bp, 10,344bp, 10,312bp, and 10,285bp, respectively and represented the high genome coverage > 95%, without gaps and 100% coverage of CoDing Sequence (CDS). The genome uniformity of DENV-1 -4 is presented in Figure 3-4. The mapping rates for all serotypes exceeded 90%, with consistently high base coverage of over 1,000 reads. The %Q30 value surpassed 90% for all serotypes (Table 3-4).

Table 3-10: Statistics table for a read mapping of DENV 1-4 sequencing using virus stocks

		DENV-1 (FJ432734)	**DENV-2 (EU482447)**	**DENV-3 (EU660409)**	**DENV-4 (KY924607)**
Mapping Rate (%)		99.1	95.2	96.8	98.6
Coverage	**Minimum**	2,021	1,159	1,653	1,780
	Maximum	10,691	15,605	11,130	19,227
	Mean	3,180	7,144	5,139	7,135
% Q30		96	92	93	98

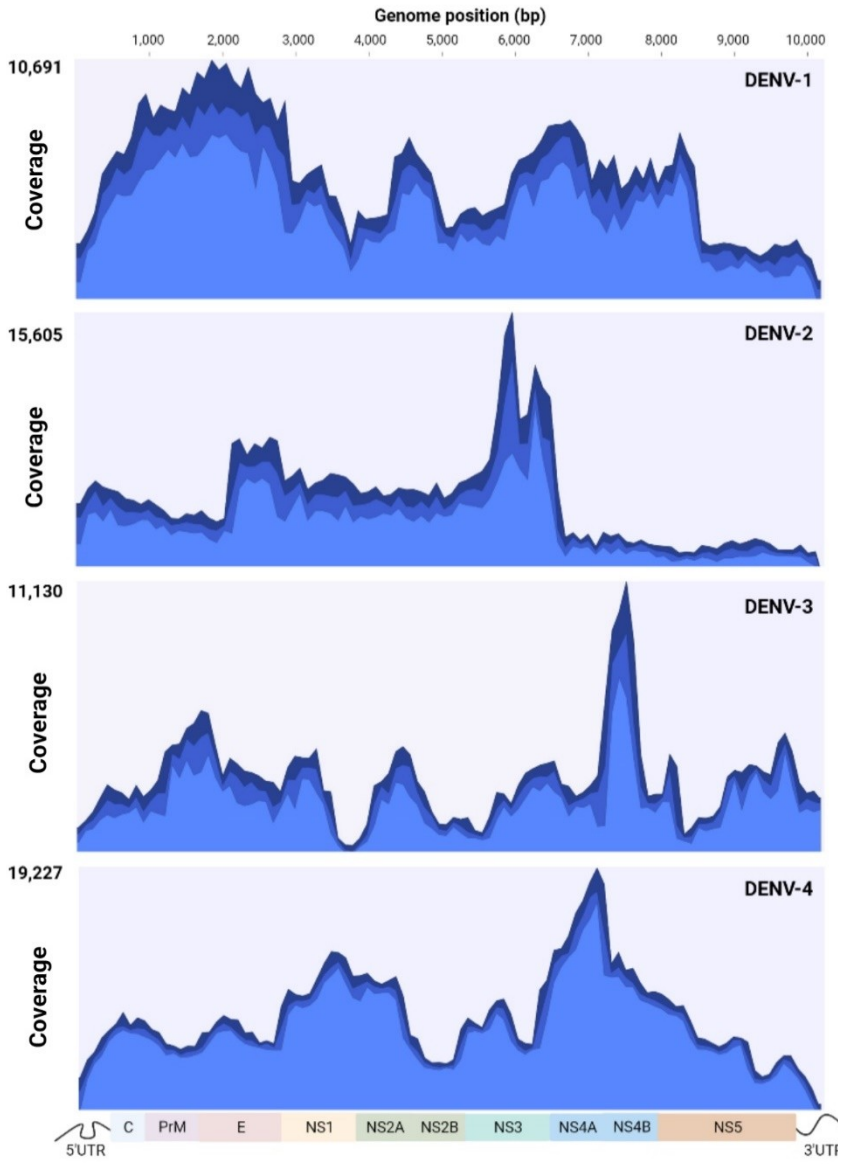


Figure 34: Reads track graph of read mapping.

The Reads Track displays three shades of blue, representing aggregated mapped reads. The top shade indicates the maximum coverage value (read count), the middle shade represents the average coverage value, and the bottom shade corresponds to the minimum coverage value within the specified aggregation bucket.

3.3.5 Assessing the capability to capture various DENV genotypes across different geographic regions

We used 10 serum samples collected from virologically-confirmed dengue cases in two cohorts: CYD14 (Indonesia, Philippines) and CYD15 (Mexico, Colombia, Brazil). The detailed mapping results are found in Appendix 2. We evaluated DENV genotypes, including DENV-1 (Genotype I, Genotype IV, Genotype V), DENV-2 (Asian/American, Cosmopolitan), DENV-3 (Genotype I, Genotype III), and DENV-4 (Genotype II).

The re-sequenced samples yielded complete consensus sequences, each exceeding 10 kb in length, and achieved an average genome coverage of 98.9%, with no gaps and complete coverage of the CDS. The mapping rate averaged at 97%, with a robust base coverage of over 1,000 reads. Additionally, %Q30 surpassed 95% for all samples. When comparing the E gene sequences, these re-sequenced samples from the WGS protocol matched the original sequences accurately. This result strongly confirms that our serotype-specific primers were strategically designed to encompass a wide spectrum of dengue genotypes and serotypes, not limited to Vietnam but also extending to other countries.

3.3.6 The capability to retrieve dengue viral genomes in virus supernatants at different levels of viral abundance

To assess the ability to recover DENV genomes at different virus abundance levels, the virus isolates of DENV-1-4 were diluted to create sets of samples. The Ct values obtained through qPCR [91] are inversely indicative of the initial viral load, with concentrations ranging from 6.75 log10 copies/ reaction (Ct = 19.1) to 2.0 log10 copies/reaction (Ct = 31.2) (Table 3-5).

The mapping data, presented in Table 3-5, revealed that mapping rates for all serotypes at low viral abundance (mean Ct =30.8) exceeded 90%, underscoring the protocol's

effectiveness. Additionally, the percentage of bases with Q30 scores consistently met or exceeded the high-quality threshold of ≥ 85%. In low viral abundance samples, genome coverage averaged 87.8% ± 1.7%, representing a notable decrease when compared to its high viral load counterparts, which achieved an impressive 98.9% ± 0.3%. Despite this decrease in genome coverage, the mean base coverage for low-abundance samples (Ct ≥ 29) across all serotypes consistently exceeded 1,000 reads. For samples with moderate viral loads, there was no significant deviation in mapping rate, %Q30 score, or genome coverage when compared to the high viral abundance group. Based on mapping quality assessments, we concluded that the protocol consistently delivered high-quality results, even when dealing with low RNA template concentrations derived from virus supernatants (Ct ≥ 29). This robustness ensured the successful recovery of DENV viral genomes.

Table 3-11: Summary statistics of read mapping of DENV virus stocks at different viral abundance levels

		DENV-1			DENV-2			DENV-3			DENV-4		
Ct value		19.1	25.2	31.2	19.5	25.3	31.0	20.1	25.3	30.9	19.8	25.7	29.9
Mapping Rate		99.2%	99.3%	98.4%	92.6%	91.2%	91.3%	97.0%	96.9%	97.6%	98.6%	95.7%	95.6%
Base Coverage	**Minimum**	1,489	2,047	319	579	1,441	229	882	1,383	521	755	2,012	228
	Mean	6,191	5,006	3,013	6,260	6,502	2,892	5,043	3,801	2,159	4,053	4,042	2,390
	Maximum	19,679	15,931	9,497	19,405	17,774	18,983	16,599	10,706	13,591	18,653	12,977	14,089
% Q30		97	94	98	97	95	89	96	92	90	96	95	90
Genome coverage (%)		98.5	99.2	88.3	99.3	98.7	89.2	98.9	98.3	88.5	98.9	99.6	85.3
CoDing Sequence (CDS) coverage (%)		100	100	90	100	100	91.5	100	100	91	100	100	87

3.3.7 DENV viral genome coverage uniformity between high and low viral abundance

The genome coverage exhibited notable differences between high and low viral abundance samples. To provide a comprehensive assessment of coverage quality and any potential regional bias relative to the viral reference genome, we calculated the average coverage for each genomic position in both high and low viral load sample.

To visualize genome uniformity across the entire genome, we generated a read track map using read mapping data from both high (Ct < 20) and low viral concentration (Ct > 29) samples of the virus supernatants (Figure 3-5). The graph demonstrates uniform coverage across the entire genome, with the majority of regions achieving approximately 1,000X coverage. Some regions even exceeded 10,000X coverage across all serotypes in both high and low viral abundance samples, particularly in high viral load samples (Figure 3-5A). Notably, variations in coverage were more prominent in low viral load samples (Figure 3-5B), suggesting that viral load may influence coverage uniformity without significantly affecting overall genome coverage. High viral load samples displayed consistent coverage across all regions, while low viral load samples exhibited multiple gaps distributed throughout the genome. In summary, low viral load samples not only had notably reduced viral genome coverage but also lacked coverage uniformity.

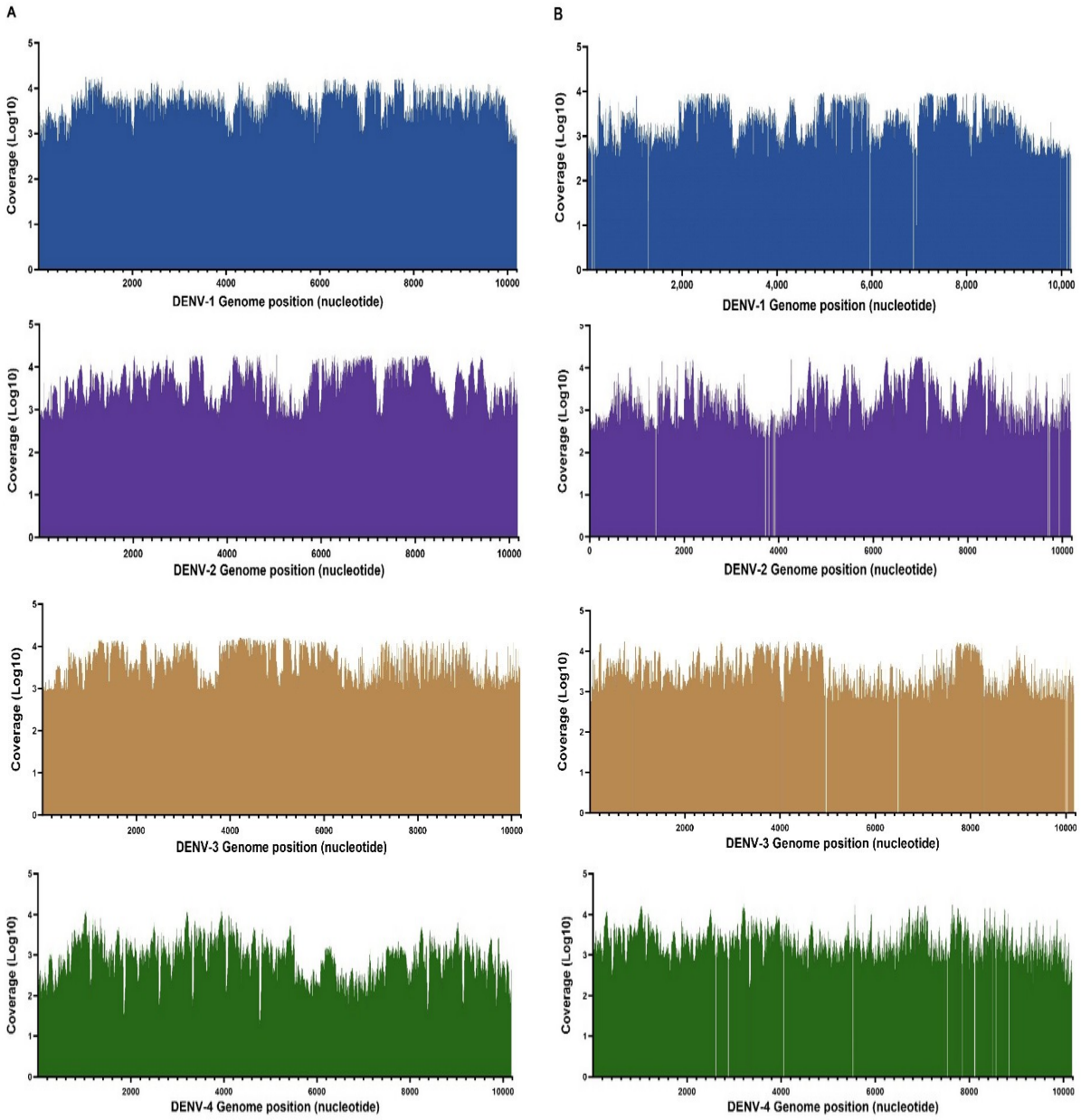


Figure 35: DENV-1-4 genome coverage uniformity.

The Reads Track illustrates the distribution of reads across the DENV genome. (A) Depicts the average DENV genome coverage in high viral abundance. (B) Depicts the average DENV genome coverage in low viral abundance. White vertical lines indicate gaps along the genome. Base coverage is presented in logarithmic scale (Log_{10} of base coverage).

3.3.8 Assessing the efficacy of the DENV WGS protocol for retrieving DENV genomes in clinical specimens

The effectiveness of the assay was evaluated using a set of 78 samples, plasma from acute dengue patients (n=65) and DENV-infected mosquitoes (n=13). The distribution of serotypes in the sample sets were as follows: viremic plasma from dengue patients (n=65, DENV-1=15, DENV-2=30, DENV-3=9, DENV-4=11), mosquito homogenate samples (n=13, DENV-1=4, DENV-2=3, DENV-3=3, DENV-4=3). The Ct values among 78 samples spanned from 17.8 to 33.5.

The assay successfully generated 66 complete consensus sequences (66/78; 84.7%) with genome coverages exceeding 98% (coverage threshold of 20X) and a minimum coverage of 20X. These sequences comprised DENV-1=16, DENV-2=30, DENV-3=10, and DENV-4=10. The complete genome group exhibited significantly lower mean Ct values in contrast to the partial consensus sequences group (23.72 ± 3.3 vs. 30.43 ± 1.3, $p < 0.0001$). Further analysis based on Ct value quartiles demonstrated a clear trend: samples with higher viral loads were more likely to result in successful complete genome generation (Figure 3-6). The subgroups with Ct < 25 achieved a 100% success rate. In the Ct range of 25 to 30, the success rate was 84%, whereas for the Ct range of 30 to 40, the success rate plummeted to 11.1%. The detailed of sequence quality is listed in Appendix 3.

Among the 66 complete genomes, an average of 96.6% of high-quality reads were successfully aligned to the reference sequences, with individual ranges spanning from 90.0% to 99.6% and the percentage of reads with a Q30 score was 96.2 % (Table 3-6). The average genome coverage was 98.8%, effectively representing 100% coverage of the ORFs within the DENV whole genomes. The pairwise analysis of the 66 consensus genomes was conducted to evaluate the accuracy and reliability of sequencing results using MEGA [474]. The variance estimation method employed was bootstrap, with 1,000 bootstrap replicates run. The average

evolutionary divergences between sequences were 0.002, 0.004, 0.006, and 0.008 for DENV-1, -2, -3, and -4, respectively.

Twelve samples exhibited low viral abundance, as indicated by Ct > 29 were partial consensuses with genome coverage from 38.3% to 79.8%.

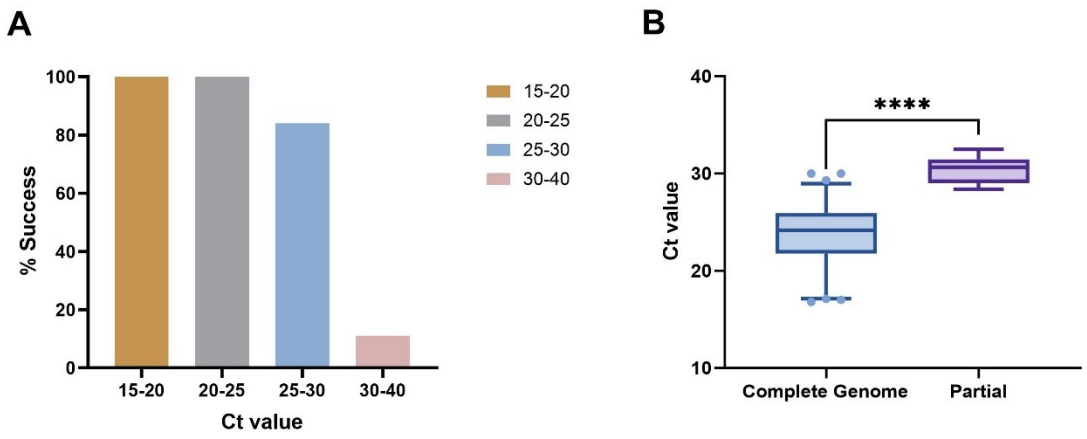


Figure 36: Effectiveness of the new WGS of DENV in clinical samples

A: Success rate of DENV WGS assay stratified by Ct value. B: The significant difference in Ct value between the complete genome and partial consensus sequences (****, P <0.0001, Mann Whitney test).

Table 3-12: Summary statistics on the mapping result of 66 completed genomes generated from clinical DENV (DENV1-4) samples

	Mapping rate (%)	Minimum coverage	Maximum coverage	Average coverage	Genome coverage (%)	%Q30
Minimum	90.0	645	3,906	1,349	98.0	90.0
Mean	96.6	1,800	10,702	4,366	98.8	96.2
Maximum	99.6	4,021	19,947	10,051	99.8	99.5

3.3.9 Viral genome coverage and CoDing Sequence (CDS) coverage and the effect of sequence read depth

To assess the impact of read depth on genome coverage and CDS coverage, we evaluated DENV viral genome coverage, CDS coverage at different sequence depths (100X, 400X, and 1,000X) across 66 complete genomes (Figure 3-7 and Appendix 4). We categorized the 66 consensus sequences into two groups based on levels of viral abundance: high (Ct <25, n=44) and low (Ct >25, n=22).

Both genome coverage and CDS coverage consistently reached significantly higher levels when a threshold of 100X reads (min100X) and min400X was applied, irrespective of the viral abundance (Figure 3-7). At a read depth of 400X, the performance of viral genome coverage in low viral abundance samples (97.0% ± 0.8%) showed no statistically significant difference when compared to high viral abundance samples, which achieved (97.9% ± 0.3%) genome coverage. The CDS coverage also exhibited no notable difference in the percentage, remaining consistently high at 99% for both groups of viral concentration.

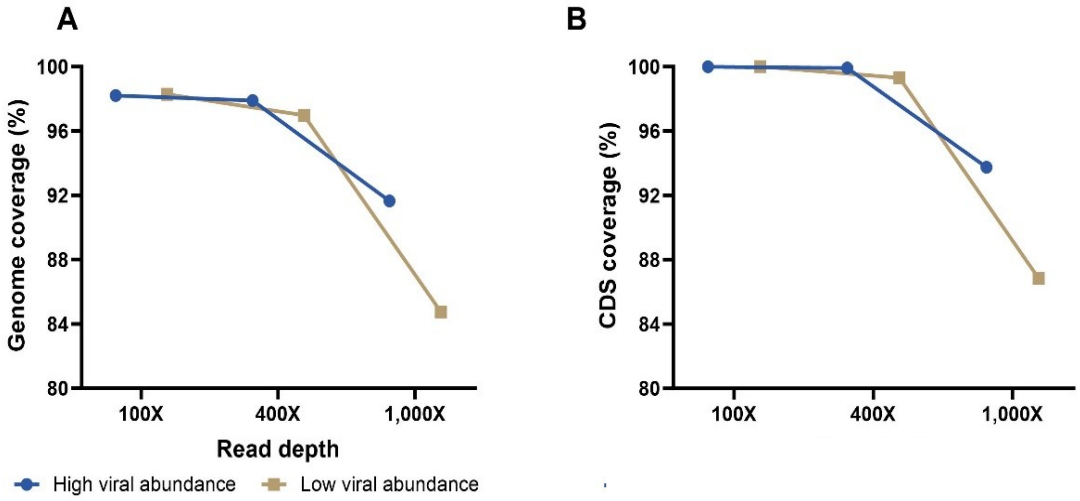


Figure 37: DENV Genome Coverage (A) and Coding Sequences (CDS) Coverage (B) among 66 DENV genomes derived from clinical specimens at read depths of 100X, 400X, and 1,000X.

The length of the DENV genome varies depending on the serotype- specific primer sets: DENV-1 = 10,372bp, DENV-2 = 10,433bp, DENV-3 = 10,462bp, and DENV-4 = 10,365bp. The CDS length for DENV-1 to DENV-4 serotypes was 10,179bp, 10,433bp, 10,173bp, and 10,164bp, respectively.

However, a significant decline in both genome and CDS coverage was observed at both high and low viral concentrations when read depth was increased to 1,000X. Specifically, the genome coverage (min1,000X) for low viral load samples decreased to 84.8% ± 2.4%, while high viral load samples maintained a coverage of 91.6% ± 2.9%. Similarly, low viral abundance samples experienced a reduction to 86.8% ± 2.4% in CDS coverage, while high viral load samples achieved a coverage of 93.7% ± 3% (Figure 3-7). Notably, at min1,000X in low viral load samples, CDS coverage exhibited a slightly higher rate compared to genome coverage, at 86.8% and 84.8%, respectively.

Based on these findings, achieving consensus-level results with a 20X read depth suggests that the multiplexing capacity could potentially exceed 78 samples per run. However, for Single Nucleotide Variant (SNV) analysis requiring a 400X read depth and

genome coverage exceeding 90%, a multiplexing capacity of up to 78 samples is feasible. Nevertheless, if the goal is to attain a read depth of up to 1,000X in both high and low viral abundance samples, reducing the number of samples per sequencing run may be advantageous. This adjustment could allow for higher sequencing depth per sample, potentially enhancing coverage metrics such as genome coverage and CDS coverage. Since our results were obtained from a sequencing run involving 78 samples, decreasing the sample count per run could facilitate deeper sequencing and improve coverage in subsequent analyses. This optimization could be particularly valuable for investigations involving variant detection, quasi-species determination, or the identification of specific mutations associated with a disease. Additionally, it's important to consider your budget and available resources, as deeper sequencing and higher coverage may incur higher costs.

For the application in Chapter 4, investigating the evolution change of DENV in the presence of *Wolbachia*, we decided to multiplex samples in each sequencing run, ensuring that the number of samples does not exceed 78 samples.

3.4 Discussion

Dengue, a global health challenge impacting millions of people, has shown a concerning spread. To better comprehend and combat DENV, enhanced viral detection, transmission tracing, and therapeutic approaches are urgently needed. DENV genome sequencing can play a role in generating data for virus genetic population studies [475]. DENV genome sequencing also contributes to vaccine development, allowing researchers to analyze various DENV strains and create vaccines with relevant coverage of contemporary viruses. Epidemiological studies benefit from DENV genome sequencing, shedding light on virus transmission dynamics and outbreak causes, which is might be helpful for forecasting in the future, and tailored interventions [476,477]. Additionally, it aids in identifying targets for therapeutics that are conserved across all serotypes and genotypes. In the context of vector

control, especially in regions employing the *Wolbachia* control method, DENV genome sequencing characterizes the viral population structure during and after release. This continuous monitoring helps assess any virus population evolution that may affect the effectiveness of control measures like *Wolbachia*. Addressing this, the development of efficient, universal, and user-friendly tools with reduced complexity becomes imperative.

Recovering viral genomes, especially from samples with low template concentrations, presents a significant challenge. To tackle these difficulties, we adopted a multiplex tiling PCR approach and developed serotype-specific primer panels for each DENV serotype. The protocol for creating tiled PCR-generated amplicon pools, initially designed by the Artic Network (https://artic.network/) for sequencing Ebola, ZIKV, and CHIKV genomes [460,478] , served as our foundation. Our approach combined PCR-tiling amplification with Miseq sequencing to analyze DENV WGS from clinical samples. We refined and evaluated this method using short (~400-bp) amplicons format. This innovative method generated amplicons through two multiplex PCR reactions, yielding a total of 36 to 42 amplicons (~400-bp). Our protocol underwent a thorough evaluation, assessing sequencing read mappability, genome coverage (percentage and uniformity), sequencing depth, and levels of viral abundance. Our success rate in accessing the DENV WGS assay reached 80%, which was impossible with our prior PCR amplicon approach that typically generated larger amplicons, requiring at least five overlapping amplicons to cover the entire genomes [475,479]. This was particularly important, as DENV, being a positive-sense single-stranded RNA virus, exhibits low stability once RNA enzymes are released after cellular destruction. High-quality virus RNA is critical for detection and genome sequencing. In our study, the majority of the samples used for protocol development and assessment were frozen samples. Additionally, we employed various types of biological matrix samples, including virus culture supernatant, dengue patient's plasma, and DENV-infected *Ae. aegypti* mosquitoes. Our analysis indicated that

there were no significant differences in DENV genome mappability among the various specimen types we employed. Additionally, we did not observe differences in mappability between samples with high and low viral concentrations. This suggests that our protocol for DENV can be effectively applied to a wide range of samples using RNA isolated from various specimen types. The assay's robustness and precision were evident, consistently achieving high mapping rates and average base coverage. During subsequent re-sequencing of the samples, our protocol consistently delivered consensus sequences that matched their GenBank-deposited counterparts.

3.4.1 Cost and platform selection

Tyson's study [480] demonstrated a significant reduction in reagent costs to approximately GBP10 per sample when applying the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using the Oxford Nanopore system. This cost-effective approach holds great potential for epidemiological surveillance during pandemics and in low-income countries. The cost savings are attributed to the simplification of sequencing library preparation, reduced hands-on time, and the capacity for enhanced sample multiplexing, accommodating up to 384 samples. Additionally, a separate study conducted a cost analysis for DENV WGS[481] directly comparing Nanopore sequencing to Illumina sequencing. This analysis revealed a significant cost difference between the two platforms. Specifically, the per-sample cost of Illumina sequencing is priced at AUD approximately AUD 100 per sample, considerably higher than the cost of Nanopore sequencing, 28 per sample. Based on our estimation, the cost for one DENV WGS sample is approximately USD 120. This estimate includes expenses from the RNA extraction step to DENV serotyping and sequencing on the MiSeq platform (estimated for our circumstances, a research unit based in Vietnam).

When assessing costs between sequencing platforms, it's important to recognize the inherent complexity of comparing them directly. Various factors come into play, including reagents, consumables, machine usage fees (which tend to be higher in low- and middle-income countries), as well as data analysis, sequencing depth, and length. These variables contribute to potential fluctuations in overall expenses, highlighting the importance of contextual factors in cost assessment.

An alternative method to evaluate cost efficiency is considering the cost per gigabyte. Oxford Nanopore sequencing generally appears to have higher prices compared to Illumina sequencing. For instance, the MinION and GridION platforms range from $50 to $2000 per gigabyte, representing a significant increase from Illumina sequencing. However, the larger-scale PromethION platform's cost is comparable to Illumina, ranging from $21 to $42 per gigabyte. Nonetheless, specific costs can vary based on factors such as applications, sequencing depth, and regional considerations.

When weighing cost, Illumina platforms emerge as the more cost-effective option per gigabyte (Gb) sequenced compared to Ion Torrent PGM and PacBio RS [482]. Notably, the MiSeq ($128 K) and HiSeq 2000 ($654 K) models exhibit particularly favorable sequencing costs per Gb. Regarding accuracy, Illumina platforms generally boast higher reported accuracy rates, with most readings exceeding Q30, compared to Ion Torrent PGM (mostly Q20) and PacBio RS (< Q10). This enhanced accuracy is particularly advantageous for applications demanding precise base calling, such as variant detection. Additionally, PacBio RS exhibits the lowest throughput (100Mb) and longest run times (10 days). Thus, if rapid sequencing is a priority, Illumina platforms like the MiSeq (27 hours) may be more suitable. Other factors, such as the availability of resources and support, also play a significant role. Illumina platforms benefit from widespread usage, resulting in more accessible resources, protocols, and support services compared to other sequencing platforms.

Beyond cost considerations, the primary factor for deliberation when selecting a sequencing platform resides in the intended purpose for its utilization. The lower DNA input requirements of Illumina sequencing was highlighted, underscoring its robustness and sensitivity [483]. Additionally, Illumina sequencers deliver high read accuracy (>99.9%), whereas Nanopore sequencers offer accuracies ranging between 87% and 98% [484]. Importantly, both Illumina and Nanopore sequencing can be used in combination to provide the most comprehensive insight into genomic data. However, in scenarios where utmost accuracy is imperative, the Illumina platform can complement the process.

3.4.2 Effective approaches for Single Nucleotide Variant (SNV) analysis

Investigating Single Nucleotide Variants (SNVs) demands a protocol that guarantees high accuracy genomic data, particularly crucial for identifying and monitoring rare and low-frequency variants. Our assay displayed resilience in recovering DENV genomes, even from samples with low viral abundance, maintaining a baseline coverage of over 400 reads across all serotypes and high viral genome coverage of approximately 97%. The CDS consistently exhibited robust coverage, reaching 99%. However, one of limitation of the multiplex tiling approach was its tendency to generate uneven coverage, resulting in regions with lower coverage, especially when dealing with low viral load samples. To rectify this imbalance in genome uniformity, we enhanced the results by increasing the primer concentration of several primer pairs to 20-40µM. Consequently, we achieved both high genome coverage and uniformity, which is essential for investigating and tracking low frequency SNVs. The assay's robustness was demonstrated in Chapter 4, investigating the DENV-1 evolution within *w*Mel-infected *Ae. aegypti*, where the protocol was used to track the adapted variant associated with *w*Mel infection.

3.4.3 Limitations

Our method relies on multiplex PCR for targeted enrichment of viral genomes and it is designed for use with the Illumina platform. As a result, direct generation of DENV genomes from RNA materials is not feasible. While our sequencing method may not be the optimal choice for resource-limited or remote areas, it offers a valuable advantage. Specifically, our designed increase the likelihood of successfully retrieving DENV genomes from samples with low viral abundance by ensuring adequate material input through prior amplification before sequencing. Furthermore, our serotype-specific primers have demonstrated their ability to effectively capture a wide range of DENV genotypes found across the globe. This versatility underscores the broad applicability of our serotype-specific primers, making our assay suitable for samples collected from diverse regions worldwide.

Despite the cost-effectiveness of primer-panel-based target amplicon sequencing [485], we have identified specific limitations with the amplicon-based whole-genome amplification protocol. Firstly, the amplicons are designed to cover genome regions starting from different positions, depending on DENV serotypes. This restricts the protocol's ability to detect SNVs outside of these PCR-amplified regions. Secondly, even a single-base mismatch between the primer and template can lead to PCR errors, including chimeric PCR amplification [486], potentially resulting in inaccurate SNV calls. Lastly, PCR amplified primer-originated 'contaminated' sequences may be introduced during PCR amplification, potentially contributing to errors in SNV calling. Therefore, proper primer trimming (as we did) becomes critical for accurately detecting SNVs within the primer binding regions.

The entire procedure, spanning from sample preparation to data analysis completion, approximately 24 to 30 working hours for execution per sample. This timeframe can vary depending on the specific sequencing kit employed and the number of samples processed in each run. Our serotype-specific primers showed its broad capturing many DENV genotypes distributed worldwide. However, continuously refining and updating the primer sets to ensure

their efficacy in capturing an expanded spectrum of genotype strains is an ongoing demand in future research.

3.5 Conclusion

In conclusion, we have developed a protocol for generating full-length DENV genomes utilizing a multiplex tiling PCR approach on the Illumina platform. We evaluated the protocol's effectiveness in samples with both high and low viral abundance, and it consistently met satisfying parameters, including mapping rate, genome coverage, and uniformity. Furthermore, its quality underscores its suitability for precise examination of intra-host diversity. This advancement not only enriches our understanding of viral evolution but also has the potential to improve diagnostic and intervention strategies in regions dealing with dengue outbreaks.

Chapter 4

DENV-1 EVOLUTION IN THE PRESENCE OF *Wolbachia pipientis* (*w*Mel) IN *Aedes aegypti*

Abstract

The investigation of DENV evolution in *Ae. aegypti* mosquitoes infected with the *w*Mel strain of *Wolbachia* is a crucial study to assess the long-term effectiveness of the antiviral *Wolbachia* introgression. Our findings reveal that an amino acid substitution, E203K, in the DENV-1 envelope protein was detected at higher frequency in *w*Mel-infected mosquitoes compared to wild-type counterparts. This positive selection at residue 203 was reproducible in different DENV-1-infected patients and in the second independent experimental system. In both wild-type mosquitoes and human cells, the E203K variant was rapidly replaced by the progenitor sequence. These results serve as proof of concept that *w*Mel-associated selection of virus populations can manifest under experimental conditions. Further investigations in field-based studies are essential to assess whether *w*Mel exerts selective pressure on DENV evolution in dengue-endemic regions where *w*Mel has been established.

4.1 Introduction

Releasing *w*Mel -infected *Ae. aegypti* mosquitoes for several months in a community results in stable introgression of *w*Mel into the *Ae. aegypti* population [358,411,435–437]. When *w*Mel establishes itself to a high prevalence in *Ae. aegypti* populations, the local incidence of dengue is decreased. The evidence for this comes from a large randomised cluster trial (RCT) and several observational studies [342,357,487,488]. Recently, the WHO's Vector Control Advisory Committee has endorsed the public health value of *w*Mel deployments [489].

Wolbachia has been suggested to inhibit the replication of DENV in *Ae. aegypti* by multi-factorial mechanisms [355,366,447,490,491], including an increase in basal expression of immune genes [396,492], modulation of the availability and trafficking of cholesterol [378], and upregulation of microRNAs [493]. Thus, one could predict that DENV resistance to *Wolbachia* likely requires several changes across the viral genome to overcome the multidimensional virus-inhibition exerted by these bacteria. The stability of this tripartite relationship, *Ae. aegypti, Wolbachia*, and the DENV, is crucial for the long-term success of this approach. Interestingly, while the *w*Mel genome remains highly stable in mosquito populations even after several years in the field [434,435,438], DENV evolve relatively more quickly due to their error-prone RNA dependent polymerase used for self-replication [60,194]. This highlights the contrasting genetic stability between *Wolbachia* and the constantly evolving DENV.

Wolbachia could reasonably be expected to exert selective pressure on DENV evolution. If *Wolbachia*-resistant variants develop in the mosquito midgut, they may have a growth advantage for dissemination into the mosquitoes' salivary glands, leading to a potential mosquito-to-human transmission. However, measuring the impact of *Wolbachia* on

DENV evolution in the field is challenging due to the complexities associated with imported DENV cases into areas where *Wolbachia* has been released. Further research is necessary to explore the potential for DENV evolution in the response to *Wolbachia* infection. The aim of the study was to investigate virus evolution in *w*Mel-infected *Ae. aegypti*. The specific aims of Chapter 4 were:

1. To test the hypothesis that DENV-1 would not adapt to the virus interference properties of *w*Mel during repeated mosquito passage.

2. To characterize, to the extent possible, the characteristics of any variants that appear from serial passage in *w*Mel-infected *Ae aegypti*

The findings of this chapter were published in the journal Virus Evolution, under the title “Genomic evolution of dengue virus type 1 under *Wolbachia pipientis* selection in *Aedes aegypti* mosquito”. Virus Evol. 2023;9(1). [190]

4.2 Materials and Methods

4.2.1 Ethic statements and Dengue patient’s cohorts

The protocol was reviewed and approved by the Scientific and Ethical Committee of the Hospital Tropical Diseases (HTD) (HTD EC CS/NĐ/16/27) and the Oxford Tropical Research Ethical Committee (OxTREC: 45-16). Inclusion criteria of the study (i) were patients older than 15 years of age, (ii) were inpatients or outpatients at HTD less than 96 hours of fever, (iii) had confirmation of DENV infection by NS1 rapid test and Dengue PCR serotyping, (iv) and provided written informed consent.

4.2.2 Virus serial passage method

We designed an experimental system that maximizes the possibility of observing selective pressure by *w*Mel on DENV-1 in mosquito tissues. DENV-1 was passaged in

parallel in wild-type (Wt) and *w*Mel- infected *Ae. aegypti* through 20 cycles of repeated passage. The first passage, Passage 0 (P0) including three replicate cohorts of *Ae. aegypti*, each cohort containing twenty mosquitoes, were generated by oral feeding with DENV-1 viremic blood. For Passage 1-20 (P1-P20), mosquito cohorts were infected using intrathoracic injection (Figure 4-1).

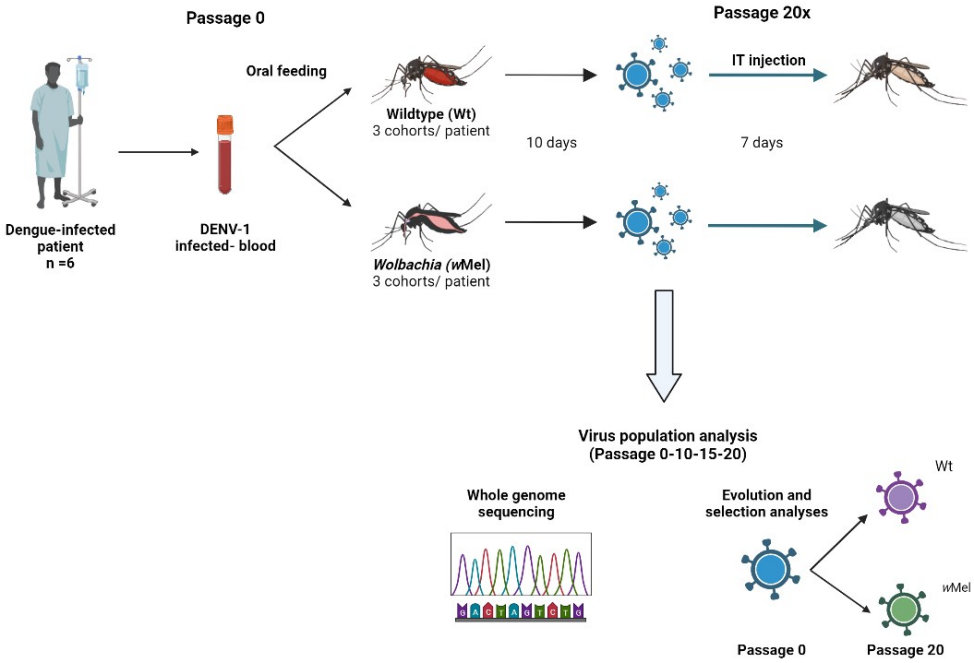


Figure 41: Schematic of DENV-1 passage system in Wt and *w*Mel-infected *Ae. aegypti*

Febrile dengue patients provided blood samples, which were fed to either wild-type (Wt) mosquitoes or mosquitoes infected with *Wolbachia* (*w*Mel). The first passage of DENV infection, P0, was established through blood-feeding. After ten days, *w*Mel and Wt mosquitoes were collected, and their virus-containing supernatants were processed. Three independent cohorts were formed by combining supernatants from 20 mosquitoes of each patient sample and mosquito line. These cohorts were intrathoracically re-inoculated into the corresponding mosquito lines, and the process was repeated up to passage 20. Viral RNA was extracted at passages 0, 10, 15, and 20 for qPCR and WGS. Three independent replicates were maintained, and each replicate was processed separately.

We measured viral RNA concentration in mosquito cohorts every ten passages to track the temporal changes in the genotype and phenotype of DENV infection in each mosquito type. As there was no existing precedent to guide a sample size estimate for this research, we adopted a pragmatic design that ensured manageability from an entomological standpoint. To serve as controls, mosquito samples were intrathoracically infected with the laboratory strain (DENV-1, Genbank Accession Number: FJ432734) for each mosquito passage.

4.2.2.1 Derivation of mosquitoes and rearing

Wt *Ae. aegypti* were derived from field-sourced materials (eggs or larvae) collected in Ho Chi Minh City (HCMC), Vietnam [494]. The HCMC *w*Mel mosquito line was produced by backcrossing Cairns *w*Mel background onto the HCMC background [366]. Wt mosquitoes used in the study derived from batch F25 to F46, and *w*Mel-infected colonies were from G28 to G49. All mosquitoes were maintained at 280C, 70-80% humidity and equal length light: darkness (12hrs: 12 hrs), with access to 10% sucrose solution. Refer to Chapter "Materials and Methods", section 2.1.2 for the procedures related to colony maintenance.

4.2.2.2 Initial exposure to virus

Patient blood samples in EDTA tubes confirmed to contain DENV-1 were used to establish infection for P0. Two to four-day-old female mosquitoes of both Wt and *w*Mel-carrying mosquitoes were orally fed 300µl of the patient's blood over 30 minutes using artificial membrane feeders. Refer to Chapter "Materials and Methods", section 2.2.3 for the procedure of mosquito oral blood-feeding using DENV-infected patient blood.

4.2.2.3 Mosquito collection into pooled cohorts

Ten days after exposure to a blood meal, 20 whole mosquitos were collected into 1mL of MD to produce one pooled cohort. Triplicate pooled cohorts were produced for each patient and mosquito line. Mosquitoes were homogenized with a Qiagen TissueLyser II at 30Hz for 2 minutes and the supernatant clarified by centrifugation for 1min, 1,400rpm.

4.2.2.4 Mosquito infection for serial passage

For generating successive passages (P1 – P20), 0.2mL of the homogenate was diluted 50x in MD and filtered using Minisart filter (Sigma- pore size 0.2µm) before injecting into naïve mosquitoes. The leftover homogenates were aliquoted and stored immediately at -80°C for virus quantification, DENV genome sequencing and virus isolation.

For P1-P20, each cohort of twenty mosquitoes was injected intrathoracically with 0.5-1µL of pooled supernatant and mosquitoes were incubated for 7 days. Twenty cycles of DENV-1 passaging were conducted using *w*Mel-infected or uninfected control *Ae. aegypti*. Refer to Chapter "Materials and Methods", sections 2.2.5 for the procedure of infecting mosquitoes by intrathoracic DENV inoculation.

4.2.3 Virus and *Wolbachia* quantification

Viral RNA was extracted from patient blood and mosquito pools using the MagNA Pure 96 DNA and Viral NA Small Volume Kit on the MagNA Pure 96 System (Roche). The extracted DENV-1 RNA was subsequently quantified using RT-qPCR assays that had been previously described [24]. Twenty mosquitoes from each cohort were homogenized in 1mL of MD, and the viral genome copy numbers per mosquito cohort were computed in terms of viral genome copy numbers/mL.

To determine the *Wolbachia* infection status in Wt and *w*Mel-infected *Ae.aegypti*, relative quantification of the *Wolbachia wsp* gene to the *Aedes rps17* reference gene was performed, following a previously described method [11]. All qPCR procedures were carried

out using a LightCycler 480 II Instrument (Roche). Refer to Chapter “Materials and Methods”, section 2.5.2 for the detailed procedure.

4.2.4 Assessing the stability of the *w*Mel-associated variant in mosquitoes with and without *w*Mel infection and in human cell lines

Serial passages were conducted in mosquito system (10 passages) and human cell lines (5 passages) to evaluate the persistence of the *w*Mel-associated variant (Figure 4-2). The details of the serial passage procedure in mosquitoes were described in section 2.2.2.

We performed five consecutive passages of DENV-1 variant in THP-1 and K-562 cells (refer to Chapter "Materials and Methods", section 2.4). The cells were exposed to the 203K and 384K variants in a total volume of 0.2 ml. Quantification of viral load using RT-qPCR [91], viral titration using TCID50 (refer to Chapter "Materials and Methods", section 2.4), and genome sequencing were performed at P21, P25, and P30 for mosquito samples, and at P1, P3, and P5 for cell samples.

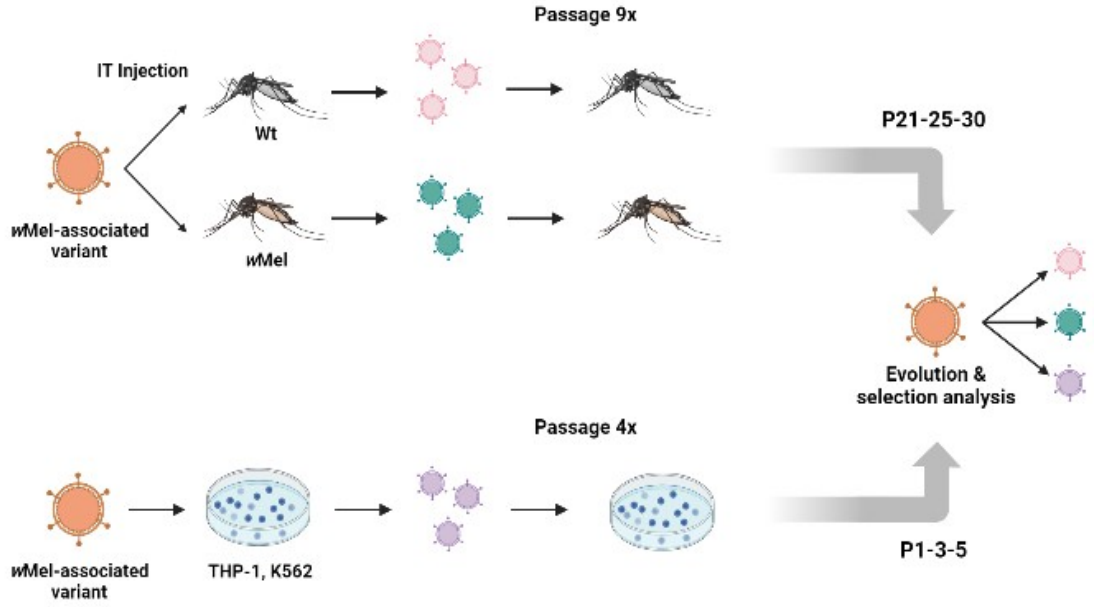


Figure 42: Stability assessment of the *w*Mel-associated variant in mosquitoes with and without *w*Mel infection and in human cells.

*w*Mel-mosquito-adapted virus were subjected to 10 serial passages in Wt and *w*Mel-mosquitoes and 5 passages in human cells lines: THP-1 and K-562. The DENV-1 genomes in mosquito pools at passages 21, 25, and 30, as well as the virus supernatants from human cell lines at passages 1, 3, and 5, were sequenced to investigate the evolutionary dynamics of the variant.

4.2.5 Viral whole-genome sequencing and NGS data preprocessing

The detailed procedure for sequencing DENV-1 and Pre-processing of sequencing reads were described in Chapter 3. Analyses were performed with CLC workbench [495]. Sequence reads from the initial oral feed, P0, were assembled de novo. The longest contiguous sequence was searched in the nr/nt nucleotide collection of Genbank to select the reference genome, which presented the highest homology. Trimmed reads were then re-

mapped to the homologous DENV-1 sequence to generate a full-length genome sequence suitable for reference-based mapping. Consensus sequences for the pooled mosquito cohorts of P10, 15 and 20 were achieved by mapping sequence reads to the P0 reference for each cognate patient (Figure 4-3). The consensus sequence incorporates nucleotides with > 10x of sequencing depth and variant frequency level > 50%.

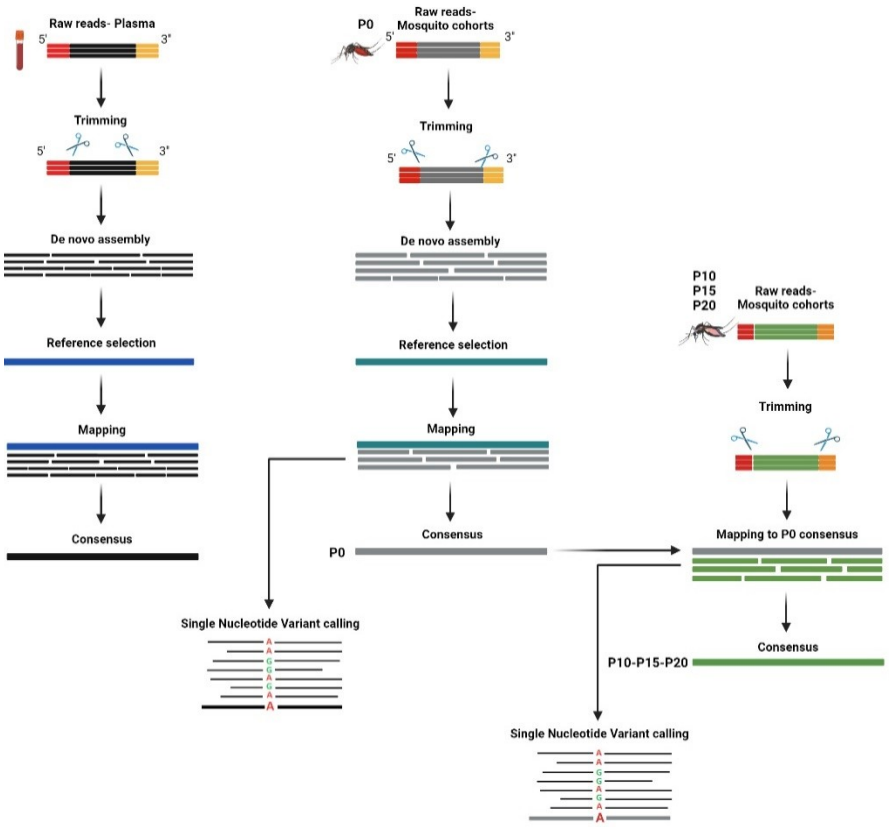


Figure 43: Schematic showing consensus sequence generation and Single Nucleotide Variant (SNV) analysis

Sequence reads from the blood meals and initial oral feed (Passage 0) were de novo assembled, and the longest contiguous sequence was selected as the reference genomes based on homology with the nr/nt nucleotide collection of GenBank. The reads were then mapped to the homologous DENV-1 sequence to generate a full-length genome for reference-based mapping. Consensus sequences for the pooled mosquito cohorts (Passages 0, 10, 15, and 20) were obtained by mapping reads to the P0 reference for each patient.

4.2.6 Phylogenetic analysis

The genome sequences derived from patients' blood were genotyping using the web-based software, Genome Detective [496]. Furthermore, the genomic sequences derived from both the mosquito cohorts and patient blood samples, which were used to generate the respective cohorts, were employed to construct a Maximum Likelihood Tree (ML tree) using IQ-tree, with a bootstrap value of 1,000 (UFBoot) [497].

4.2.7 Single Nucleotide Variants (SNV) calling:

Low frequency variants were detected using a Neighbourhood Quality Standard model [498]. To be included in the analysis, variants had to meet the following criteria: a minimum coverage cut-off of 1,000, a minimum variant frequency cut-off of 0.5% [172,452,499], and presence in both forward and reverse reads. Genome coverage analysis was performed to track the locations of SNVs and determine their association with low or high coverage regions (having a coverage > 1,000X). The algorithm employed a Poisson distribution to fit observed coverages at mapping positions. The parameters "Minimum length" and "P-value threshold value" were set to 50 and 0.0001, respectively.

4.2.8 Fast Unconstrained Bayesian Approximation for inferring selection (FuBAR):

A nucleotide alignment of consensus sequences of all sequenced samples from all patients, cohorts and mosquito lines included in the study were aligned with MUSCLE. The multiple alignment and ML phylogenetic tree were uploaded to the software package Hypothesis Testing Using Phylogenies to determine individual sites under positive selection with a FuBAR analysis [500]. In this analysis, non-neutral evolution is predicted using an empirical Bayes approach. The specific values used for the analysis included 5 MCMC chains of length 2×10^6 with a final 1×10^6 chains and a final draw of 100 samples. The concentration parameter of the Dirichlet prior was 0.5.

4.2.9 Envelope protein alignment and structural visualization:

Thirteen E sequences representing common DENV genotypes were downloaded from Virus Pathogen Database and Analysis Resource (ViPR) and were aligned with MUSCLE [501]. The PyMOL molecular graphics system (version 2.3.2) [502] was used to visualize DENV-2 Envelope protein (protein data bank ID: 3J2P). Site directed mutagenesis in PyMol was used to alter residues 200-210 from QMENKAWLVHR in the native sequence to a DENV1-like sequence of TMKEKSWLVHK.

4.2.10 Statistical analyses and visual representation

Analyses were conducted and plotted in RStudio (version 1.2.5033), R (version 3.4.4) and GraphPad Prism (version 9.5.1), and the visual illustrations of Image Graphs were created using Biorender.com.

4.3 Results

4.3.1 Patient characteristic and viremia measurement

Infectious DENV-1 blood was used to feed mosquitoes, and the plasma from these patients was serotyped and quantified. The six patients were enrolled in the study on their third day of illness. Viral RNA levels were quantified in the plasma of these six DENV-1 patients, all of whom (#172, 176, 180, 185, 193, and 197) exhibited high viremia (Appendix 5), with viral loads exceeding 7.7 log10 copies/mL.

4.3.2 Viral load in mosquito cohorts

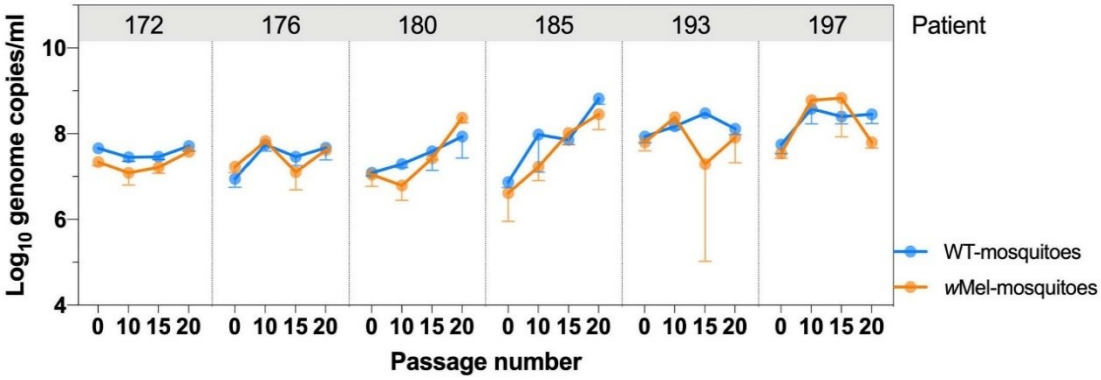


Figure 44: DENV copy number throughout serial-passage in WT and *w*Mel-infected

Symbols represent geometric mean log_{10} viral genome copy numbers geometric SD of triplicate mosquito pools for passage numbers 0, 10, 15 and 20. Viral genome copies/mL were determined after 10 days of IT inoculation. Color depicts the mosquito line used for virus passage (WT-mosquitoes shown in blue and *w*Mel-mosquitoes shown in orange).

DENV copy number in mosquito cohorts at P0, P10, P15, and P20 was determined by absolute quantification. DENV-1 genome copies (10 days after inoculation) varied between 4.75-9.0 log_{10} (copies/mL) between different passage numbers and different patients (copies/ mL), shown in Figure 4-4 and Appendix 5. Importantly, sufficient copies were maintained for the purpose of viral genome sequencing in both mosquito lines for all patients and passages. This enabled us to search for sites of the viral genome that could be adapting to the selective pressures imparted by *w*Mel.

4.3.3 *w*Mel density across passages

*w*Mel density in the mosquito pools was also tested to ensure maintenance of *Wolbachia* throughout the rearing process and that it presented a selective pressure. The mean relative densities of *w*Mel in the supernatants from passage material were 1.250 (SD = 0.148) and did not fluctuate between passage numbers (Figure 4-5, Appendix 5). According to Kruskal-Wallis test (p=0.202), *w*Mel levels were homogeneous between passages.

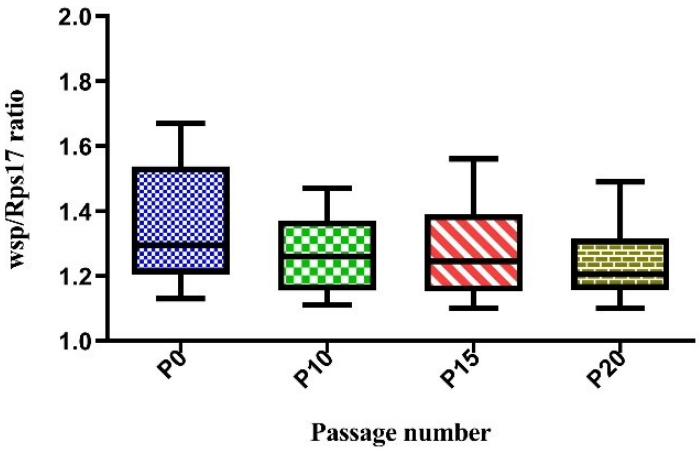


Figure 45: *w*Mel density in mosquito pools across passages

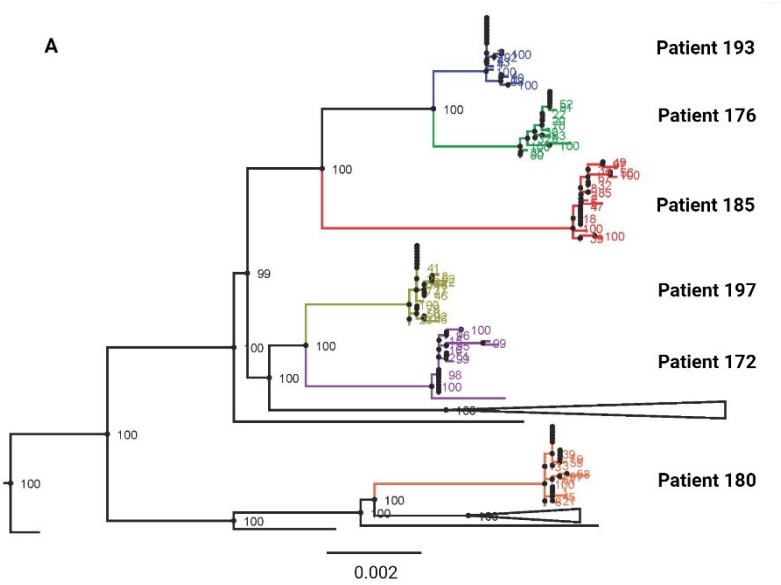
The *Ae. aegypti rps*17 housekeeping gene and the *w*Mel *wsp* target gene were amplified by qPCR. The ratio of *wsp/rps17* was calculated using the delta CT method and is depicted for *w*Mel mosquito pools at P0, 10, 15, and 20. *w*Mel levels exhibited the homogeneity among passages (p=0.202, Kruskal-Wallis test)

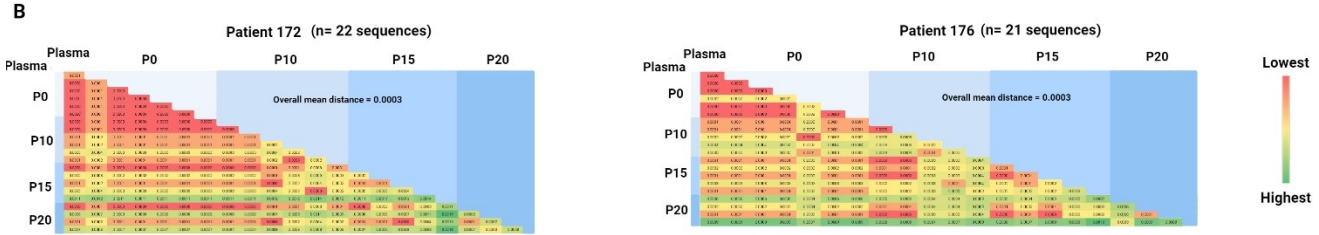
4.3.4 DENV-1 genomes in infectious blood feeds and mosquito cohorts

The six DENV-1 infectious blood samples belonged to Genotype I of DENV-1. Based on the assessment of read quality, mosquito cohorts from patients #172 (passage 15 (1 cohort) & 20 (2 cohorts)), #176 (passage 10 & 15 (1 cohort) & 20 (2 cohorts)), and #193 (passage 10, 15 & 20 (1 cohort)) were excluded from the analysis due to not meeting the satisfactory quality requirements. The 140 qualified full-length DENV-1 genomes (Genbank Accession Number: MN912109.1-MN912248.1) were utilized for subsequent analysis. The Quality control metrics for DENV-1 sequencing of patient-derived passage material and mosquito controls was presented in Appendix 6. Full-length DENV-1 genomes derived from six infectious blood feeds and P0 cohorts were re-constructed through a two-steps procedure. The highest homology genome - accession number JQ045668.1, was selected as reference genome. The trimmed reads of six infectious blood feeds and P0 cohorts were re-mapped to JQ045668.1 to generate a full-length genome sequences. The ML tree included 207 DENV-1 sequences in Vietnam from 2003 – 2017 and 140 sequences from the present study. The majority of P0-P20 DENV1 sequences clustered according to patient number, with a bootstrap support of 100% and a substitution/site value of 0.002 (Figure 4-6A).

The heat map in Figure 4.6.B provides insight into the pairwise genetic distances among DENV-1 sequences isolated from various passages during mosquito passaging across different patients. Remarkably, minimal genetic divergence was observed within sequences from different passages within the same patient, indicating limited changes in the DENV-1 consensus over the course of mosquito passaging. This consistency suggests a high degree of

accuracy in the data obtained. Similarly, the genetic distances between sequences from different patients were also notably small, reflecting the observations within the same patient. However, the genetic distances between DENV-1 genomes at P20 and their original genomes (plasma and P0) were progressively increased. This indicates mutation accumulation during mosquito passaging, suggesting that genetic distances could be even greater with continuous passages. It's noteworthy that the intrathoracic method utilized in this in vivo system bypassed bottlenecks typically encountered in natural transmission scenarios. However, the heat map only provided partial insight into overall genetic diversity. Complementing pairwise genetic distance analysis with Single Nucleotide Variant (SNV) analysis would provide a more thorough assessment of the genetic dynamics and diversity of the virus population.





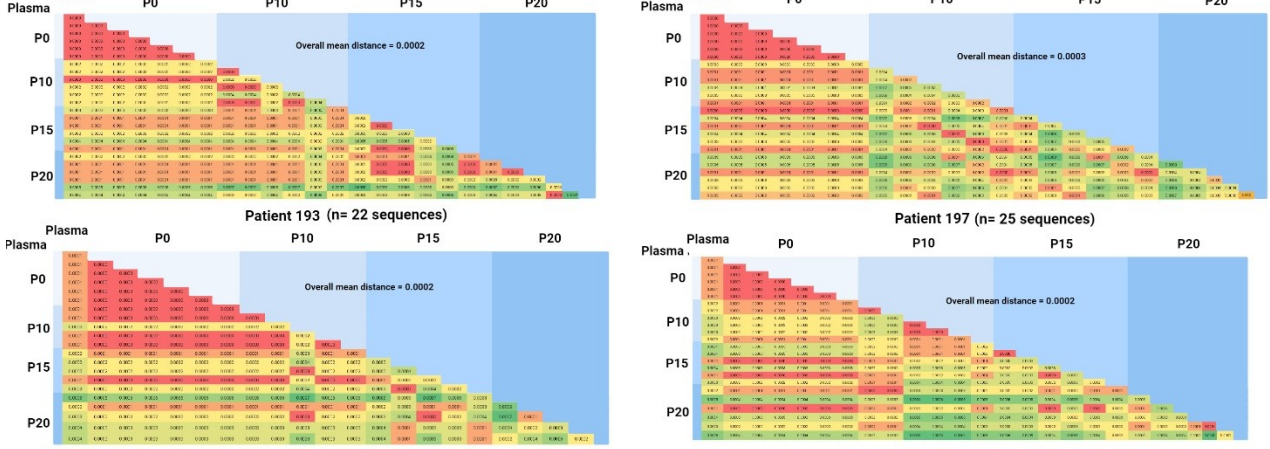


Figure 46: DENV-1 genomes in infectious blood feeds and mosquito cohorts

(A) Maximum Likelihood phylogenetic tree of DENV-1 genomes from patient plasma samples and pooled mosquito cohorts. The rooted maximum likelihood phylogenetic tree displays the genetic distance of DENV-1 consensus sequences obtained from six patient plasma samples and pooled mosquito cohorts at Passages 0, 10, 15, and 20. Additionally, the tree incorporates 206 complete DENV1 genome sequences collected in Vietnam from 2003 to 2017. (B) shows the pairwise genetic distances between DENV-1 sequences isolated from different passages. Each square in the heat map represents the genetic distance between two sequences, with green colors indicating greater divergence.

4.3.5 Adaptive evolution of DENV-1 to *w*Mel-mosquitos features positive selection of codons in envelope

4.3.5.1 SNV analysis in mosquito cohorts by passages and mosquito lines

Low-frequency SNVs were identified in DENV-1 sequences at P0 and P20 in both Wt- and *w*Mel-mosquitoes (Figure 4-7). Compared to P0 (A-B), the number of SNVs increased dramatically by P20 (C-D) in both mosquito lines (2-way ANOVA, p = 0.0264).

The number of SNVs increased by approximately 1.3 fold in both Wt- and *w*Mel-mosquitoes by P20. Notably, the SNVs were distributed across the entire DENV1 genome and were not concentrated in a specific viral gene. Among the total of 14,520 SNVs identified across all passages, thirteen SNVs resulted in nonsynonymous mutations. Two specific SNVs, 1447G>A and 1990G>, led to amino acid changes in the E gene. These thirteen nonsynonymous mutations were localized in high coverage regions (Table 4-1).

Table 4-13: Genome coverage analysis of sequences containing the thirteen nonsynonymous mutations.

CDS Position	Coding region	Nucleotide change	Protein change
214	C	T > C	Phenylalanine > Leucine
595	PrM	G > A	Glutamic acid > Lysine
770	PrM	C > T	Alanine > Valine
1447	E	G > A	Glutamic acid > Lysine
1990	E	G > A	Glutamic acid > Lysine
2540	NS1	C > T	Serine > Leucine

2708	NS1	T > C	Valine > Alanine
3007	NS1	T > A	Serine > Theronine
3062	NS1	T > C	Isoleucine > Theronine
3497	NS2A	T > C	Valine > Alanine
5419	NS3	G > A	Valine > Isoleucine
5489	NS3	T > C	Phenylalanine > Serine
9142	NS5	A > G	Isoleucine > Valine

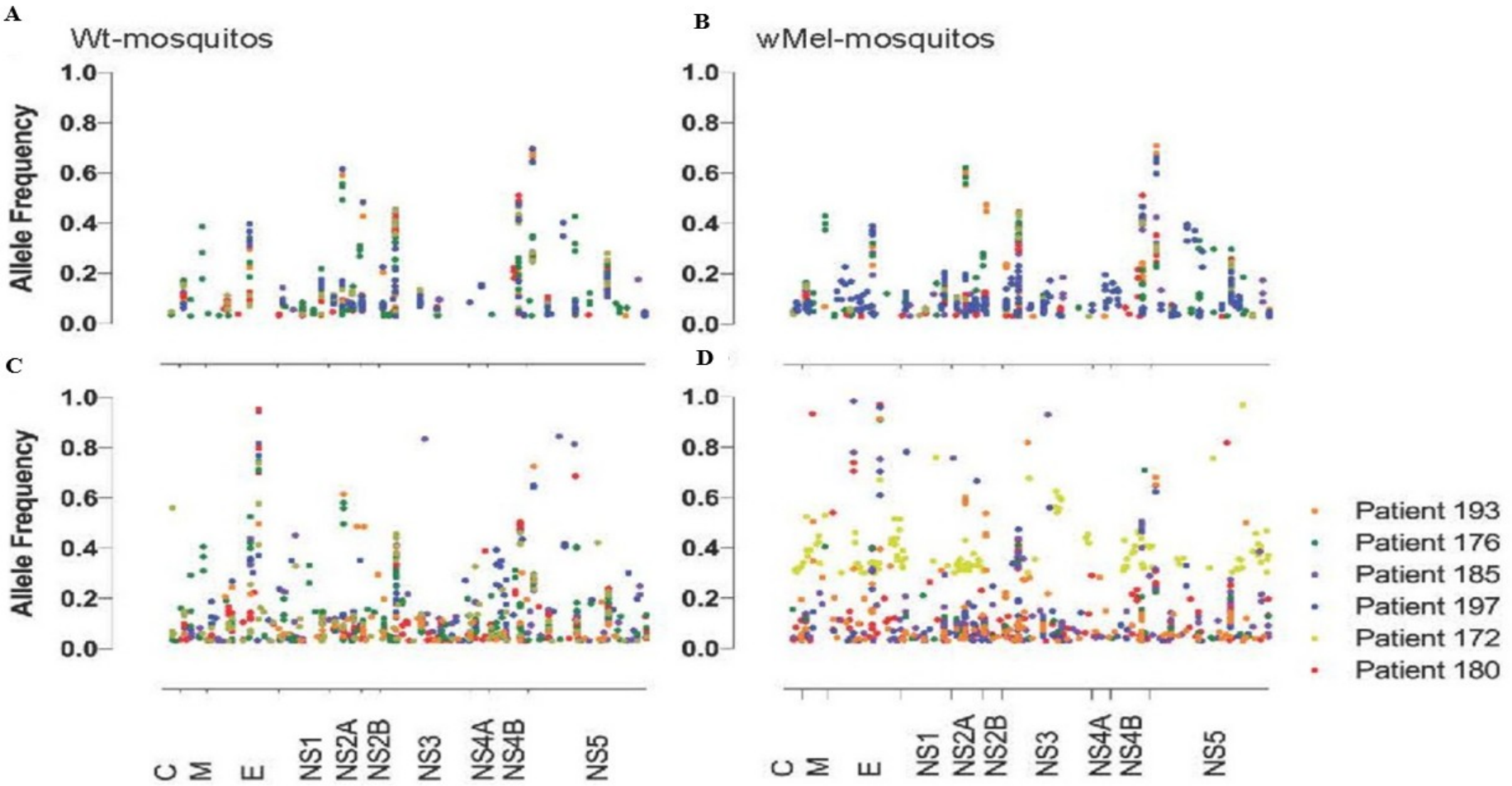


Figure 47: Single Nucleotide Variants (SNVs) in mosquito cohorts across passages (P0, P20) and mosquito lines

Dot plots depict allele frequencies of SNVs in DENV1 sequences in both Wt- and *w*Mel-infected mosquitoes for each patient. Only SNVs with frequencies exceeding 0.5% are displayed

4.3.5.2 Adaptive evolution of DENV-1 to *w*Mel-mosquitos features positive selection of codons in envelope

We hypothesized that SNVs providing a selective advantage in the presence of *w*Mel would increase in frequency over passage in *w*Mel and not Wt-mosquito cohorts, and ultimately appear in the consensus sequence by P20 (> 50% frequency) [456].

Two SNVs, located at positions 1447 and 1990 in E gene, met the predefined criteria for temporal and prevalence thresholds. The 1447G>A SNV was found at a frequency above 50% in 7 out of 18 *w*Mel-mosquito cohorts originating from 4 of the total 6 independent patient plasma samples (Figure 4-8 and in Appendix 7). However, this SNV was not detected above 50% in any Wt-mosquito cohort at any passage number or from any patient plasma sample. In Wt-mosquitoes, the 1447G>A SNV was only transiently present and at a low frequency (Figure 25 and in Appendix 7). Similarly, the 1990G>A SNV was present at a frequency above 50% in 9 out of 18 *w*Mel-mosquito cohorts and 13 out of 18 Wt-mosquito cohorts across all 6 independent patient samples (Figure 4-9 and Appendix 7).

The selection pattern related to 1447G>A and 1990G>A was also observed in an independent experimental system using DENV-1 (performed after the work described in this thesis) whereas the frequency of the 1447G>A SNV was below the 50% threshold [190].

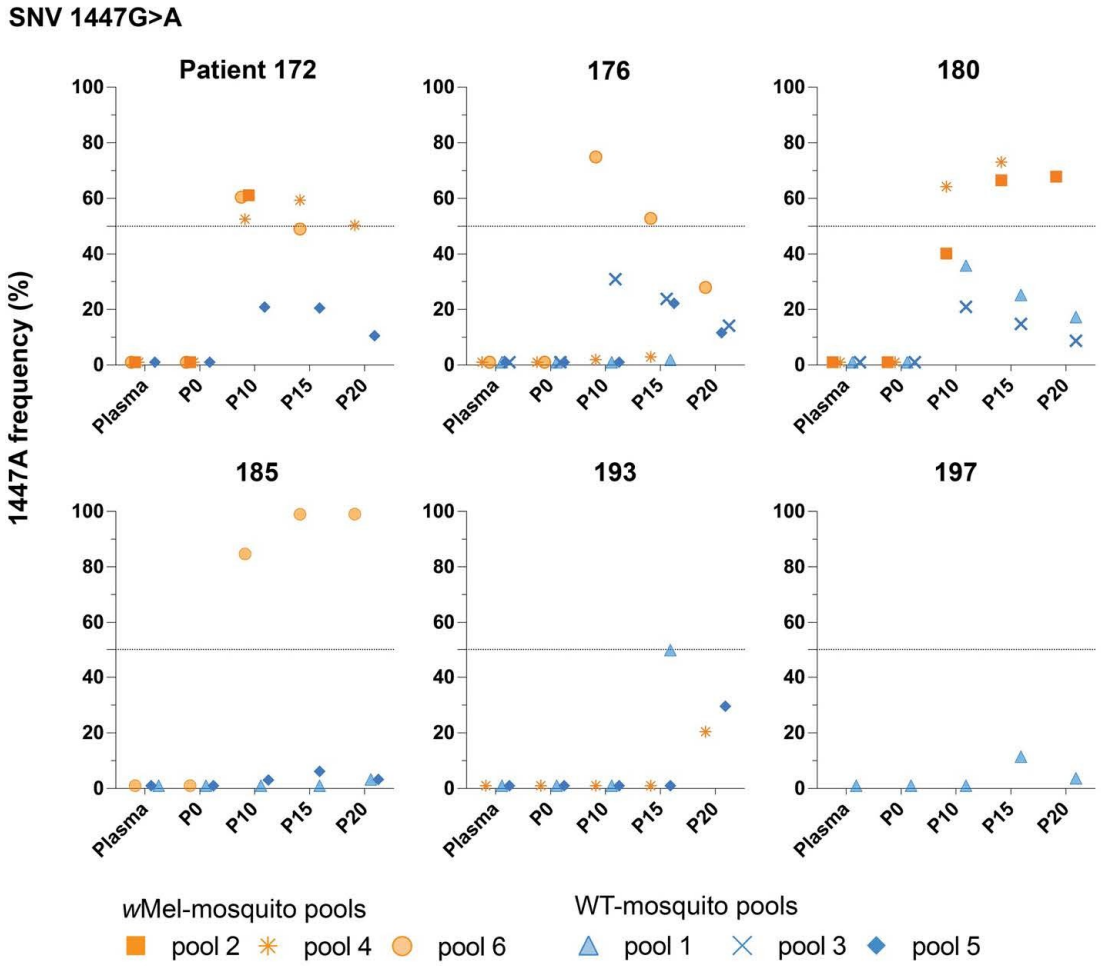


Figure 48: Evolutionary dynamics of the 1447G>A DENV-1 genomic variant in *w*Mel- and Wt-mosquitoes

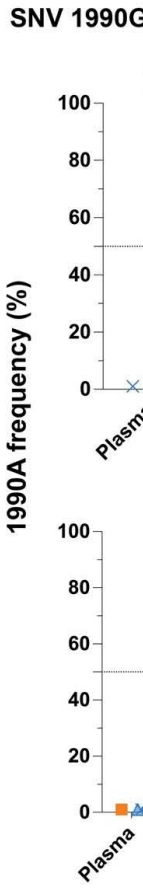


Figure 49: E
Wt-mosquito

The adaptive evolution of DENV-1 in *w*Mel and Wt mosquitoes was investigated through 20 passages of virus derived from patient blood. The SNV, 1447G>A (E203K), exhibited an increasing frequency from P0 to P20, resulting in a consensus change in the DENV-1 genome with a frequency exceeding 50%. The SNV frequency of 1447G>A in plasma and in *w*Mel (orange) or Wt (blue) mosquito cohorts at P0, 10, 15, and 20 is depicted for each patient. Unique symbols represent the frequency in individual mosquito cohorts. The cohorts lacking the 1447G>A SNV throughout the entire passage are excluded from the figure

The 1990G>
increased in
of 1990G>A
and 20 is sh
symbols. Co
shown.

The SNVs 1447G>A and 1990G>A exhibited frequencies above 50% at passage 10, and their frequencies fluctuated over the subsequent 10 passages, either increasing or plateauing (Figure 4-8, 4-9). This indicates the stability and competitiveness of these SNVs within the mosquito lines over time. These SNVs resulted in amino acid substitutions E > K at residues 203 and 384 of the E protein, respectively. The over-representation of E203K in the *w*Mel-mosquito line suggests its involvement in adaptation to *w*Mel. The emergence of E384K in both mosquito lines suggests its potential advantage for DENV-1 infection in mosquitoes. Interestingly, the 1447G>A and 1990G>A SNVs were not simultaneously

present with a frequency ≥ 50% in the same mosquito cohort, indicating the possibility of a fitness cost when both variants are present in the virus population.

4.3.6 Positive selection of codons in the DENV-1 envelope

SNVs subjected to positive pervasive selection were determined with Fast unconstrained Bayesian AppRoximation for Inferring Selection (FuBAR) based on P0 and P20 consensus sequences. FuBAR identified four separate codons under positive selection and two of these include nucleotides changes 1447 (dN/dS = 4.2) and 1990 (dN/dS = 5.5) resulting in nonsynonymous amino acid changes at position E483K and E664K, according to numbering of the entire DENV polyprotein. These two codon changes are located within the E protein (Figure 4-10).

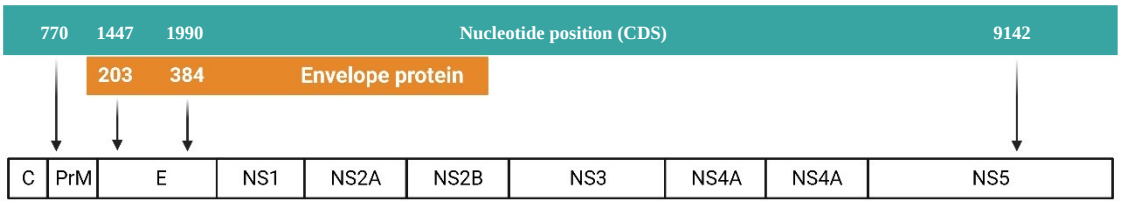


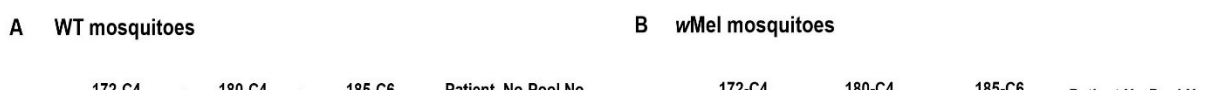
Figure 410: A selection analysis was performed with FuBAR and residues under positive selection are indicated on the DENV-1 genome

Four codons were identified under positive selection, with two of them involving nucleotide changes at positions 1447 and 1990, resulting in nonsynonymous amino acid alterations.

4.3.7 Persistent viral infection of 1447A (E203K) and 1990A (E384K) variants

4.3.7.1 Stability of 1447A (E203K) in mosquitoes in the presence and absence of *w*Mel selective pressure

We tested our hypothesis that the frequency of the 1447A variant would decline in the absence of *w*Mel selection. To do this, we conducted serial passages of P20 virus populations from *w*Mel mosquito cohorts obtained from patients 172, 180, and 185. These virus lineages initially exhibited a frequency of the 1447A variant above 50% and underwent



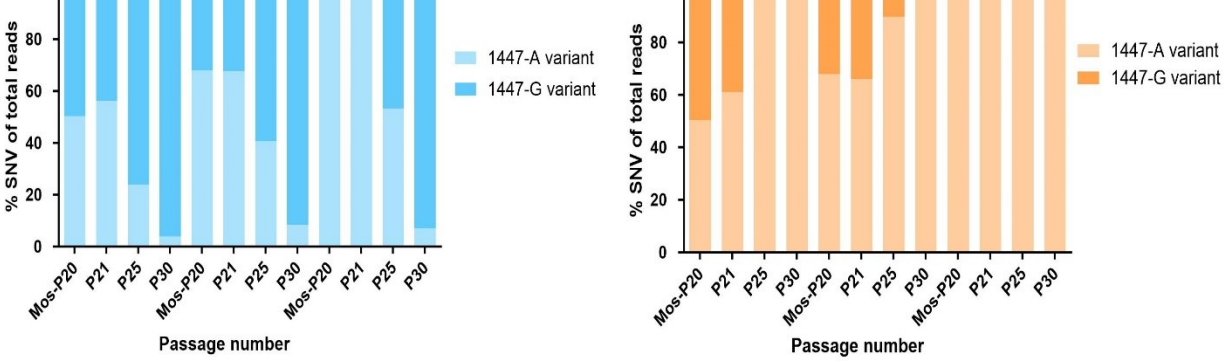


Figure 411: Decline in 1447A and 1990A frequencies throughout passages in Wt-mosquitoes

After one passage in Wt mosquitoes (P21), the frequencies of the 1447A variant remained above 50% at consensus levels. However, by P25, these frequencies started to decline and eventually dropped to less than 10% in all three virus lineages by P30, with the 1447A variant being replaced by the wild-type progenitor sequence, 1447G (Figure 4-11A). In contrast, the 1447A variant persisted over 10 passages when the same three P20 virus lineages were passaged in *w*Mel-infected mosquitoes (Figure 4-11B). By P30, the 1447A variant became fixed at a frequency greater than 99% in all three lineages in *w*Mel-infected *Ae. aegypti*. These findings provide strong evidence that the presence of *w*Mel was the driving force behind the selection and persistence of the 1447A (E203K) variant. In the absence of *w*Mel, the 1447A variant was negatively selected and replaced by the wild-type 1447G variant.

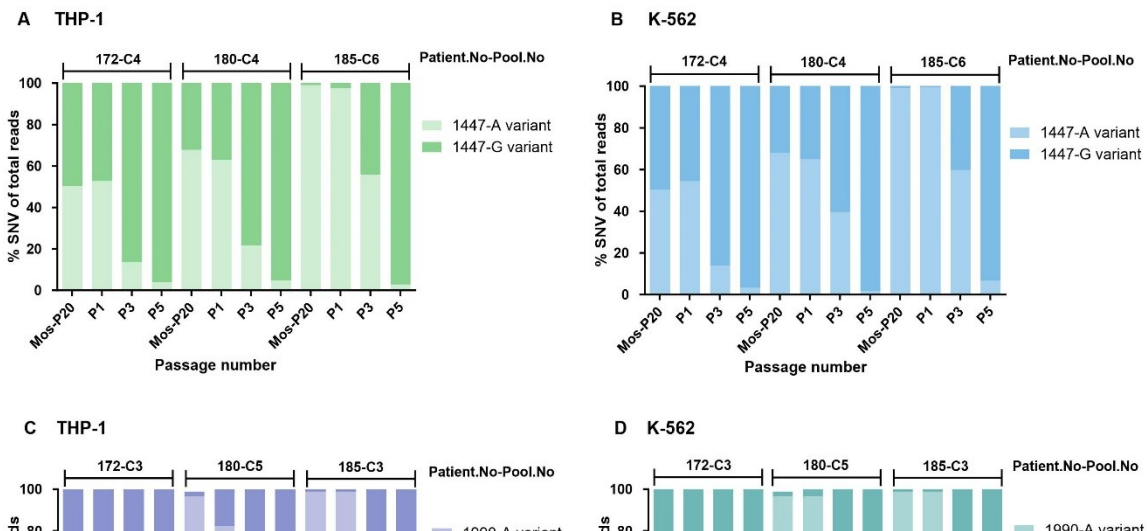
*w*Mel-mosquito-adapted virus, containing the 1447A variant at a frequency > 50%, were subjected to 10 serial passages in Wt (A) and *w*Mel-mosquitoes (B). The SNV frequencies were determined by sequencing whole DENV-1 genomes in mosquito pools at P21, 25, and 30 mosquito pools. The bar charts represent the frequencies of the G form (dark colors) and the A form (light colors) of each SNV.

4.3.7.2 Transience of 1447A (203K) and 1990A (384K) in human cell lines:

We investigated the stability of the 1447A variant in human cell lines lacking *w*Mel infection. In THP-1 and K-562 human cell lines, the frequencies of 1447A declined rapidly (< 10%) within 5 passages for all three virus lineages. This decline was attributed to the reversion to 1447G as the consensus within the virus population (Fig 4-12A, B). We also quantified the DENV genome copies in the supernatants of P1, P3, and P5 to assess the magnitude of DENV infection in human cells. The DENV genome copies within the *w*Mel-adapted virus lineages exhibited comparable levels to those observed during the passage of the progenitor virus. The DENV copy numbers displayed an overall increase with each subsequent passage number (Table 4-2). To assess the viral titers, the supernatants of P1, P3,

and P5 were titrated, revealing no significant difference in titers between the *w*Mel-adapted virus lineages and the progenitor virus (Table 4-3). This finding suggests that the variation in variants was not attributable to the reduced susceptibility of the cell lines to DENV-1 infection.

We further investigated whether the rapid frequency decline observed in human cells was specific to 1447A or common to other variants identified in the passaging system. To explore this, we passaged three supernatants from P20 Wt-mosquito lineages that had high frequencies of 1990A (> 50%), the selected SNV in both *w*Mel- and Wt-mosquito pools. Similar to 1447A, the frequency of 1990A also declined to < 10% by P5 (Figure 4-12C, D).



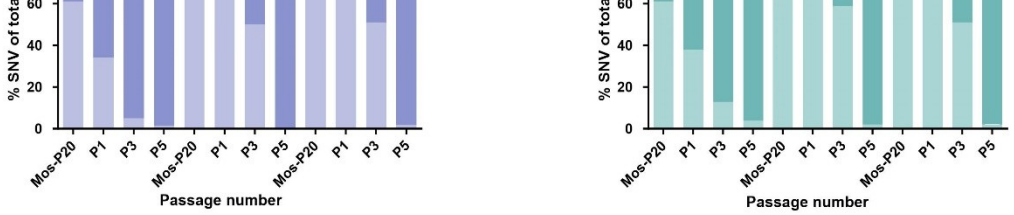


Figure 412: Decline in 1447A and 1990A frequencies throughout passages in human cell lines

These findings suggest that both 1447A (203K) and 1990A (384K) are not competitive enough to persist in the consensus sequence during multiple rounds of infection in human cells.

(A-B) wMel-mosquito-adapted virus, with the 1447A variant at a frequency >= 50%, underwent five serial passages in human cell lines, THP-1 and K-562. (C-D) Similarly, the Wt-mosquito-adapted virus supernatants, containing high frequencies of 1990A, were passaged five times in THP-1 and K-562 cells. The SNV frequencies were determined by sequencing whole DENV-1 genomes in mosquito pools at P1, P3, and P5 virus supernatants. The bar charts illustrate the frequencies of the G form (represented by dark colors) and the A form (represented by light colors) of each SNV

Table 4-14: DENV genome copies during passage of three 1447A and three progenitor lineages in human cell lines.

Cell line	Cell-passage Number	Log_{10} geometric mean DENV genome copies/ml ± SD of three lineages: Passage of *w*Mel-adapted virus (1447A)	Passage of progenitor virus (1447G)	Ratio of *w*Mel-adapted/progenitor DENV copies
THP-1	1	5.14 ± 0.17	5.13 ± 0.18	1.00
	3	7.07 ± 0.98	7.10 ± 0.91	1.00
	5	10.11 ± 0.56	10.20 ± 0.59	0.99
K-562	1	5.32 ± 0.51	5.31 ± 0.53	1.00
	3	7.89 ± 0.057	7.89 ± 0.07	1.00
	5	10.22 ± 1.06	10.23 ± 1.07	1.00

Table 4-15: Log10 Geometric mean titer (TCID50/mL) during passage of three 1447A and three progenitor lineages in human cell lines.

Cell line	Cell-passage Number	Log_{10} Geometric mean titer (TCID50/ mL) ± SD of three lineages: Passage of *w*Mel-adapted virus (1447A)	Passage of progenitor virus (1447G)	Ratio of *w*Mel-adapted/progenitor DENV (TCID50/ mL)
THP-1	1	4.67 ± 0.56	5.01 ± 0.89	0.93
	3	6.98 ± 0.89	7.34 ± 0.83	0.95
	5	10,09 ± 0.93	9.89 ± 0.89	1.02
K-562	1	3.93 ± 0.91	4.01 ± 0.84	0.98

	3	5.78 ± 1.02	6.51 ± 0.98	0.89
	5	9.37 ± 1.23	8.43 ± 1.07	1.11

4.3.8 Prevalence and structural analysis of E protein mutations at residues 203 and 384 in the Envelope protein of DENV-1

To assess the prevalence of mutations in existing dengue viruses, we against the complete E protein sequences from this study to protein database. The complete E protein sequences of thirteen dengue strains covering various genotypes across the four serotypes were selected to present the location of the residues 203 and 384. Notably, both residues 203 and 384 code for different amino acids across serotypes (Figure 4-13A). Analysis of DENV-4-II sequences revealed that the presence of K at residue 203 is commonly observed for this specific genotype. However, further examination of DENV-1-I sequences at residue 203 did not reveal the presence of K at this site.

To further investigate the potential impacts of the E203K and E384K change on the structure and function of the E glycoprotein, the DENV-2 E glycoprotein cryo-EM structure of mature E was analysed in PyMol [502]. To compensate for the difference between DENV-1 and DENV-2 five residues were mutated within E to recapitulate a DENV-1 structure surrounding residue 203. Residue 203 is located in DII of E (Figure 4-13B) and lies in close proximity to H261 (Figure 4-13C).

Residue 384 is located in DIII of E protein (Figure 4-13B), is highly surface exposed and constitutes part of a 10 amino acid lateral loop that has been implicated in mediating attachment to mosquito cells for the DENV-2 serotype. Considering several of the amino acids differ between DENV-1 and DENV-2 at this site it remains unclear as to whether its role in attachment would be comparable for DENV-1 E protein.

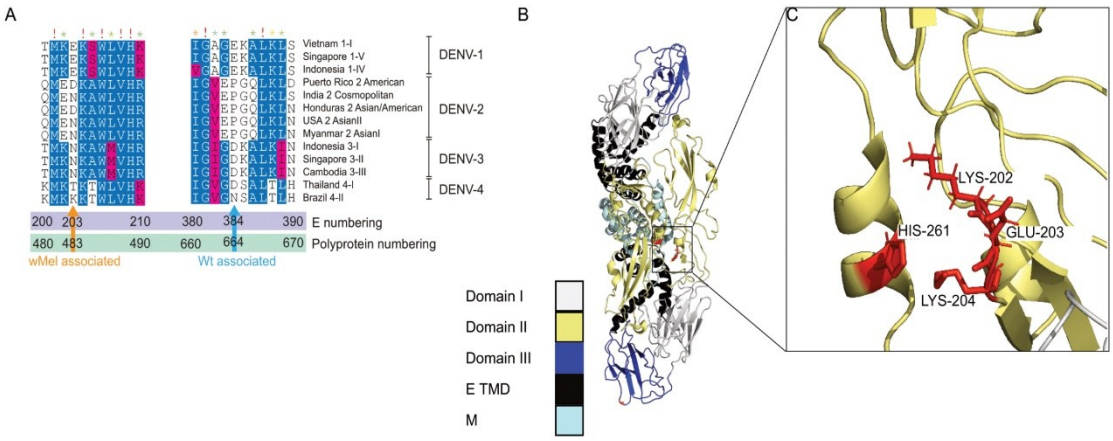


Figure 413: Prevalence and location of positively selected amino acids in Envelope protein

(A) Thirteen E sequences were downloaded from ViPr and aligned with MUSCLE. Conserved residues are indicated in blue while variable residues are not colored. (B) The CryoEM structure of the mature, dimeric DENV2 E protein (PDB: 3J2P) was visualized using PyMol, with different domains color-coded. (C) An 8Å image of residue 203 highlights proximal residues (C).

4.4 Discussion

We employed a mosquito serial passage approach to study the genome evolution of DENV-1 in *w*Mel-infected *Ae. aegypti* mosquitoes. In the following discussion, I discuss the above results and explore the implications of these findings for understanding *w*Mel-viral population interactions, their relevance to field-based investigations and the limitations of the study.

4.4.1 Virus passage studies and *Wolbachia*

In vitro (cell culture) serial passage systems used to study DENV evolution in the presence of *w*Mel have faced challenges, often resulting in viral extinction and leaving the

impact on the DENV genome uncertain [503]. In our study, we developed a DENV-1 serial-passage system in mosquitoes that provided a viable approach to explore the effects of *w*Mel on the DENV genome. Through multiple replicate passages of patient-derived DENV-1, we identified changes in the envelope protein that were under selection in *w*Mel-infected mosquitoes but not in Wt mosquitoes. Although our findings were derived from a laboratory mosquito serial-passage system, they provided insights into the ability of *w*Mel can drive viral evolution.

Comparisons between previous *w*Mel serial-passage systems (in cell culture), which experienced viral extinction and the system described in our study suggest that several factors influence virus survival during serial passage, including virus serotype, culture system, virus origin, and injection volume. A previous study passaged DENV-3, a serotype that is less easily isolated in cell lines [504] and less infectious for mosquitoes compared to DENV-1 and 2 [494]. In our study, we selected to passage DENV-1 due to its lower inhibition by *w*Mel compared to DENV-2, 3, and 4 [490], and DENV-1 was predominant in Vietnam during the time we conducted this study. Instead of using cell lines as the virus culture system, we employed mosquitoes, which are more susceptible to DENV infection [505]. The presence of *w*Mel in cell lines may impair the infectiousness of nascent virions [506], potentially leading to the attrition of viral particles over successive passages. However, whether this phenomenon is relevant for *w*Mel-infected mosquitoes remains unknown.

To have a “real-world” population of viruses to input into this experimental system we utilized blood samples obtained from febrile DENV-1 patients to feed mosquitoes and establish infection at P0. In contrast, previous passage studies relied on cell-grown virus stocks, which are more likely to contain non-infectious viral particles compared to viremic plasma from a patient [507] and may have acquired genetic changes over time due to their passage history [508]. Furthermore, our experimental design utilized intrathoracic injection of

filtered mosquito homogenates to passage the virus population; this might have prevented bottlenecks caused by low virus titers. The use of low virus titers in previous studies may have contributed to the *w*Mel-driven viral extinction observed in *D. melanogaster* [509]. In our serial-passage system, we obtained intriguing insights into the *w*Mel-mediated blockade of virus replication. Notably, we observed the absence of the typical inhibitory effect of *w*Mel on viral load, suggesting that a substantial viral inoculum can overcome this restriction.

4.4.2 Exploring genetic outcomes and selection signals of *w*Mel in DENV-1 using a mosquito serial-passage system

The potential development of resistance by DENV to the antiviral effects of *w*Mel, a critical component of the *Wolbachia* introgression method for public health use, remains uncertain. In our study, we employed a mosquito serial-passage system to examine the genetic changes occurring in DENV-1 in the presence of *w*Mel and to identify indications of *w*Mel-associated selection. Within this passage system, we observed positive selection for specific amino acid changes in the E protein of DENV-1 in both *w*Mel-infected mosquitoes (203 and 384) and Wt mosquitoes (384). These substitutions emerged independently in different cohorts during the passage of DENV-1 obtained from different patients infected with DENV-1. Interestingly, an independent experiment conducted at Monash University involving different DENV-1 strains and *w*Mel *Ae. aegypti* with an Australian genetic background also demonstrated the emergence of both substitutions (E203K and E384K) in multiple technical replicate mosquito pools [190].

The recurring nature of these substitutions (E203K and E384K) might be linked to their central position within homopolymeric (HP) A tracts in the viral genome, known to be susceptible to mutations induced by RNA-dependent RNA polymerases [510,511]. Despite its recurrence, E203K was weakly selected by *w*Mel in the replicate experiment. Inter-

experimental variation could be due to stochastic competition between the variant and wild-type viruses during replication in *Wolbachia*-free mosquito cells. Alternatively, the E203K substitution may impose fitness costs on different genetic backgrounds of DENV-1, leading to the reversal of its selective advantage.

To understand the dynamics of viral evolution and host-virus interactions, exploring the interplay between *w*Mel, the viral genetic background, and the fitness costs associated with the E203K variant in diverse genetic contexts would be valuable. Mechanistic studies focusing on the impact of *w*Mel on RNA-dependent RNA polymerase-induced mutations within HP A tracts, particularly involving the 1447A variant, could enhance our understanding of how *w*Mel influences viral fitness and adaptation.

4.4.3 Postulated effects of E protein mutation, E203K on DENV infection and *w*Mel blocking

Across the DENV1 genome, four amino acid changes are subject to positive pervasive selection over 20 passages in mosquitos and only one, E203K in the E glycoprotein is clearly associated with the presence of *w*Mel. The 203K variant are rarely observed in public viral genome repositories, constituting less than 5% of the sequences, whereas 92% exhibit the 203E variant (4100 sequences). This low prevalence has hindered the study of the functional implications of the 203K mutation in viral infection, specifically for the DENV-1 serotype.

Residue 203, located in DII of the DENV1 E protein, plays a role in viral fusion and virion maturation [512]. While the exact function of amino acid residue 203 in DENV replication remains unclear, neighboring residues have been studied. Amino acid changes from K to R at residue 202 were observed during DENV3 passage in Vero cells, and plaque purification of both virus variants revealed that the 202R variant reduced the pH threshold

required for fusion by 0.6-0.8 pH units [513,514]. This suggests that viral variants with modified fusion pH thresholds might follow an unconventional endocytic pathway.

The current model of DENV entry suggests that fusion primarily occurs in late endosomes due to a reliance on low pH and high concentrations of anionic lipids that serve as fusion co-factors [131]. Sufficient lipid concentration in late endosomes is crucial for DENV uncoating, and potentially lipid dysregulation might inhibit this process [131]. Hypothetically, the E203K substitution could alter fusion pH thresholds, resulting in viral uncoating occurring in early endosomes rather than late endosomes. Such a shift in uncoating location could offer distinct advantages for viral entry into *w*Mel-infected cells. Interestingly, studies have shown that *w*Mel can disrupt lipid metabolism and vesicular trafficking [378], potentially offering an advantage for DENV mutations that alter the typical endosomal fusion process in the context of a *Wolbachia* infection.

The E → K substitution at residue 203, located in DII of E gene, would increase local positive charge and position cationic sidechains proximal to a conserved histidine (261H) based on the DENV-1 E gene 3D structure. Histidine–cation interactions are known pH-sensing elements [515], triggering the E protein's conformational change from dimeric to trimeric through electrostatic repulsion [512,514,516,517]. This pH-induced positive charge facilitates viral-endosomal membrane fusion. The presence of additional positive charges due to the E203K substitution near the conserved histidine residue 261H may amplify repulsive forces between these residues, promoting the conformational changes necessary for fusion. This raises the hypothesis that the E203K substitution could affect E protein trimerization kinetics and essential pH thresholds for fusion.

The evasion of *w*Mel-induced virus blocking by the E203K variant has not been investigated in this study. This substitution may be “without phenotype” and represent an

initial step in the selection process because potentially evasion of *w*Mel-induced blocking could involve the accumulation of multiple mutations in the viral genome.

4.4.4 Proposed influence of E384K mutation on DENV infection

We hypothesize that the E384K variant, found in both Wt- and *w*Mel-infected mosquitoes could confer a general advantage for DENV attachment to mosquito cells at 28^0C. The residue 384 is situated in DIII of the E protein, which is prominently exposed on the surface and forms part of a 10-amino acid loop recognized for facilitating specific binding to mosquito cells, particularly concerning DENV-2 [518].

Within this loop, four out of the ten residues (382-385) have been identified in mosquito-borne flaviviruses but are absent in tick-borne ones, suggesting a potential role in determining host tropism. The binding of DENV to mosquito cells at a constant temperature like 28°C might preferentially favor E proteins with specific thermodynamic properties. Furthermore, limited exposure to human receptors may be a driving force for selecting variants with a high affinity for mosquito receptors, potentially explaining the observed prevalence of the E384K variant in mosquito-infected hosts.

The persistence of viruses carrying the 384K variant was abolished upon transition to human cell hosts, underscoring the variant's high affinity for mosquito receptors. Thus, we propose that the E384K variant likely augments the virus's capability to infect and replicate within mosquitoes, thereby playing a pivotal role in facilitating efficient transmission of DENV within mosquito populations.

4.4.5 Limitations and relevance to the field

*w*Mel, the symbiotic bacterium exerts a suppressive effect on DENV-1 by impeding its dissemination from the mosquito midgut, which serves as the initial site of DENV

exposure, to the salivary glands, thereby reducing the potential for mosquito-to-human virus transmission through saliva [490]. However, our serial-passage system, involving the passage of viral filtrate derived from the entire mosquito body, does not exhibit the typical inhibitory influence of *w*Mel on DENV-1. Although this system does not fully recapitulate the complex dynamics of field DENV ecology, specifically pertaining to salivary virus populations and the natural mosquito-to-human transmission cycle, it affords a valuable opportunity to investigate DENV evolution under optimal conditions for adaptive processes. Consequently, we were able to discern distinct genetic signatures of DENV-1 evolution driven by *w*Mel, contrasting them with the background evolutionary changes observed in wild-type mosquitoes.

Our primary objective centers on the assessment of the risk posed by the 203K and 384K variants to the *Wolbachia* method. While these variants were consistently generated during our passage experiments, they readily reverted to their progenitor forms (203E and 384E) in human cells and, in the case of 203, also in Wt mosquitoes. Thus, it is plausible that purifying selection mechanisms operative during mosquito-to-human transmission would effectively eliminate the 203K and 384K variants.

This study determined an amino acid alteration in the E protein of DENV-1 that exhibited consistent selection throughout serial passage in *w*Mel-infected *Ae. aegypti* mosquitoes. These findings establish proof of concept regarding the selective pressure exerted by *Wolbachia* on the viral population under controlled laboratory conditions. The potential emergence of such variants in areas where *Wolbachia* has been introduced into the *Ae. aegypti* population remains uncertain. Nonetheless, our laboratory-based observations concerning virus evolution can provide valuable insights for future investigations into the population genetics of DENV in *Wolbachia* release sites.

4.5 Conclusion

We examined the genetic outcomes of serial passage of DENV-1 in *w*Mel-infected *Ae. aegypti*. The results showed a significant amino acid substitution, E203K, in the DENV-1 Envelope protein, which exhibited higher frequency in virus populations passaged with *w*Mel-infected mosquitoes. The E203K substitution demonstrated evidence of adaptive selection, consistently emerging in virus populations during serial passage with *w*Mel infection. However, the 203K variant reverted to the progenitor sequence in wild-type mosquitoes and human cells, providing evidence of two factors affecting virus evolution: negative fitness trade-offs from alternating host replication (human-to-mosquito) and competition from other viral variants (lack of *w*Mel infection). These findings demonstrated the occurrence of *w*Mel-associated selection in virus populations under experimental conditions, highlighting the potential role of *Wolbachia* in shaping the genetic diversity and evolutionary dynamics of DENV-1. Further field-based studies are required to investigate the selective pressure exerted by *w*Mel on DENV evolution in regions where *w*Mel is established, providing valuable insights for disease control strategies.

Chapter 5

EVALUATING VERTICAL TRANSMISSION OF DENGUE VIRUS IN FIELD-COLLECTED *Aedes aegypti* AND THE EFFECTIVENESS OF *Wolbachia, w*Mel STRAIN IN REDUCING VERTICAL TRANSMISSION TO OFFSPRING

Abstract

Vertical transmission (VT) of mosquito-borne viruses has traditionally been considered negligible in natural transmission dynamics. In Chapter 5, we investigated the potential for VT of DENV in field-sampled *Ae. aegypti* populations fed upon dengue patient-derived blood meals. Our findings revealed that VT is a rare event, suggesting a limited role for vertically transmission in the natural history of DENV transmission. Additionally, we demonstrated a significant lower rate of VT in *w*Mel-carrying *Ae. aegypti* compared to wild-type counterparts The results of the study indicated that VT becomes even more improbable in the presence of *Wolbachia*, revealing an additional protective mechanism against the transmission of DENV between generations. The combined effect of *w*Mel infection in reducing both horizontal and vertical transmission can significantly contribute to decreasing the incidence of dengue.

5.1 Introduction

DENV is primarily transmitted between humans through the bite of infected female *Aedes sp.* mosquitoes, known as horizontal transmission (HT). In addition to this, DENV can also be transmitted vertically from infected female mosquitoes to their offspring, a process known as transovarial transmission or vertical transmission (VT) [270,271,519]. The virus can be transmitted from female mosquitoes to their offspring during follicle development or during oviposition. Robust evidence of VT in nature is the presence of DENV in wild-caught males of *Ae. aegypti* and *Aedes albopictus* (*Ae. albopictus*) [270,271,276].

The determination of VT has been considered through various approaches, depending on the study methods employed, which include examining the presence of infected male mosquitoes and detecting viruses in both mature and immature mosquito stages of any sex [271,276,288,520]. Due to various methodological approaches, the determination of DENV VT frequency in nature can vary, ranging from 0% [286,288] to approximately 20% in pooled larval samples [521] or based on the Ovitraps Index [289,290] (number of ovitraps with positive eggs divided by the total number of ovitraps installed). These varying results lead to contradictory conclusions about the role of VT in DENV maintenance in nature. Therefore, it is crucial to interpret these findings cautiously.

Vector control has been the mainstay of dengue control efforts for decades. However, existing vector control methods have not sufficiently reduced the public health burden of dengue. *Wolbachia*-based biocontrol relies on CI and the inhibition of viral replication within mosquitoes to mitigate DENV transmission[522–524]. *Wolbachia* is hypothesized to inhibit DENV replication in mosquito cells through several mechanisms, such as disrupting intracellular membranes, changing the host cell cytoskeleton, altering lipid homeostasis, triggering the innate immune response, and interacting with the cellular machinery involved

in RNA virus infection [525]. The *Wolbachia pipientis w*Mel strain has been deployed for population replacement in several countries [340,355,358,411,437], showing a decrease in dengue incidence in treated communities compared to non-treated areas. The WHO's Vector Control Advisory Group have recognized the public health value of the *Wolbachia* replacement method [489]

To achieve a better understanding of transmission of DENV in *Ae. aegypti* (Vietnamese genetic background), this chapter focuses on estimating the frequency of VT of DENV in field-derived *Ae. aegypti* and colonized *Ae. aegypti* with and without *w*Mel infection. The aims of this chapter are:

1. To measure the frequency for vertical transmission of DENV and potential for subsequent horizontal transmission.

The findings of the first aim were published as a research article in the Vector and Parasite Journal under the title 'Assessing the vertical transmission potential of dengue virus in field-reared *Aedes aegypti* using patient-derived blood meals in Ho Chi Minh City, Vietnam.' Parasite Vectors. 2020/09/16. 2020;13(1):468. [526] .

2. To test the hypothesis that *Wolbachia, w*Mel could reduce vertical transmission of DENV in *Ae. aegypti*.

The results of the second aim were reported in a research report in the Vector and Parasite Journal under the title *'Wolbachia w*Mel-mediated effects on dengue virus vertical transmission from *Aedes aegypti* to their offspring. Parasite Vectors. 2023 Aug 31;16(1):308. [527].

5.2 Material and Methods

5.2.1 Dengue patient's cohorts and healthy volunteers

The study's protocol received approval from both the Scientific and Ethical Committee of the Hospital Tropical Diseases (HTD) (HTD EC CS/NĐ/16/27) and the Oxford Tropical Research Ethical Committee (OxTREC: 45-16). Inclusion criteria for participants were: (i) age 15 years or older, (ii) admitted as inpatients or outpatients at HTD with less than 96 hours of fever, (iii) confirmed DENV infection by NS1 rapid test and Dengue PCR serotyping, and (iv) provided written informed consent.

The venous blood from patients was drawn into two tubes: one for blood-feeding mosquitoes and the other for DENV serotyping and quantification [91]. Healthy volunteers also provided venous blood samples for experimental feeding after providing written informed consent, and their tympanic temperature was measured prior to blood draws to confirm they were afebrile.

5.2.2 Experimental approach and methods

Experiment One investigated wild type (Wt) *Ae. aegypti*, with Vietnamese background, which were maintained under field-reared conditions as described in Section 5.2.3 below (Figure 5-1). We estimated the proportion of F0 females transmitting the virus to their progeny after viremic blood meals from acutely infected dengue patients and the proportion of vertically infected F1 females with infectious saliva.

Experiment Two estimated the VT rates of DENV-1 in a laboratory colony *w*Mel-infected and Wt *Ae. aegypti* with Vietnamese genetic background, (Figure 5-1). Both mosquito lines were infected with approximately 1µL of a 10^5pfu/mL propagated DENV-1 culture supernatant (GenBank Accession Number: FJ432735) via intrathoracic inoculation

DENV-1 was used to challenge mosquitoes because it is less inhibited by *w*Mel compared to the other three serotypes [490]. The F1 adults were tested in pooled samples, and the VT rate in F1 progeny per 1,000 mosquitoes was assessed by Maximum Likelihood Estimation (MLE). The presence of DENV and *w*Mel was detected using real-time RT-PCR [355,445].

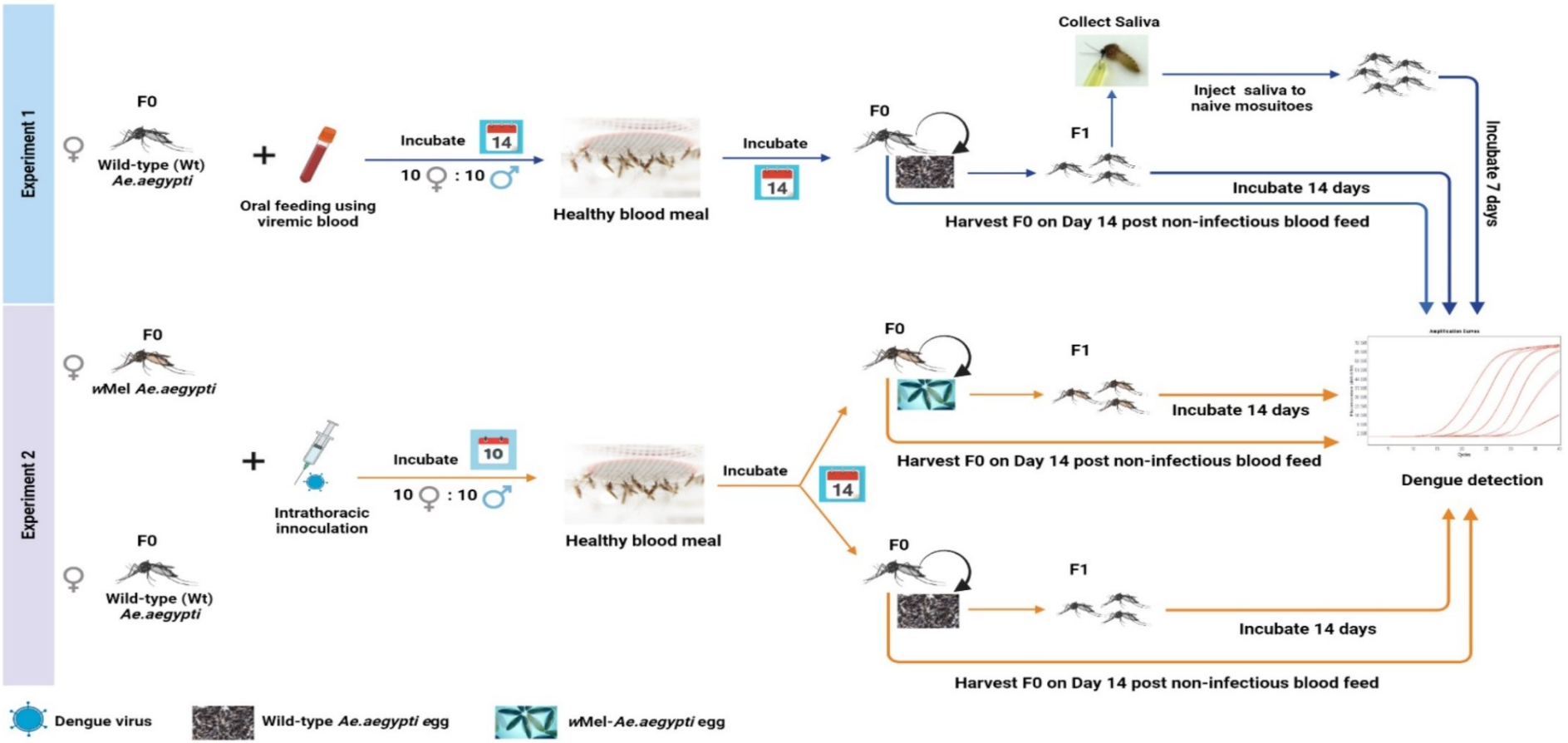


Figure 51: Experimental study of vertical transmission (VT) of dengue virus in *Ae. aegypti*, Vietnamese genetic background with and without *Wolbachia*, *w*Mel infection

In Experiment 1, wild-type (Wt) *Ae. aegypti* were maintained under field-reared conditions to estimate the proportion of F0 females transmitting the virus to their F1 offspring after viremic blood meals from acutely infected dengue patients. Experiment 2 estimated the VT rates of laboratory colony *Ae. aegypti* with a Vietnamese genetic background, including both *w*Mel-infected and Wt mosquitoes, by infecting them with an adapted DENV-1 strain through intrathoracic inoculation. The F1 adults were subsequently tested in pooled samples, and the VT rate in F1 progeny per 1,000 mosquitoes was assessed. The presence of DENV and *w*Mel was detected by real-time RT-PCR.

5.2.3 Mosquito's derivation, rearing and maintaining

For the first VT experiment with field-collected *Ae. aegypti*, weekly collections of fourth instar larvae or pupae were carried out from households in District 8, Ho Chi Minh City (HCMC), Vietnam. Larvae and pupae were kept in the same water that they were living and not provided with additional food during emergence. The colonized *Ae. aegypti* and field-collected mosquitoes in the two experiments share the same genetic background [494,526].

The colonized wild type and *w*Mel-infected *Ae. aegypti* were reared and maintained under laboratory conditions [494]. The *w*Mel- infected *Ae. aegypti* females were generated by repeated backcrossing of *w*Mel infected males msoquitoes from Cairns, Australia with wild type females from HCMC as previously described [261]. The generations from G59 to G61 colonized *w*Mel *Ae. aegypti* were used in the study. The colonized wild type *Ae. aegypti* was derived from generations F54 and F55, and the procedure and origin of the colony were described previously [494,526]. The presence of *w*Mel in each *w*Mel-carrying *Ae. agypti* generation was tested [355]. All mosquitoes were maintained at 26-28 °C, 65-85% rH, and a 12:12 hrs light:dark cycle, with access to 10% sucrose solution *ad libitum*.

5.2.4 Generating DENV-infected F0 mosquitoes

In Experiment One, twenty field *Ae. aegypti* females were offered the blood of dengue patients via artificial membrane feeders and maintained in cups (1:1, ratio of females: males). After 30 minutes of viremic blood-feeding, only fully engorged females were retained and maintained under controlled conditions: 28 °C; 65–85% relative humidity; and a 12:12 hrs light: dark cycle with sugar and water ad libitum.

In Experiment Two, two- to three-day-old colonized female adults were intrathoracically injected with 1µL of laboratory-adapted DENV-1 (10^5pfu/mL, Gen bank

Accession Number: FJ432735). Repeated: DENV-1 was selected for infection establishment due to its lower inhibition by *w*Mel compared to the other three serotypes [490]. Ten injected female adults were kept in cups with ten male mosquitoes (1:1 ratio of females: males). The F0 females of both wild type and *w*Mel lines were maintained on sucrose for ten days in separate chambers.

The second blood meal was given to surviving F0 adults on Day 14 (Experiment One) and Day 10 (Experiment Two). After 30 minutes of blood feeding, fully engorged females were retained and placed into separate cups (isofemales) containing wet cotton balls for oviposition. Sugar (10% sucrose) was provided for 14 days. On Day 14 post non-infectious blood meal, individual F0 females were harvested for DENV-1 screening.

5.2.5 Hatching and harvesting F1 mosquitoes

F1 eggs were hatched five to seven days after the second blood meal. F1 eggs from the same F0 mother were floated in the same tray, and larvae or adults from DENV-negative F0 females were immediately discarded. F1 adults from Experiment 1 and 2 were maintained for 14 days after emergence. They were subsequently stored individually and sorted by sex within cohorts originating from the same mother. Refer to Chapter "Materials and Methods", section 2.1 for the procedures of floating eggs, rearing larvae, and pupae collection, and refer to section 1.2.6 for mosquito tissue collection.

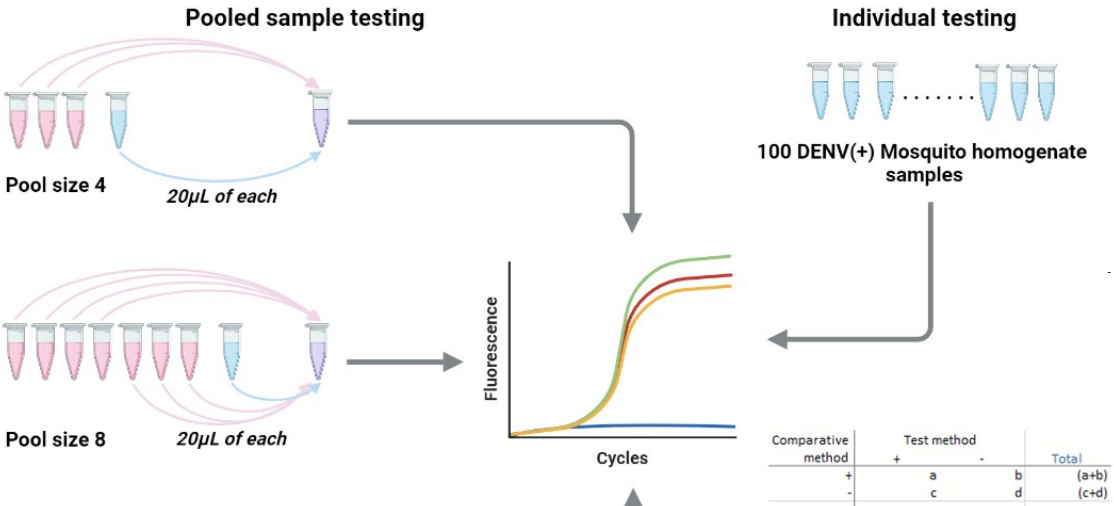
For the field-reared mosquito group, saliva from F1 mosquitoes was collected and inoculated into five naïve mosquitoes before collecting their whole bodies. Only inoculated mosquitoes derived from infected F1 adults were harvested and tested for DENV infection in pools according to the saliva source on Day 7 post-injection. Refer to Chapter "Materials and Methods", section 2.2.5 for the procedure of infecting mosquitoes by intrathoracic DENV inoculation.

5.2.6 Mosquito homogenization and pooling

All mosquitoes were homogenized individually in 100µL of MD. In Experiment 1, F0 and F1 (at Day 14 of emergence) mosquitoes were individually tested for DENV. In Experiment 2, F1 adults were pooled (20µL from each sample) for generating pooled samples that used for testing. Following the initial detection of positive DENV pooled samples, we proceeded to un-pool these samples to determine the number of infected individuals. This was accomplished by using the remaining volume of mosquito homogenate. The mosquito homogenates were subjected to heating, following the outlined methodology in Chapter "Materials and Methods", section 2.2.6. The presence of DENV in *Ae. aegypti* and the density assessment of *w*Mel in *Ae. aegypti* were determined using specific procedures, outlined in Chapter "Material Method", section 2.5.2.

5.2.7 Determining the pool size for pooling strategy

To accurately estimate the proportion of infected individuals in a population using Maximum Likelihood Estimate (MLE), determining the appropriate pool size is crucial to minimize the likelihood of false-negative results. In this study, the pool size of pooled testing was examined using a sample-media pooling approach, adapted from the Clinical and Laboratory Standards Institute (CLSI), CLSI EP12 protocol [528]. One hundred mosquito homogenates containing DENV RNA, ranging from Ct values 28-35, were utilized to form pools of different sizes: four, eight, and sixteen (Figure 5-2). The Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) values were calculated for different pool sizes. The PPA of 85% was selected as the cut-off value [529].



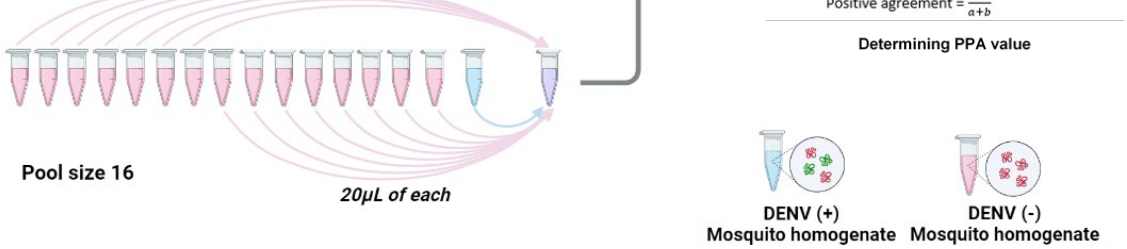


Figure 52: The procedure for determining the pool size that achieves an 89% Positive Percent Agreement (PPA) between pooling and individual testing

5.2.8 Estimating the VT rate of F1 progeny under field-reared conditions

using three key indices. Firstly, we quantified the number of F0 mothers capable of transmitting the virus to any number of their progeny. Secondly, we calculated the proportion of their progeny that were DENV infected. Lastly, we determined the proportion of infected progeny with infectious virus in their saliva. These indices were calculated using two different approaches with distinct denominators. One approach involved a restricted subset of DENV-infected F0 mosquitoes and their eggs, while the other encompassed all F0 females that fed on infectious blood meals and laid eggs, irrespective of their infection status.

5.2.9 Estimating the prevalence of DENV-1 vertical transmission in a laboratory colony of *Ae. aegypti* with and without *w*Mel infection

To estimate the VT rate of DENV-1 in a large number of F1 mosquitoes, we utilized MLE to determine the proportion of infected individuals in pooled samples. This method calculates the most likely infection rate based on the testing results and an assumed probabilistic model of binomial distribution of infected individuals in a positive pool [298,530–532]. We employed bias-corrected likelihood methods and computed a skew-corrected score confidence interval (95% CI) to account for biases [299]. The infection rate was expressed as the number of infected mosquitoes per 1,000 individuals. We used the Wilson score-based interval of the Newcombe method [533] to compare confidence intervals and assess statistical significance in infection rate differences between mosquito lines. Subsequently, the positive pooled samples were un-pooled to ascertain the number of positive F1 adults.

5.2.10 Statistical analysis and graphing

The statistical analysis was performed using Prism 9.5.1 software and the R package for PooledInfRate estimation [530]. Visual illustrations were created using Biorender.com

5.3 Results

5.3.1 Vertical transmission of DENV-1 in field-collected wild-type *Ae. aegypti*

5.3.1.1 Characteristics of the confirmed dengue patients enrolled and the viral load of DENV-1 strain (GenBank Accession Number - J432735)

The mosquito samples and corresponding data from ten patients who tested negative for DENV were removed. Out of the remaining 30 patients who were virologically confirmed to have dengue, DENV-2 was the most prevalent serotype at 50%, followed by DENV-1 at 30% and DENV-4 at 20%. The median viremia level across all patients was 7.2$\log_{10}$ copies/mL, with DENV-2 having the lowest median value of 6.92 $\log_{10}$ copies/mL. 62.5% of patients were enrolled on Day 4 of their illness (Table 5-1). The VT experiment utilized DENV-1 culture supernatant (J432735) for intrathoracic inoculation in colonized *w*Mel-infected *Ae. aegypti* with a Vietnamese genetic background, having a $\log_{10}$ viral titer of 5.01 (pfu/mL).

Characteristics	**Undefined (n = 10)**	**DENV-1 (n = 9)**	**DENV-2 (n = 15)**	**DENV-4 (n = 6)**	**All patients (n = 30)**
Age (years)	28.0 (25.5–34.7)	33.5 (25.2–35.0)	34.0 (27.0– 39.0)	36.5 (27.7– 37.7)	34.5 (26.0– 38.0)
Day of illness					
2	0	0	4 (26.7)	0	4 (10.0)
3	3 (30.0)	3 (33.3)	2 (13.3)	3 (50.0)	11 (27.5)
4	7 (70.0)	6 (66.6)	9 (60.0)	3 (50.0)	25 (62.5)
Viremia	<LOD	7.5 (5.3–	6.9 (5.7–	7.6 (6.7–	7.2 (6.3–

(log_{10}copies/ml)		8.0)	7.5)	8.3)	8.1)

Table 5-16: Characteristics of the enrolled dengue patients whose blood samples were used to feed mosquitoes

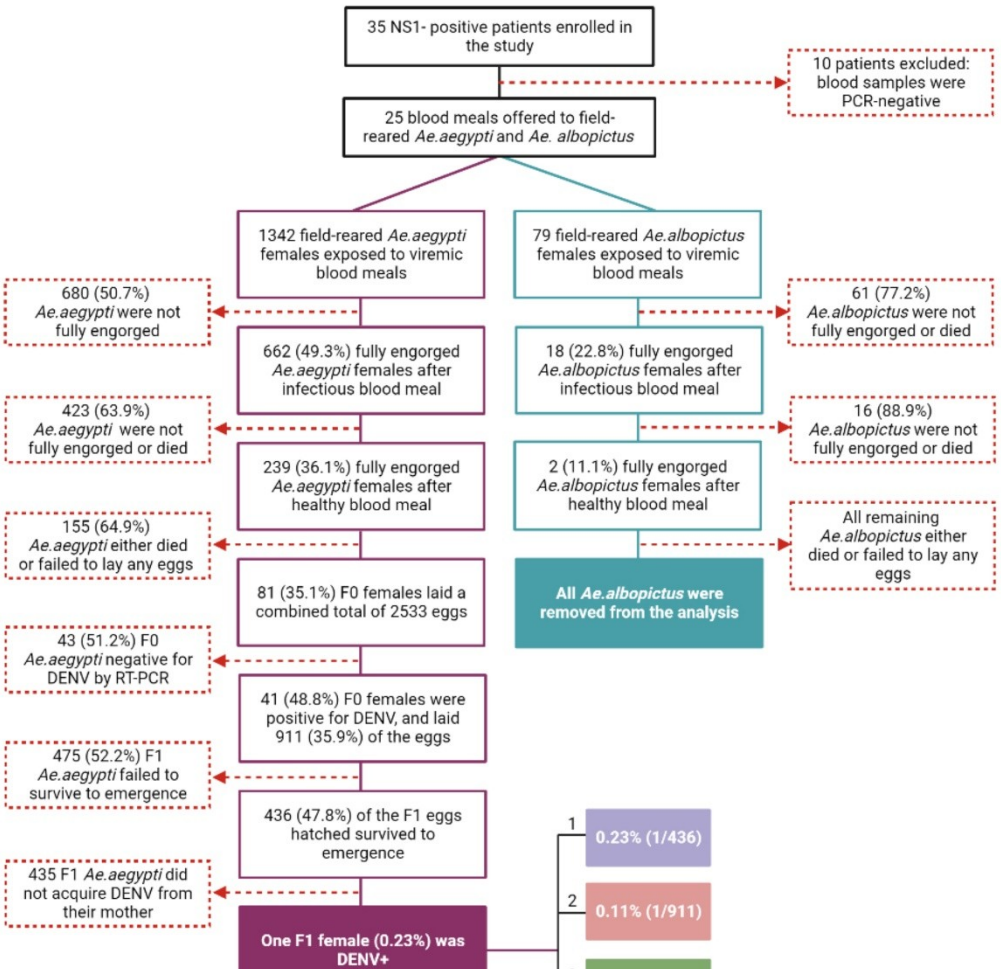
5.3.1.2 Species composition of field-reared mosquitoes

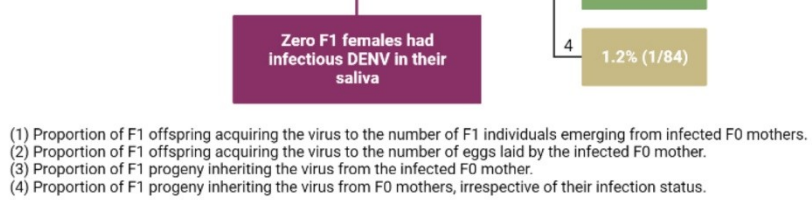
Over a period of 38 weeks, 33,720 pupae and larvae of *Aedes* were collected from four sites in District 8, HCMC. 33,720 *Aedes* larvae/pupae were collected over 38 weeks from four sites in District 8, HCMC. Of these, 94.8% (3,528) were *Ae. aegypti*, with 1,802 females and 1,726 males. On average, 47 female *Ae. aegypti* mosquitoes were collected weekly. *Ae. albopictus* accounted for 5.16% (192) of the total, while *culicine* mosquitoes were the majority of remaining individuals.

5.3.1.3 Estimates of vertical transmission in field-reared *Ae. aegypti* and *Ae. albopictus*

We investigated the VT of DENV in field-derived wild-type *Ae. aegypti* mosquitoes following their feeding on 25 independent DENV viremic blood meals. Among 1,342 female *Ae. aegypti* mosquitoes, 662 (49.3%) successfully engorged after blood feeding. Of these engorged mosquitoes, 239 (36.1%) females fed on a subsequent (non-infectious) blood meal, and out of them, 81 (35.1%) survived long enough to lay a combined total of 2,533 eggs. Among these 84 F0 female mosquitoes, 41 (48.8%) were found to be infected with DENV. Subsequently, 911 eggs from the DENV-infected F0 mothers developed into 436 (47.8%) F1 adults. Interestingly, none of the 214 (49.1%) male mosquitoes from the F1 generation tested positive for DENV infection. Among the 222 F1 females tested, only one individual (95% CI: 0.00-0.03) was found to be infected with DENV-2, with a Ct value of 33.11. The F1 female that was vertically infected with DENV-2 had her saliva tested, but none of the saliva samples

collected from any F1 female contained infectious virus via an in vitro transmission assay (Figure 5-3).





(1) Proportion of F1 offspring acquiring the virus to the number of F1 individuals emerging from infected F0 mothers.
(2) Proportion of F1 offspring acquiring the virus to the number of eggs laid by the infected F0 mother.
(3) Proportion of F1 progeny inheriting the virus from the infected F0 mother.
(4) Proportion of F1 progeny inheriting the virus from F0 mothers, irrespective of their infection status.

Figure 53: The flowchart outlines the sequential processing stages employed to estimate the frequency of vertical transmission of DENV in *Ae. aegypti* and *Ae. albopictus* mosquitoes maintained under field-reared conditions

Excluded samples are visually represented in red box

The occurrence of VT was determined by analyzing both the frequency of mothers transmitting the virus to their progeny and the proportion of F1 progeny inheriting the virus from infected mothers. Our results, obtained under conditions similar to the natural environment, demonstrate the rarity of VT. Among the group of 41 F0 females that tested positive for DENV and laid eggs, only one (2.4%.) F1 female was found to be vertically infected. When considering 84 F0 females regardless of the infection status, the transmission frequency of the virus to F1 increased slightly to 1.2%. If the numbers of F1 progeny that emerged to adults (n = 436) were counted, the percentage of DENV-infected progeny from a DENV-infected mother dropped to 0.23%. Furthermore, the analysis of the proportion of progeny born from a DENV-infected mother revealed that only 0.11% (n = 911) of progeny

acquired the virus during the second gonotrophic cycle (Figure 5-4). These observations collectively indicate that VT of DENV in field-collected *Ae. aegypti* mosquitoes is a rare event under conditions resembling natural habitats.

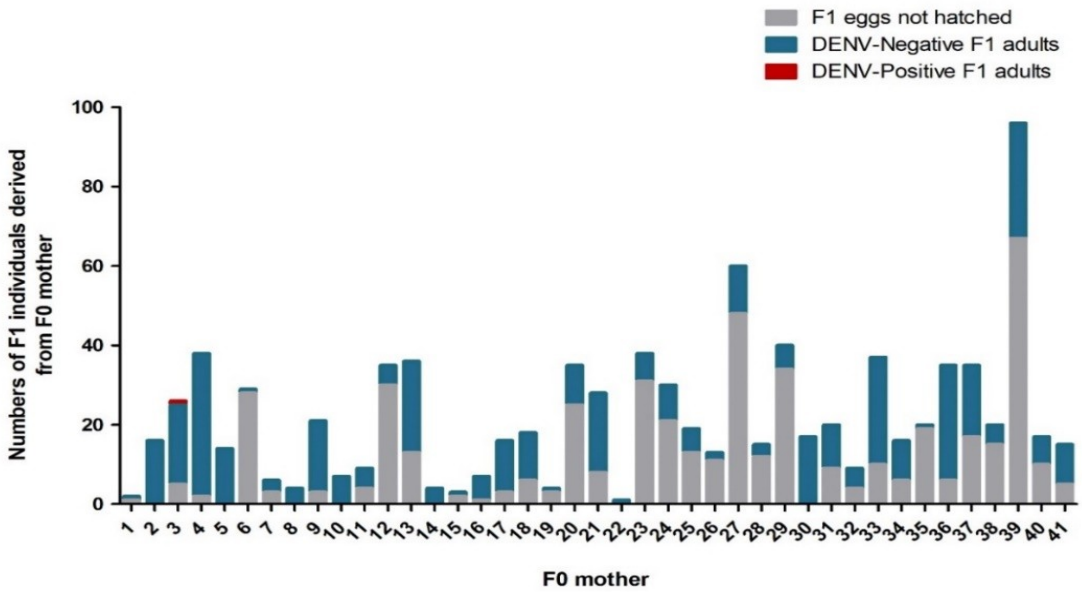


Figure 54: The histogram illustrates the distribution of F1 offspring derived from individual DENV-infected *Ae. aegypti* female mosquitoes that were field-reared.

Each bar on the histogram corresponds to one of the 41 DENV-positive F0 females and is categorized based on the outcome of their progeny. The light-grey portion represents the number of eggs that did not hatch, the dark-grey portion represents the F1 offspring that tested negative for DENV, and the black portion represents the F1 offspring that tested

Field-collected *Ae. albopictus* mosquitoes were utilized in this study to investigate the VT rate of DENV. These mosquitoes were exposed to the same 25 viremic blood meals as the field-reared *Ae. aegypti* mosquitoes in the study. Among 79 female *Ae. albopictus* mosquitoes, 18 (23.0%) individuals survived and fed on the infectious blood meal. However, only two out of the 18 engorged females (11.1%) were able to complete a second gonotrophic cycle, while the remaining individuals failed to produce subsequent generations. Therefore, the investigation of the VT of DENV in *Ae. albopictus* was limited due to the absence of viable progeny resulting from the restricted number of engorged females.

5.3.2 Vertical transmission in colonized *w*Mel-infected *Ae. aegypti*

5.3.2.1 Optimal pool size for mosquito pooling testing

The PPA values were computed for three different pool sizes: four, eight, and sixteen, and the highest PPA value of 96% was observed for a pool size of four mosquito homogenates. A pool size of eight demonstrated a PPA of 89% (95% CI = 0.81-0.94) (Table 5-2), and a pool size of sixteen showed a lower PPA of 68%. All three pool sizes exhibited a 100% agreement in the NPA value. Based on these results, a pool size of eight was selected [529], allowing a maximum of eight mosquitoes in each pool sample.

Table 5-17: The Positive Percent Agreement (PPA) results for Pool Size of eight mosquito homogenates. (+) Positive result, (-) Negative result

	Pool size- 8		
Individual testing	**-**	**+**	**Total**
-	15	0	15
+	9	76	85
Total	24	76	100
	Proportions		
	Agreement		**Wilson 95% CI**
-	1.000		0.79 – 1.0
+	0.894		0.81 – 0.94

5.3.2.2 Vertical transmission in colonized *w*Mel-infected *Ae. aegypti* versus their wild type counterparts

Our study aimed to measure whether VT was less likely to occur in *w*Mel-infected *Ae. aegypti* mosquitoes versus their wild type counterparts. We conducted VT estimation using 480 *w*Mel- *Ae. aegypti* females and 480 Wt females. Ten days after intrathoracic inoculation with DENV-1, non-infectious blood meal was provided to 420 (87.50%, N=480) surviving Wt females and 389 (81.04%, N=480) *w*Mel females. Approximately 10,328 eggs were collected from 343 (89.09%, n=385) virus-infected Wt F0 females, while approximately 12,027 F1 eggs were collected from 304 (91.84%, n=331) virus-infected *w*Mel F0 females. A total of 6,047 (58.55%) F1 adults emerged from the approximately 10,328 eggs collected from Wt F0 females. In the *w*Mel mosquito group, 5,500 (45.73%) *w*Mel F1 adults emerged from the approximately 12,027 F1 eggs. Wt F1 adult mosquitoes were grouped into 785 pools, while *w*Mel F1 adults were grouped into 712 pools (Figure 5-5).

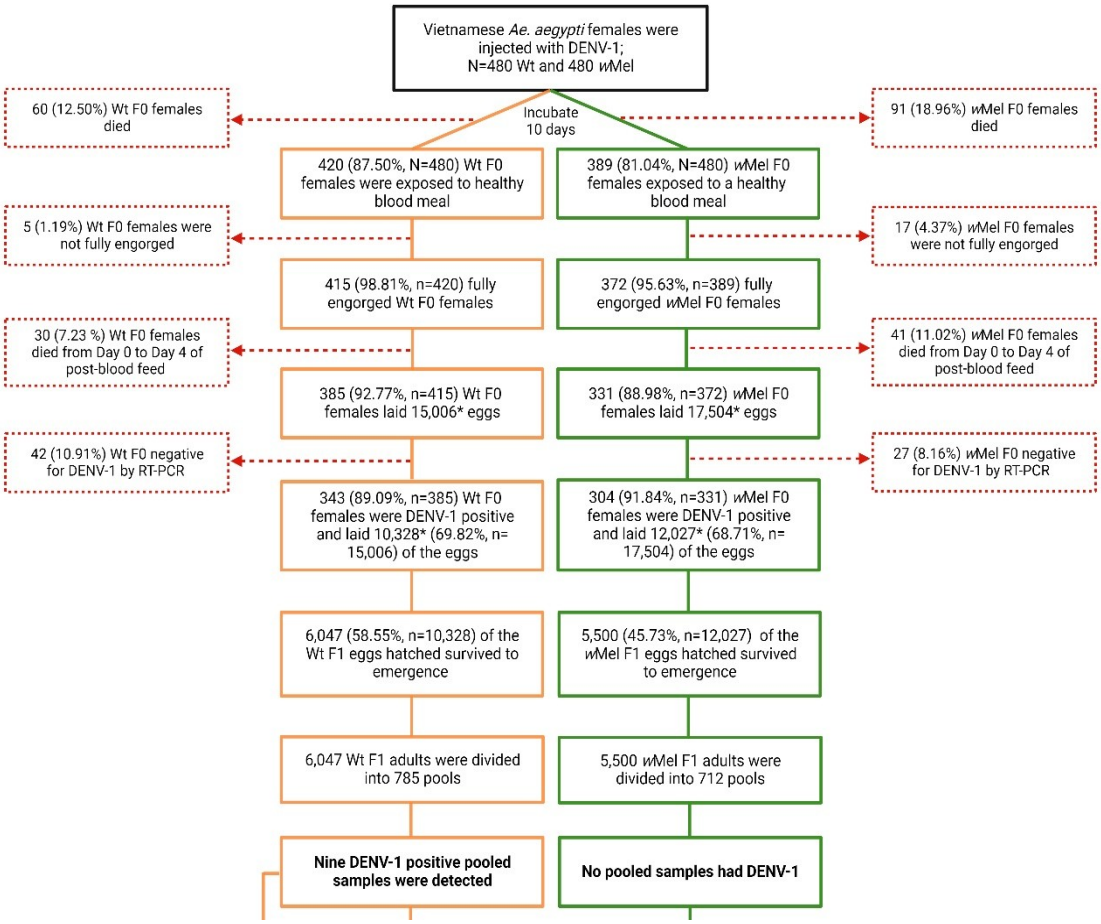


Figure 55: Flowch Vild type (Wt) and *w*Mel-infected *Ae.*

This flowchart illustrates the fate of mosquitoes as they were processed to estimate the vertical transmission rate of DENV-1 in two mosquito types with and without *w*Mel infection, using intrathoracic injection. The orange boxes represent Wt *Ae. aegypti*, the green ones represent *w*Mel-*Ae. aegypti*, and the red boxes indicate excluded samples. * Estimated numbers)

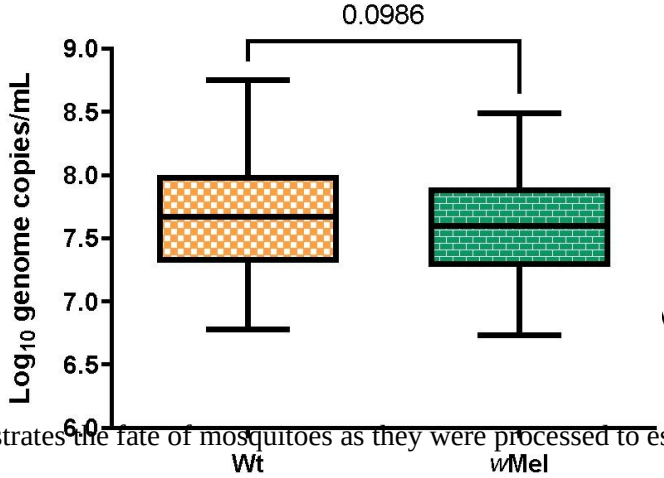


Figure 56: DENV-1 copy numbers in F0 female *Ae. aegypti* with and without *w*Mel infection

The infectivity of DENV-1 by intrathoracic injection in F0 females represents log10 viral genome copy numbers. Wt-mosquitoes shown in orange and *w*Mel-mosquitoes shown in green

In both mosquito lines high parental infection rates of DENV-1 were observed, with 89.09% (n=385, surviving mosquitoes) Wt mosquitoes and 91.84% (n=331, surviving mosquitoes) *w*Mel mosquitoes, respectively. Consistent with previous findings, intrathoracic injection resulted in a higher prevalence of DENV-1 compared to oral feeding [526,534] due to intrathoracic inoculation with a large inoculum of the virus. The DENV-1 RNA concentrations in F0 Wt and *w*Mel-infected *Ae. aegypti* whole bodies were similar and high, measuring 7.7 (95% CI = 7.63-7.72) and 7.6 (95% CI = 7.57-7.67) $\log_{10}$ copies/mL, respectively (Figure 5-6). The copy numbers detected in whole bodies of both mosquito lines showed no significant difference (Mann-Whitney test: P= 0.09) [190]. The *w*Mel density was also tested and it remained stable among generations from G59 to G61 (Kruskal-Wallis test: P=0.3), highlighting the consistent impact of *w*Mel infection within these maternal lines.

F1 mosquitoes were pooled for generating pooled samples, resulting in 785 pools for Wt and 712 pools for the *w*Mel strain. Subsequently, the mosquito pools tested for DENV-1 (Table 5-3). Nine DENV-1 positive pools were detected in the Wt group, while no positive

pools were found in the *w*Mel group. The VT rate of DENV-1 in Wt mosquitoes was calculated to be 1.49/1,000 adults (95% CI=0.73-2.74). In contrast, the VT was 0/1,000 (95% CI = 0-0.69) adults in *w*Mel mosquitoes. Based on the MLE of DENV-1 infection rate in F1 progenies (1.49 per 1,000 Wt mosquitoes), an estimated 10,328 eggs laid by infected mothers would likely result in 7.54 to 28.30 infected progenies. The most probable value is around 15.38 mosquitoes (calculated using the infection rate). Furthermore, we conducted pairwise comparisons between MLE frequency distributions for Wt *Ae. aegypti* and *w*Mel-*Ae. aegypti*. The 95% CI for the MLE frequency distribution comparison was (95% CI = 0.78-5.06). The reported CI, not containing zero (0), indicates the VT rate of DENV-1 in Wt group is significantly higher than in *w*Mel group.

Table 5-18: Summary of pooled samples and DENV testing results

In the group with a pool size of 8, nine DENV-1 positive pooled samples of the Wt mosquito line were detected.

Mosquito type	Pool size	Numbers of pooled samples	Numbers of DENV-1 infected pooled samples
Wt	1	7	0
Wt	2	9	0
Wt	3	8	0
Wt	4	10	0
Wt	5	9	0
Wt	6	8	0
Wt	7	7	0
Wt	8	727	9
*w*Mel	1	16	0
*w*Mel	4	21	0
*w*Mel	8	675	0

The frequency of mothers transmitting the virus to any of their progeny and the percentage of vertically infected progeny showed a slight increase based on the positive pooled samples containing thirteen DENV-1 infected Wt F1 individuals [526]. Our findings indicate that among 343 DENV-infected Wt *Ae. aegypti* mosquitoes that survived and reproduced within a 14-day period, 11 (3.21%) F0 mothers were capable of transmitting the virus to their offspring. However, the proportion of virus-carrying progeny inheriting the virus from infected mothers was remarkably low, with only 0.13% of the offspring (13 out of 10,038 eggs) acquiring the virus. This proportion increased to 0.21% when calculated based on 6,047 F1 adults. The higher frequency of mothers transmitting the virus to any of their progeny in Wt *Ae. aegypti* (3.21%), compared to the frequency identified in field-collected *Ae. aegypti* (2.43%) using patient-derived blood meals [526], might be attributed to the direct introduction of virus into systemic tissues via intrathoracic inoculation.

Among the nine positive pools, thirteen DENV-1 infected Wt F1 individuals were identified, of which ten were females. The mean DENV-1 RNA concentration in F0 Wt *Ae. aegypti* whole bodies was 7.7 ± 0.4 log10 copies/mL, indicating a relatively consistent viral load among the F0 mothers. Initially, we hypothesized that this consistency in viral load would extend to vertically infected F1 individuals. However, our data did not support this hypothesis. The thirteen F1 mosquitoes exhibited a range of viral loads, as indicated by DENV-1 Ct values, ranging from 17.08 to 34.97, with a mean Ct of 27.66. In contrast, the mean Ct of F0 mothers was 21.63, signifying a higher viral load in the F0 generation compared to their F1 progeny. Statistical analysis confirmed a significant difference in viral load between F0 and infected F1 mosquitoes ($P = 0.0029$) (Figure 5-7 and Table 5-4). This observation suggests that viral abundance levels in F1 progeny may be influenced by various factors, not solely dependent on the viral abundance of their mothers.

Table 5-19: Ct values of real-time RT-PCR for DENV-1 in F0 mothers and their F1 progenies

	Ct values of real-time RT-PCR for DENV-1		
F0 Mosquito code	**F0**	**F1**	**F1 gender**
Wt-35	21.29	25.45	Female
Wt-69	21.81	24.01	Female
Wt-120	21.89	34.44	Female
Wt-120	21.34	32.24	Female
Wt-134	21.78	20.19	Male
Wt-205	21.5	29.11	Female
Wt-234	21.61	29.46	Female
Wt-249	20.67	31.15	Female
Wt-278	21.19	29.24	Female
Wt-301	22.64	30.02	Female
Wt-321	20.6	17.08	Male
Wt-321	23.99	22.2	Female
Wt-332	20.95	34.97	Male

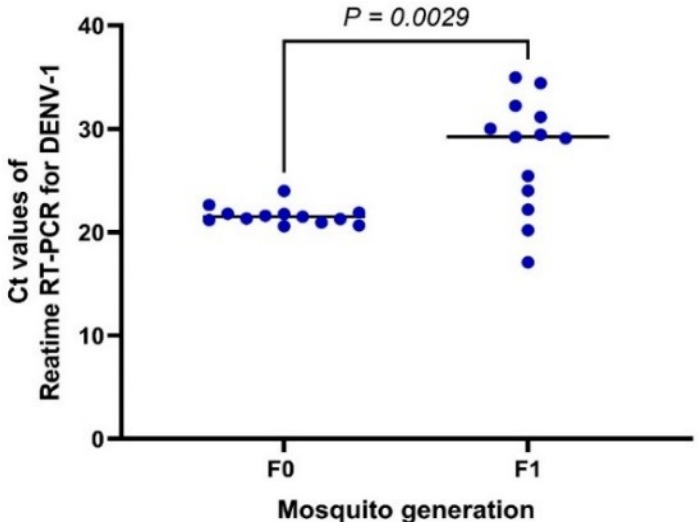


Figure 57: Cycle threshold (Ct) values of real time RT-PCR for DENV-1 in F0 wild type (Wt) and their F1 progenies

The median Ct value in F1 mosquitoes was 29.24, while in F0 mothers, it was 21.5, indicating a significant difference in viral load between F0 and F1 adults (P = 0.0029, Mann-Whitney test).

5.4 Discussion

Our study investigated the VT of DENV in *Ae. aegypti* with a Vietnamese genetic background with the two approaches: (1) mimicking, within reason, natural transmission using field-collected mosquitoes orally fed on viremic blood from dengue patients, and (2) examining the VT rate of DENV-1 in a colony of *Ae. aegypti* with and without *w*Mel infection through intrathoracic injection.

5.4.1 Vertical transmission of DENV in *Ae. aegypti* and factors influencing it

VT provides a possible mechanism supporting DENV persistence during unfavorable periods such as low mosquito density periods (e.g., dry season diapause) [275,284,535,536]. However, the findings from the first experiment did not support the role of vertical virus transmission in dengue transmission dynamics. The results from the initial experiment indicated that VT of DENV is indeed a relatively rare event, consistent with several previous studies [275,537,538]. Notably, our study diverged from previous research by replicating natural conditions using field-derived mosquitoes and introducing viremic blood from dengue

patients through oral feeding. The successful and systemic dissemination of viruses acquired via oral feeding within *Aedes sp.* mosquitoes plays a crucial role in determining the occurrence of VT to subsequent generations [521,539]. This process can span several days to weeks [540,541], indicating that the majority of VT likely transpires during later gonotrophic cycles in female mosquitoes [538,539,542].

To ensure the systemic viral dissemination and the attainment of high viral titers in our infection model, we allowed for a 14-day virus replication period before initiating a second gonotrophic cycle with a healthy blood meal [261,494,543]. The VT rate was estimated in the second gonotrophic cycle, with mosquitoes averaging 18-20 days old. Despite these measures, the results revealed that less than 3% of field-reared females successfully transmitted DENV to any of their progeny. Previous studies have suggested that the relatively small sample size of the third gonotrophic cycle may be attributed to decreased survivorship and fecundity of female mosquitoes [266]. While the gonotrophic cycle significantly influences infection rates in progeny [265].], it's important to balance its timing with mosquito longevity and fecundity. Thus, the second gonotrophic cycle is likely the appropriate time point to observe vertical transmission phenomenon.

Another concern in the VT studies is the variability in VT rates observed across different studies. A previous published study suggested that VT rates ranged from 14.8% to 23.5% in *Ae. aegypti* population collected using ovitraps [289]. The high prevalence of VT from this study suggesting a significant role for VT in maintaining DENV in nature. However, these high values were derived from calculations based on the number of positive ovitraps, rather than the number of positive individuals contained within those traps. In a study similar to ours, Sánchez-Vargas et al. [521] reported VT rates ranging from 55% to 68.6% in F1 larvae from infected mothers. Comparing VT rates between their study and ours shows a significant difference. Possible explanations for their observed high VT rate can be

attributed to three factors: (1) their initial infection of mosquitoes using a blood meal spiked with DENV-2, (2) the amplification of the virus derived from a pool of larvae by infecting C6/36 cells before conducting DENV testing, and (3) the use of larvae, an immature stage of the mosquito, which could potentially overestimate the likelihood of the adult mosquito being infected [544].

Our findings contrast with previous studies reporting higher VT rates, but we attribute this difference to several factors. Firstly, our method mimicked natural conditions more closely, with fourth-instar larvae experiencing an extended period under field conditions. Secondly, the high mortality rate observed among field-reared mosquitoes after the second blood meal, the second gonotrophic cycle, suggests potential limitations in mosquito fitness and lifespan due to suboptimal rearing conditions experienced during larval development in the field. These conditions, including nutritional stress, overcrowding, and fluctuating temperatures, which can impact mosquito fitness and lifespan [261–263], likely contribute to the rarity of VT events observed in our study. While the strict conditions imposed in the first experiment resulted in a small sample size, we remain confident that our experimental design and the resulting data accurately capture field.

In the second experiment, we addressed the limitations in our first investigation. We conducted a challenge that resulted in virtually 100% of F0 females being infected. This challenge aimed to test the hypothesis that *w*Mel would reduce the VT rate relative to the WT rate. The efforts involved increasing the sample size and conducting the test to calculate the most likely infection rate based on the test results via MLE estimate. The MLE infection rate in colonized Wt *Ae. aegypti* was 1.49 per 1,000 mosquitoes. This low VT rate persisted despite favorable conditions such as intrathoracic inoculation of the virus with a large volume and an extended extrinsic incubation period. In the second experiment, although there was an increase in the frequency of mothers transmitting the virus to their progeny in Wt *Ae. aegypti*

(3.21%) compared to field-reared *Ae. aegypti* (2.43%), the proportion of progeny inheriting the virus from infected mothers remained consistent. In field-reared *Ae. aegypti*, it was 0.11% (1/911), and in colonized Wt *Ae. aegypti*, it was 0.13% (13/10,328). This means that although more infected F0 mothers possibly transmitted the virus in the second experiment, the overall proportion of progeny inheriting the virus did not significantly change. The F1 adult mosquitoes in the second experiment were conducted during the first gonotrophic cycle. Exploring whether additional gonotrophic cycles could increase the detection of DENV transmission from infected mothers to progeny should be explored in future experiments.

5.4.2 The potential of horizontal transmission (HT) from vertically infected *Ae. aegypti*

The potential re-entry of vertically acquired viruses into the human population is an interesting question [545,546]. No studies have successfully explored the important hypothesis of whether vertically infected females can reintroduce an infection into the mosquito-human transmission cycle [275,537]. If the vertically infected females fail to attain viral loads sufficient for DENV transmission to a human host, they cannot contribute as a source for subsequent HT.

In this study, we determined the frequency at which F0 females transmitted the virus to their offspring after feeding on blood from acutely infected dengue patients. We also examined the proportion of vertically infected F1 females with infectious saliva. Our findings revealed that the vertically infected mosquito did not exhibit the presence of DENV in their saliva. Furthermore, the relatively low viral load detected in her whole body (Ct = 32) supported the hypothesis that the likelihood of reintroducing the virus into the human population depends not only on the infection status of progeny but also on viral loads sufficient for DENV transmission in saliva.

Moreover, our investigation in Wt *Ae. aegypti* unveiled an interesting phenomenon: while F0 mothers displayed comparable virus levels based on Ct values (mean Ct = 21.63, CV = 4.13%), a distinct variance in virus load was observed among F1 progeny (mean Ct = 27.66, CV = 19.85%). This observation suggest that other barriers, such as ovarioles [547], along with the mosquito's immune response [548–550], would also contribute to the successful transmission of the virus from mosquito mothers to their progeny and potentially influence the viral abundance inherited by F1 adults.

By consolidating the results of VT rates from the two experiments, especially the second one where favorable conditions were provided to observe VT, we reached a conclusive determination that VT of DENV in *Ae. aegypti* is rare. Furthermore, our findings provide no substantiation for the notion that vertically infected mosquitoes can serve as reservoirs for subsequent HT of the virus in a natural setting. Virus persistence within human populations is likely influenced more by other mechanisms, such as virus introduction from external sources or continued “asymptomatic” transmission (i.e. below the radar of surveillance systems) in the human population [551].

5.4.3 The impact of *w*Mel infection in reducing vertical transmission

In the second experiment, our goal was to enhance our ability to observe VT, particularly within *Wolbachia*-carrying mosquitoes. Despite creating favorable conditions including intrathoracic virus inoculation with large volume, a long extrinsic incubation period and large sample size (5,500 *w*Mel F1 adults), no vertically DENV-1 infected F1 adults were detected in *w*Mel-*Ae. aegypti*. This observation is consistent with previous observations in *w*Mel-infected *Ae. aegypti* populations from Brazil [534], where five DENV-positive pools were found among the Wt group out of 256 (DENV-1: 1 pool; DENV-4: 4 pools), and three dengue-positive pools were identified among the *w*Mel group out of 354 (all DENV-4). In the DENV-1 infection group, which consisted of 154 pooled samples (Wt = 68 pools, *w*Mel = 86

pools) derived from ~ 665 larvae, the *w*Mel group had zero DENV-positive pooled samples, resulting in an MLE of 0 per 1000 larvae. In contrast, the Wt group in the DENV-1 infection group had one DENV-positive pool and an MLE of 3.63 per 1000 larvae.

Our study was conducted with a large sample size, ~11,547 total F1 *Ae. aegypti* (6,047 Wt and 5,500 *w*Mel). The difference in infection rates between the Wt group and the *w*Mel group suggests the effectiveness of *w*Mel in reducing VT of DENV. However, our assessment of VT in *w*Mel-infected *Ae. aegypti* was limited to the first gonotrophic cycle. This limitation could prevent us from fully captured the complete impact of *w*Mel infection on the rate of VT of DENV. Further investigation across multiple gonotrophic cycles is needed for a comprehensive assessment. The distribution of *w*Mel in various mosquito tissues, notably abundant in ovaries [366,552], offers a potential insight into how *w*Mel blocks DENV transovarial transmission. Mejia et al. [552] described a strong positive association between *Wolbachia (w*AlB*)* densities in ovaries and eggs, suggesting a potentially significant impact on DENV transovarial transmission. These findings highlight the role of *Wolbachia*'s localization in reproductive tissues, particularly the ovaries, in preventing vertical DENV transmission. Additionally, Pacidônio et al. [534] further supported this by demonstrating lower DENV-1 viral loads in the ovaries of *w*Mel-infected mosquitoes compared to Wt mosquitoes.

In another study, researchers specifically investigated the mechanisms underlying the inhibition of viral replication in mosquito ovaries in the presence of *Wolbachia*, triple *Wolbachia* infection (*w*AlA, *w*AlB and *w*Pip), focusing on ZIKV [264]. They discovered that the *Wolbachia* enhances the expression of immune genes, including antimicrobial peptides, in mosquito ovaries. These genes are governed by key immune pathways like JAK/STAT, Toll, and IMD pathways [553–556]. This mechanistic insight sheds light on how host immune responses in *Wolbachia*-infected mosquitoes may significantly reduce VT of flaviviruses like

DENV and ZIKV by curbing virus replication and persistence in mosquito ovaries. While our study demonstrated a significant reduction in VT rates in *w*Mel-infected mosquitoes, our focus was primarily on the observed effects. Further research is warranted to elucidate the mechanisms responsible for the reduction in DENV VT rates in mosquitoes mediated by *w*Mel.

The release of *w*Mel-carrying mosquitoes and subsequent introgression into mosquito populations has proven effective in reducing the incidence of dengue in endemic regions [341,342]. This study contributes evidence supporting the capacity of *w*Mel to also diminish VT of DENV and thus play a small, but helpful role, in reducing the burden of dengue.

5.5 Conclusion

In conclusion, our study reveals the rarity of vertical transmission of DENV in *Ae. aegypti* within two complementary experimental systems. Our study provides substantial evidence for the effectiveness of *w*Mel infection in reducing the vertical transmission of DENV in *Ae. aegypti* with a Vietnamese genetic background. These findings underscore *w*Mel's role in decreasing both horizontal and vertical transmission of DENV. Implementing the *w*Mel replacement method therefore presents a comprehensive approach to mitigate dengue incidence and reduce the overall disease burden.

Chapter 6

SUMMARY AND FUTURE DIRECTIONS

The expanding geographic range of arbovirus vectors, *Aedes sp.* mosquitoes, underscores the significant and increasing threat of dengue transmission globally. As vector control remains the primary preventive strategy, strengthening methods for integrating *Wolbachia* into the wild *Ae. aegypti* population, becomes paramount for effective disease management. *Wolbachia* introgression stands out as a promising and sustainable disease control measure, with evidence this method can reduce dengue incidence in communities where *Wolbachia*-carrying mosquitoes have been released.

Reduced dengue disease burden, in addition to the health benefits to individuals and health systems, also carries a health economic benefit, with a recent study showing *Wolbachia* intervention was found to be cost-effective [557]. The *Wolbachia* introgression approach was estimated to cost $420 per DALY averted, and it also demonstrated economic benefits from a societal standpoint. This cost-benefit was contingent on a sustained 20-year effectiveness, but remained favorable even when assuming only 10 years of benefit. This underscores the importance of assessing the long-term viability and sustainability of the *Wolbachia* approach. Such an evaluation not only highlights its potential as a cost-effective strategy but also emphasizes its ability to deliver significant benefits extending beyond health gains.

The overarching aim of my PhD project was to investigate potential factors that could compromise the efficacy of the *Wolbachia* introgression approach as a public health prevention strategy for dengue. I had several objectives. Firstly, I set out to understand how

DENV adapts to the antiviral properties induced by *w*Mel in *Ae. aegypti*. Secondly, I investigated whether vertically acquired DENV could be transmitted horizontally to the human population. Lastly, I explored the potential influence of *w*Mel on the vertical transmission of DENV. Overall, this thesis has bridged some major knowledge gaps in our understanding of DENV transmission and has provided new evidence regarding the broader implications of *Wolbachia* introgression.

The DENV WGS assay developed in Chapter 3 served as the central tool for subsequent investigations in Chapter 4. Furthermore, our serotype-specific primers have demonstrated their ability to effectively capture a wide range of DENV genotypes found across the globe. The protocol will be published in an open-access format to allow its use by the broader research community. This comprehensive whole genome sequencing assay for DENV (DENV-1-4), employing an amplicon multiplex tiling PCR method enabled accurate sequencing of DENV genomes extracted from both human plasma and mosquito samples with moderate to low viral loads. Moreover, the sequencing protocol's quality ensured its applicability in investigating the intra-host diversity. It allowed us to observe DENV-1 SNVs in *w*Mel-infected *Ae. aegypti* and track the frequency of DENV-1 *w*Mel-associated variant during repeated mosquito passages and cell passages. Additionally, the protocol can be adapted to run in the Nanopore platform, making it suitable for use in remote areas.

Through repeated passage in *w*Mel-infected mosquitoes, we observed DENV-1 evolving to adapt to *w*Mel's virus interference properties, resulting in the emergence of the E203K variant, a substitution in the Envelope protein. This study is the first to demonstrate the direct impact of *w*Mel on shaping DENV evolution. Our findings have highlighted the need for further field studies to detect and manage *Wolbachia*-associated variants, especially in *w*Mel-established areas. Our research findings identified positive selective pressure on the E gene in the DENV genome driven by *w*Mel. This insight allows us to predict a higher

likelihood of mutations in the E gene in *w*Mel-established areas. Thus, focusing on screening mutations in the E gene could provide a preliminary assessment and reduce the screening costs. To investigate potential genetic changes that could lead to viral resistance, we propose sequencing circulating DENV isolates from both human and mosquito hosts over the course of a seasonal outbreak. Subsequently, these isolates will be compared with recent historical isolates, enabling us to analyze any genetic variations that may contribute to viral resistance. Field investigations can provide a real-world understanding of changes in the viral population before, during, and after the releases. If persistent local DENV transmission chains were to potentially emerge in areas where *w*Mel is established, it might necessitate considering factors such as viral resistance. Early identification and causes of underlying any breakthrough transmission events will be of paramount importance for the future success of this method.

In Yogyakarta, Indonesia, where *Wolbachia* was introduced into the *Ae. aegypti* population, a project investigated the genetics of the DENV population before releasing [558]. This study provided essential baseline information on the genetic characteristics of the DENV population before its release. Thus, we could observe and compare the DENV population genetics between the intervention and control areas before and after the release of *w*Mel-infected *Ae. aegypti*.

While in the absence of *w*Mel infection, the E203K variant lost its persistence, primarily due to fitness trade-offs and competition from wild-type variants, several important questions remain unanswered. Firstly, we have not yet assessed the resistance potential of the E203K variant against *w*Mel-mediated viral inhibition, and further studies on the vector competence for the DENV-1 E203K variant need to be conducted. If the proportion of mosquitoes carrying the E203K variant in their saliva is similar in both Wt and *w*Mel-infected mosquitoes, it would indicate that this E203K variant possesses resistance to *w*Mel. Secondly, the capacity of the E203K variant to persist in the transmission cycle between mosquito and

the human host remains unexplored. Our observations of the E203K variant were primarily conducted in repeated passages in mosquito or human cells, which do not fully model the natural mosquito-to-human transmission cycle. Although purifying selection may slow down the emergence of the E203K variant, it may not completely eliminate them. Over time, the E203K variant could accumulate and potentially dominate in transmission cycles. Thus, understanding the evolutionary processes that influence the maintenance of the E203K variant is essential for a comprehensive assessment.

Furthermore, it is important to note that several *Wolbachia* strains, including *w*Mel and *w*AlbB, provide only partial protection against DENV, creating opportunities for the virus to rapidly evolve and potentially escape blocking. Therefore, understanding the complete profiles of DENV's adaptive evolution in response to different *Wolbachia* strains becomes imperative. Such understanding will play a pivotal role in guiding the selection of *Wolbachia* strains for deployment, ensuring that strains are chosen with a clear awareness of their potential to shape the evolution of DENV.

Long-term monitoring efforts are essential for tracking the persistence and evolution of *w*Mel-associated DENV variants. Continuous surveillance is crucial for detecting changes in viral populations and assessing the effectiveness of *Wolbachia*-based interventions. Genetic characterization of DENV isolates, including whole-genome sequencing, can identify mutations associated with *w*Mel adaptation, providing insights into viral evolution.

My work has also shown the frequency of VT of DENV in both field-reared and colonized *Ae. aegypti*, with a comparison between those with and without *w*Mel infection. I have demonstrated that VT of DENV in *Ae. aegypti* mosquitoes is rare, irrespective of whether they were in natural or laboratory settings, and *w*Mel infection reduces this even further. This work suggests that vertically infected mosquitoes are unlikely to serve as a

source of subsequent horizontal transmission. By extension, in dengue-endemic regions like Vietnam, DENV transmission is therefore likely to be predominantly a result of HT, without a VT component. Dengue seasonal outbreaks are likely the results of introduction from external sources, increased mosquito activity, DENV serotype/ genotype changes, and continued transmission within the human population, which may include asymptomatic carriers. These findings underscore the importance of focusing our efforts on interrupting HT as a crucial component of effective public health strategies to reduce the prevalence of DENV infection.

In the context of *w*Mel infection, the significant further reduction in VT of DENV underscores the multifaceted influence of *w*Mel on DENV transmission, extending its efficacy beyond controlling HT. Recognizing *w*Mel's role in reducing VT adds to our comprehensive understanding of *Wolbachia*'s impact on DENV transmission. Supporting this, previous data has shown that *w*Mel infection results in an estimated 42% reduction in the prevalence of abdominal DENV infection just 14 days after oral infection [490]. Moreover, in a study involving field-collected *w*Mel-infected *Ae. aegypti*, a notably higher efficacy of *Wolbachia*-mediated DENV transmission blockage was observed when compared to their laboratory-reared counterparts [261]. The implication of these findings is that the introduction of *Wolbachia* to a mosquito population could significantly reduce the number of individual mosquitoes capable of potentially transmitting DENV to their offspring. By curbing both horizontal and vertical transmission, *Wolbachia*-carrying mosquitoes hold substantial promise as a comprehensive strategy for reducing dengue incidence and its associated burden.

While the *Wolbachia*-introgression approach has demonstrably reduced dengue transmission, its effectiveness alone is insufficient for complete elimination. A multi-pronged strategy that seamlessly integrates vaccination, anti-viral treatments, and prophylactic measures alongside *Wolbachia*-vector control is crucial to achieve this ambitious goal. Evaluating the long-term efficacy of dengue vector control measures presents many

challenges, requiring substantial resources for monitoring and assessment. While evidence exists for the persistence of *Wolbachia*-vector control after demonstrating short- to medium-term effectiveness, significant questions and challenges remain. Their long-term impact remains a crucial point of research.

It's also important to consider the ecological dynamics between *Wolbachia*-infected mosquitoes, DENV, and other pathogens in the same ecosystem. Understanding how *Wolbachia* affects the dynamics of other arboviruses and vector populations is essential for comprehensively assessing the impacts of *Wolbachia*-introgression approach. Community engagement and collaboration are critical in field monitoring efforts. Engaging local communities can enhance data collection, promote awareness of vector-borne diseases, and garner support for intervention programs. Ongoing risk assessments are necessary to evaluate potential risks associated with the *Wolbachia*-introgression approach, including the emergence of viral variants and any unintended consequences on public health and vector control efforts.

Exploring opportunities for integrating *Wolbachia*-introgression with existing vector control strategies can enhance overall effectiveness and sustainability. Furthermore, global collaboration and knowledge-sharing among researchers, public health agencies, and community stakeholders are vital for monitoring and managing *Wolbachia*-associated variants on a broader scale.

In summary, my research has contributed to our understanding of the molecular evolution of DENV within *Wolbachia*-infected *Ae. aegypti* mosquitoes and highlights the inhibitory role of *Wolbachia* on vertical DENV transmission. These findings provide valuable insights into the potential long-term advantages and sustainability of *Wolbachia* introgression

field releases. Ongoing work is essential to develop effective field monitoring strategies to ensure the continued success of this innovative dengue biocontrol initiative.

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APPENDICES

Appendix 1: The list of primers using for generating full length of DENV1-4 genomes

The primer working stock was 10µM and prepared into 2 primer pools by mixing equal volume of each 10µM primers. Primers indicated by "*" was pooled at a concentration of 20µM and primers indicated by "**" was pooled at a concentration of 40µM to help normalize sequencing coverage, CDS: coding sequences. DENV-1 genome: 10, 372bp (complete CDS: 10,179bp), DENV-2 genome: 10,433bp (complete CDS: 10,176bp), DENV-3 genome: 10,462bp (complete CDS: 10,173bp), DENV-4 genome: 10,365bp (complete CDS: 10,164bp)

1-DENV-1-Pool 1

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV1-400bp-F1	GTTGTTAGTCTAYGTGGACCG	DV1-400bp-R1	AACAAGAGTGACTTTCCTCTTTC
DV1-400bp-F3	GCGATGGAYTTGGGAGAG	DV1-400bp-R3	CTATTCCCACGCATCGCAT
DV1-400bp-F5	GCAAACTGTGCATTGAAGC	DV1-400bp-R5	CCATTGTTTGTGGACTAGCCA
DV1-400bp-F7	GTCATATGTGATGTGCACAGG	DV1-400bp-R7	TATTCCTATTTTCATGGTCCAGGA
DV1-400bp-F9	ACAAATTTCAAGCTGAYTCCCC	DV1-400bp-R9	TCCTTGATGGCAGCTGACAT
DV1-400bp-F11	CACTCTATGGAGYAATGGAGT	DV1-400bp-R11	CCATCTGGATCTCATCACYTC
DV1-400bp-F13	GCATCATGGTYGGAGCYAA	DV1-400bp-R13	CGGAAGCCATGTTGTTTTTTG
DV1-400bp-F15	TCCCAAAAAACAACRTGGCT	DV1-400bp-R15	GGTCGCTGGTATTGATAATGG
DV1-400bp-F17	TCCAAGATGATGGAACCATGA	DV1-400bp-R17	ATCACCTGCACTTCTTCTCC
DV1-400bp-F19-Ver2	CATCYGGATCTCCYATYGTGAA	DV1-400bp-R19-Ver2	AARATTGCAGCYGCYTCACCC
DV1-400bp-F21	CCTTTCCACAGAGYAAYGC	DV1-400bp-R21	TTGTTTAAAGGCTGTCCCATGTA
DV1-400bp-F23	GTGGAGCTCATGAGAAGAGG	DV1-400bp-R23	TCTGGCAGTTCTTCCATTGC
DV1-400bp-F25	GCTGGCATATGTGGTGATAGG	DV1-400bp-R25	CTTGCAGTCCAGGTCCAATTA
DV1-400bp-F27	GATGCGGACYACATGGGC	DV1-400bp-R27	TAGTATGACCAGCCACCTCT
DV1-400bp-F29	GATGGTGGARCCATGGCT	DV1-400bp-R29	CATATGATCCATGATAGGCCCA
DV1-400bp-F31	AAGTTGACACRCGCACACC	DV1-400bp-R31	TCCACTCCGCTGAGTGAATT
DV1-400bp-F33	TGGTAAGGGTRCARAGACCA	DV1-400bp-R33	TTTCCCTCCCATCCTTCATAAT
DV1-400bp-F35	GTCATATGCACAAATGTGGCA	DV1-400bp-R35	ACATCCCCACGATGGAGC

2-DENV-1-Pool 2

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV1-400bp-F2	TACCCCCAACAGCAGGART	DV1-400bp-R2	ATGTYCCATARGTCACCCA
DV1-400bp-F4	CCYTGAGACACCCAGGATT	DV1-400bp-R4	GAAGAGCCCACAGCCATT
DV1-400bp-F6	AGCTGACCGACTACGGAG	DV1-400bp-R6	ATGGTGCGTCTGTTCCTTC
DV1-400bp-F8	AGACACCGCATGGGACTT	DV1-400bp-R8	CATGATGTTCTCGAGACGAGT
DV1-400bp-F10	ACCTTCATCATYGAYGGCC	DV1-400bp-R10	ACTGTGGTACCYTCACAYA
DV1-400bp-F12	GGAGARGATGGGTGYTGGTA	DV1-400bp-R12	CAAGTCCATCCCCCAGCTC
DV1-400bp-F14	TTCCTTGGAGGAGCTGGG	DV1-400bp-R14	CGGCTGAGCTTCCAGATATAAC
DV1-400bp-F16	GCTGGAGGCATGYTRATAGC	DV1-400bp-R16	CTGGTGACRTGCCACATTGT
DV1-400bp-F18	AGRGGAGCTGTNCTYATGTA	DV1-400bp-R18	GTTTTCCCYGATCCTGGRTG
DV1-400bp-F20	TTCCGAAATGGCAGAGGC	DV1-400bp-R20	CACCCGTTTCCCATTCTTTCT
DV1-400bp-F22	AAAGATGGTCCAGAGCGYG	DV1-400bp-R22	GGTTGTTCCTTTCCCCRTC
DV1-400bp-F24	ATAGGGAAGCTTCCACAACAC	DV1-400bp-R24	CAGTCCCATCTCATTGGCTG
DV1-400bp-F26	TTGACAAAGGATGGCCAATATC	DV1-400bp-R26	TTTGCCATGGAKACYGCTAT
DV1-400bp-F28	CGCTAAACTGAGGTGGTTYGT	DV1-400bp-R28	TTTCGCACTAGCATTCCTCC
DV1-400bp-F30	CTAGATATCATTGGCCAGAGGAT	DV1-400bp-R30	GCTGAGTTCCATTGATTTTCATCA
DV1-400bp-F32	CGTGCAATATGGTACATGTGG	DV1-400bp-R32	TTAAGCCATAAGTTCCGACCTG
DV1-400bp-F34	GGGAACCTTCAAAAGGATGGAA	DV1-400bp-R34	ACCATTGGTCTTCCCTTTTYCC
DV1-400bp-F36	TACCACCTGGTCRATCCA	DV1-400bp-R36	CTCACAGGCAGCATAGCT

3-DENV-2-Pool 1

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV2-400bp-F1	GTCTACGTGGACCRACAAAG	DV2-400bp-R1	ACGATCATGTGTGGTTCTCC
DV2-400bp-F3	GGGTTCAGGAAAGAGATYGG	DV2-400bp-R3	GCCCCTTCTGATGACATCC
DV2-400bp-F5	GTATCAGGAGGRAGYTGGGT	DV2-400bp-R5	CCATGTTTTCCTGTGTCATTWCC
DV2-400bp-F7	TGGTCACTTTCAAAAATCCYCA	DV2-400bp-R7	CGAATGGAGGTTCTGCTTCTAT
DV2-400bp-F9	TCCACCAAGTYTTYGGAGC	DV2-400bp-R9	TCTGGTGTTATTTGTYTCCACAT
DV2-400bp-F11-Ver2	AAYCACACCTTTCTYATTGATGG	DV2-400bp-R11-Ver2	CCWTCGCAGARATCRAAGTCCAT
DV2-400bp-F13	AATGGTGCTGYCGATCTTG	DV2-400bp-R13	TCATCAATTCCTTGGAGGTCA
DV2-400bp-F15	GACCTCCAAGGAATTRATGATG	DV2-400bp-R15	GCCACTAATGGWCCTGTCAT
DV2-400bp-F17	GGATTGCTAGTGATCTCAGGA	DV2-400bp-R17	CCAGAGATACRGCRCCTAT
DV2-400bp-F19**	TCCAGGAGCRGGAAARAC	DV2-400bp-R19**	ACGAACGCTCAGGGATTTC
DV2-400bp-F21	AGACGCTGCATGAAACCAG	DV2-400bp-R21	ATCAAAACACCACYTTCTGTCT
DV2-400bp-F23	GGTAGACTTCCAACWTTCATGAC	DV2-400bp-R23	TCTCGTTTGCCATGGTTGC
DV2-400bp-F25	TGTCAAAAATGGAYATYGGAG	DV2-400bp-R25	AATGTTAGCCATTGACACYGC
DV2-400bp-F27-Ver2	GGAGAAACGGACCAYCAYGC	DV2-400bp-R27-Ver2	GATTCCTCACCAARGCTCCTCC
DV2-400bp-F29	TGGCACTATGAYCAAGAYCA	DV2-400bp-R29	CACATGTTTCACACTTYCCTTC
DV2-400bp-F31	AAGGAGGAGCMATGTAYGC	DV2-400bp-R31	TCCACTGATGGCCATTCTTG
DV2-400bp-F33	TYCCATGCAGAAAYCAAGATGA	DV2-400bp-R33	GGATGTTCTTTGCCCAKGT
DV2-400bp-F35	AGAAAACCCATGGATGGAAGA	DV2-400bp-R35	CCTCCTCAGGTGGAGCTAC

4-DENV-2-Pool 2

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV2-400bp-F2	GCTAAGCTTAACGTAGTTCTAACAG	DV2-400bp-R2	ACGATCATGTGTGGTTCTCC
DV2-400bp-F4	AGAAGAGAAAAAAGATCAGTGGC	DV2-400bp-R4	TTGGCTTCCGTTTTTRTCAGTTC
DV2-400bp-F6	GCATTGTGACCTGTGCTATGT	DV2-400bp-R6	TCTGTGGCTCCTGTGAGTG
DV2-400bp-F8-Ver2	AAATAGCAGAAACACAACATGGAAC	DV2-400bp-R8-Ver2	GCTRCGTGARTTCATTCCTATCC
DV2-400bp-F10	GCAYACGTGGACAGARCA	DV2-400bp-R10	GYGAGTTCCAAGCTCTRTT
DV2-400bp-F12	CACCCTCTGGAGYAATGRAGT	DV2-400bp-R12	TCGTGTCCTGAGCATTTCYTC
DV2-400bp-F14-Ver2	TGCTYAGGACCCGARTRGGAA	DV2-400bp-R14-Ver2	GTGCAGCTCACYTTCCATGC
DV2-400bp-F16	CATGGCAGTYGGGATGGT	DV2-400bp-R16	GTGAAGGGACRTCCCACA
DV2-400bp-F18	CTCGAAGGAGAATGGAAGGA	DV2-400bp-R18	CTCTGAGAGCTTCYTCCATTTC
DV2-400bp-F20	TTTCAACTCGAGTRGAGATGGG	DV2-400bp-R20	CCTCTTCTTTGYGCTGCRCT
DV2-400bp-F22-Ver2	TAYCGCTTGAGAGGRGARGC	DV2-400bp-R22-Ver2	CATYCCCAGGGTCATYTTYCC
DV2-400bp-F24	TGCTCATTCCAGARCCAGAAAA	DV2-400bp-R24	TCCTGGCCCTATRATRGCRTA
DV2-400bp-F26	ATGATGAGGACYACATGGGC	DV2-400bp-R26	CAATAGTATGACCAGCCYCC
DV2-400bp-F28	ACACCCAATTTTGCRTAAARGT	DV2-400bp-R28	ACCATGGATGATGCTGATCC
DV2-400bp-F30-Ver2	AYGAGAACAAGTGGAARTCRGC	DV2-400bp-R30-Ver2	TRTCCATTACTGTKCCTCTTGG
DV2-400bp-F32	GACAGATGGAGGGAGAAGGA	DV2-400bp-R32	CCACATTTGGGCGTAAGACTT
DV2-400bp-F34	TCCCATCACAYTGGGTTCC	DV2-400bp-R34	ATGGCTTAATCCGACCTGAC

5-DENV-3-Pool 1

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV3-400bp-F1	ACTCGGAAGCTTGCTTAACG	DV3-400bp-R1	TGARGTCARGTGGAAAGCAAGTG
DV3-400bp-F3	ACYTAYGGAACGTGCAAYCAAGC	DV3-400bp-R3	TRCATARYTTCCTTAGGGTYG
DV3-400bp-F5**	ATCATYACAGTGCACACAGG	DV3-400bp-R5**	TCCCCTTGAGTTCYARTTTGTCC
DV3-400bp-F7	TTGGAATTGGAGACAAMGCCTTG	DV3-400bp-R7	ATTCCRCTTCCACATTTGAGTTC
DV3-400bp-F9	AACACCACARCCCATGGAGC	DV3-400bp-R9	TAYCCRGGYCTGTRGTTGTG
DV3-400bp-F11	ACATGGTAAAGTCTTTAGTCTCAGC	DV3-400bp-R11	TTTWAGAGYCATGAGCCCCARRG
DV3-400bp-F13**	ACYHAAAAGGARRAGCTGGCC	DV3-400bp-R13**	TTTCTGTGTYTCTGGRGGGC
DV3-400bp-F15*	ARGAGGTGCAGGTYATYGC	DV3-400bp-R15*	TCTTCCATYTCAGCTGCAAC
DV3-400bp-F17	TGGGAGARGCAGCYGCAATYTTC	DV3-400bp-R17	TTCCTGCCRACTCTCCCTCTCC
DV3-400bp-F19	AGCCCAYAAAGTAGCMTCAGAAG	DV3-400bp-R19	AGTCTTTCCAAYCCCTTTRCCTG
DV3-400bp-F21 ver2	ACCAGCTATTTRGATGTRGAYTTGC	DV3-400bp-R21 ver 2	TARAGCTTCACAMARKGCCCATG
DV3-400bp-F23	TCACARGGYGAAACYTTRGGAG	DV3-400bp-R23	TTCCACYGTTGGRCTTGGTG
DV3-400bp-F25	TYACGATGACACACAGRAGACC	DV3-400bp-R25	TTCCCTTGTGCAYAAYCTRGG
DV3-400bp-F27*	ATATGGTACATGTGGTTGGGAG	DV3-400bp-R27*	ACRCCTTCTCCYTCCATYTG
DV3-400bp-F29	TTTCTGCTCCCACCACTTYCATG	DV3-400bp-R29	ARGTTGCTCTGGARGTRAGAC

6-DENV-3-Pool 2

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV3-400bp-F2	TCAAGAAGTCRGGGGCYATYAAG	DV3-400bp-R2	TTTGGGYRCGTGTGTCYAGTC
DV3-400bp-F4	TGYGTGACYACYATGGCTAAG	DV3-400bp-R4	TTCRRYGGTTGATGCCTGRGG
DV3-400bp-F6	AYACMGCRYTGACAGGRGC	DV3-400bp-R6	AAAGTCCCARGCTGTGTCTC
DV3-400bp-F8	ATYATYACACTCTATCTGGGAGC	DV3-400bp-R8	TTGGACACTCYGGTGTGYTTG
DV3-400bp-F10	ACACACYCTYTGGAGYAATGGTG	DV3-400bp-R10	TCCCAAATTTTCCTCTCATYACCTC
DV3-400bp-F12	AAATTTRYTRTTRGGAGTTGGG	DV3-400bp-R12	AGCCATGGGYACRTCATTYC
DV3-400bp-F14	AGGMATYTTTCCATACTCCATACC	DV3-400bp-R14	TYGCTCCTATYTCCCCYGTWG
DV3-400bp-F16	AGACRCGGAAATAYCTTCCAGC	DV3-400bp-R16	TGARTTCCATGAGCGTTCYGG
DV3-400bp-F18	AGTGATYGACCCAAGAAGATGTC	DV3-400bp-R18	TCCACRTCCATRTTYTCCTC
DV3-400bp-F20*	TRGAGGAAYTACCAGARACAATG	DV3-400bp-R20*	TGGTGTTATTACTGTWGTGGCC
DV3-400bp-F22*	TAATGAAGAATCCAACRGTGG	DV3-400bp-R22*	TCTTTTYARCCCTTCYTTGGC
DV3-400bp-F24 ver2	TGGAAAGGAYGTGTTYTATCTYCCAC	DV3-400bp-R24 ver 2	AGTGCCATGTTGARYTRTGCTCCTC
DV3-400bp-F26	AAGAGAAAGTGGACACCAGGAC	DV3-400bp-R26	TCCTTCYCCTTCYACTCCACTG
DV3-400bp-F28	AAGACCARAGRGGCAGYGGAC	DV3-400bp-R28	AGGCTCCATCCYGCTCCYTG
DV3-400bp-F30	MGAGGAMAATCCATGGATGGAAG	DV3-400bp-R30	TCTCCTCTAACCRCTAGTCTGC

1-DENV-4-Pool 1

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV4-400bp-F1**	TCGGAAGCTTGCTTAACACAG	DV4-400bp-R1**	AYGCCATTRYGGTGGGAATC
DV4-400bp-F3	TTTCCATCCCRCCAACAGCAG	DV4-400bp-R3	AATCCCATTCCTGAATGTGGTG
DV4-400bp-F5	TGGATACTCAGAAACCCAGG	DV4-400bp-R5	TCYCTCCGGCARATGTACTG
DV4-400bp-F7	TTGGRAAAGGAGGAGTTGTGAC	DV4-400bp-R7	TCCYAGCACTGTCACATCCTGTC
DV4-400bp-F9	ATGGAAAYCAYATGTTTGCAGG	DV4-400bp-R9	ATGGCCATKCGYTTTGCRCC
DV4-400bp-F11	TGAAACAGCYTGGGATTTTGG	DV4-400bp-R11	ACATRACATTTTCCAGCCTCG
DV4-400bp-F13	TGGGAAGGAGGACAYGAYCTCAC	DV4-400bp-R13	TTGGGCCACAGRCAWGTTTTYAC
DV4-400bp-F15	ATTGTGAYCAYAGAGGCCCATC	DV4-400bp-R15	TAYCCTGGTGACATCTTRAACAC
DV4-400bp-F17	AAGAGAGACAGCGCTAATGG	DV4-400bp-R17	ACCATTGGRCCAGCYAAAG
DV4-400bp-F19	AAYGTGCARTGGGATGARATGG	DV4-400bp-R19	ATGTCRTTCCTGACGTCRGC
DV4-400bp-F21	TTCAAACCCGGAACRTCYGG	DV4-400bp-R21	AAGTCTYGTTGTRAAGGTTGC
DV4-400bp-F23	ACCAGGGTRGAAATGGGAG	DV4-400bp-R23	ACTCTCTCTGGCCCATCWG
DV4-400bp-F25	AYTGTTTGGYCCRGAAAGGG	DV4-400bp-R25	TGTGGAGCATRACTATGTTRTC
DV4-400bp-F27	ACTRGAAACACTCATGCTTGTAG	DV4-400bp-R27	TATGGTGTGYCTCAGCATGGGAG
DV4-400bp-F29	ARGCAGCCGTCYTAATGG	DV4-400bp-R29	TGTTCCAAAACCTTCCCGG
DV4-400bp-F31	AGAAGTGGAYAGGACYGAAGCC	DV4-400bp-R31	TYGGCATGTAGGGRTTRAGGAC
DV4-400bp-F33	ATGAGATGTATTGGGTGTCAG	DV4-400bp-R33	TCCACCTTCTCYTTGAAYAC
DV4-400bp-F35	TGCACAAGGGAAGAGTTCATCTC	DV4-400bp-R35	TCCCAGCCTGCTGTGTCRTCAG
DV4-400bp-F37	AACTCATCCGCCAAATGGAAG	DV4-400bp-R37	ATCTGGGCRTAAGCTTTGCC
DV4-400bp-F39	AAAGCTTAYGCCCAGATGTGGTC	DV4-400bp-R39	ACAGAACTCCTTCACTCTCKRAAGG

8--**DENV-4-Pool 2**

Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence
DV4-400bp-F2	AGCAGATCTCTGGAAAAATGAACC	DV4-400bp-R2	ACAAGAGAGGTCTCCCYCTTTC
DV4-400bp-F4	ATTGCCATGGACYTRGGTGARATG	DV4-400bp-R4	AYGCAYCGCATTCCGTARGATGG
DV4-400bp-F6**	TGGCCCAGGGAAARCCAAC	DV4-400bp-R6**	AGTCCGGCARTTTRACTTCWAC
DV4-400bp-F08 ver4	AAGTCAAATTGCCRGAYTATGG	DV4-400bp-R08 ver4	GRACTTTACACGGAGCYCCAG
DV4-400bp-F10	TYTCAKCYACCCCTTTKGCTG	DV4-400bp-R10	TCCATGACACCRCACAMCCC
DV4-400bp-F12	ACAACGTGCACACTTGGACAG	DV4-400bp-R12	TCTGARCTTCCTTCTCGRAATTTC
DV4-400bp-F14 ver2	AGCTCAAAAAACCAGACCTGGC	DV4-400bp-R14 ver2	ATCCCRTACCARCAYCCATCYTC
DV4-400bp-F16	AGAACATGGTYAAATCACARG	DV4-400bp-R16	TAARGTTCCCACTTGRGTG
DV4-400bp-F18	ATGAAAGGAGCCTCAARAAGATC	DV4-400bp-R18	ACGTCCCACAGRGCTCCTG
DV4-400bp-F20	TYGGGAAAACYCAGGTTGGAG	DV4-400bp-R20	TCCTCATCCACYTCATAATCTGGC
DV4-400bp-F22	TGGCRGCCGARATGGAAG	DV4-400bp-R22	TGCTGGGAACAAACCACACAG
DV4-400bp-F24	AGYAGRAAAACCTTTGAYACRG	DV4-400bp-R24	TCTTCCTTTGTTCCCCTCTGAG
DV4-400bp-F26*	AYCGRGAATGGTGCTTYACWGG	DV4-400bp-R26*	TGACARTTTCCCTATYCCTTTYCC
DV4-400bp-F28	TAGCAGCCAAYGAGATGGGG	DV4-400bp-R28	TTCATGATYCCAGCAGCTGTC
DV4-400bp-F30	ACTYTTRATGAGAACAACATGGGC	DV4-400bp-R30	ARCCAAGATCYACRACYTTYCC
DV4-400bp-F32*	AGGATGGTCYTATTACATGGC	DV4-400bp-R32*	TGGGTTTCCTATGCCTTGTTG
DV4-400bp-F34	AAACTGCTRACAAAACCYTGG	DV4-400bp-R34	ARCCACATRTACCAGATTGCTC
DV4-400bp-F36	AGAGAAAAYTCATGGAGTGGAG	DV4-400bp-R36	ACACTCTYTCAGCCATTTCTC
DV4-400bp-F38	TTGAAYGACATGGGAAAGGTGAG	DV4-400bp-R38	ATRTCTTCCCACGAATGRACTG
DV4-400bp-F40	TCACCARTGGATGACCACTGAAG	DV4-400bp-R40	TACAGCTTCCGTGGCGCATGG

Appendix 2: Characteristics and detailed statistics of read mapping for 10 serum samples from virologically-confirmed dengue cases in the phase III trials of the tetravalent dengue vaccine, CYD-TDV

Sample Name	Serotype	Genotype	Mapping rate	Minimum coverage	Maximum coverage	Average coverage	%Q30	Genome coverage (%)	CDS coverage (%)	GenBank. Accession No
DV1/INDONESIA/364/2013	DENV-1	Genotype I	98.82	3,013	13,023	7,032	99.3	99.7	100	KY818145
DV1/PHILIPPINES/ 003/2011	DENV-1	Genotype IV	97.43	4,012	16,324	6,032	98.4	99.7	100	KY818102
DV1/MEXICO/357/2013	DENV-1	Genotype IV	97.32	2,940	13,943	4,920	97.42	99.0	100	KY818277
DV1/COLOMBIA/134/2013	DENV-1	Genotype V	98.12	2,395	12,094	5,902	99.1	99.3	100	KY818060
DV2/BRAZIL/229/2013	DENV-2	Asian/American	95.34	3,021	14,923	6,032	98.53	98.9	100	KY851515
DV2/PHILIPPINES/ 051/2013	DENV-2	Cosmopolitan	98.39	3,920	17,932	5,902	99.18	98.6	100	KY851505
DV3/PHILIPPINES/ 638/2011	DENV-3	Genotype I	97.45	2,932	13,035	5,023	98.04	98.6	100	KY851591
DV3/COLOMBIA/613/2013	DENV-3	Genotype III	96.94	3,234	16,932	6,224	99.03	98.4	100	KY851557
DV4/PHILIPPINES/ 109/2013	DENV-4	Genotype II	95.9	2,884	13,902	5,299	97.32	98.4	100	KY851666
DV4/BRAZIL/576/2013	DENV-4	Genotype II	94.24	3,001	12,894	4,114	98.91	98.6	100	KY851665

Appendix 3: Characteristics and detailed statistics of read mapping from DENV 1-4 sequencing for the 78 samples used in this study

Sample Number	Specimen type	Serotype	Genotype	Ct value	Log 10 Copies/ reaction	Mapping rate	Minimum coverage	Maximum coverage	Average coverage	%Q30	Genome coverage (%)	Reference for mapping	Result
3	Plasma	DENV-1	Genotype I	23.7	4.85	98.22%	1,208	13,518	4,198	98.0	98.4	FJ432734	Complete genome
4	Plasma	DENV-1	Genotype I	23.8	4.71	98.42%	2,449	17,890	4,384	99.0	98.8	FJ432734	Complete genome
19	Mosquito	DENV-1	Genotype I	23.7	4.83	98.05%	2,014	13,110	4,082	95.0	98.1	FJ432734	Complete genome
20	Mosquito	DENV-1	Genotype I	24.9	3.80	98.12%	2,020	15,269	4,774	95.0	98.6	FJ432734	Complete genome
21	Mosquito	DENV-1	Genotype I	23.5	4.97	98.04%	3,017	17,993	5,270	93.0	98.8	FJ432734	Complete genome
22	Mosquito	DENV-1	Genotype I	24.7	3.85	97.97%	1,109	14,009	4,220	94.0	98.5	FJ432734	Complete genome
28	Plasma	DENV-2	Asian I	20.7	5.79	93.19%	2,191	18,739	5,159	97.0	98.7	EU482447	Complete genome
29	Plasma	DENV-2	Asian I	24.5	4.10	90.01%	3,218	19,947	3,896	91.0	98.4	EU482447	Complete genome
40	Mosquito	DENV-2	Asian I	23.6	4.88	92.39%	1,475	14,363	5,467	95.0	98.3	EU482447	Complete genome
41	Mosquito	DENV-2	Asian I	25.2	3.73	91.47%	2,827	7,694	3,203	92.0	98.6	EU482447	Complete genome
42	Mosquito	DENV-2	Asian I	24.6	4.08	93.19%	1,042	7,225	3,508	95.0	98.7	EU482447	Complete

													genome
50	Mosquito	DENV-3	Genotype II	23.7	4.82	97.68%	1,742	18,049	7,678	93.0	98.4	EU660409	Complete genome
51	Mosquito	DENV-3	Genotype II	24.3	4.26	97.15%	1,243	19,757	7,692	90.0	98.5	EU660409	Complete genome
52	Mosquito	DENV-3	Genotype II	26.4	3.26	97.63%	2,074	16,731	4,916	92.0	98.1	EU660409	Complete genome
53	Plasma	DENV-3	Genotype II	29.6	1.86	93.57%	2,082	7,510	5,791	91.0	66.7	EU660409	Partial Consensus
56	Plasma	DENV-4	Genotype I	24.0	4.62	95.03%	2,097	15,093	7,811	92.0	99.5	JX024758	Complete genome
57	Plasma	DENV-4	Genotype I	24.9	3.75	94.85%	3,235	18,346	10,051	92.0	98.4	JX024758	Complete genome
65	Mosquito	DENV-4	Genotype I	23.6	4.92	94.38%	2,045	8,627	3,835	98.0	98.4	JX024758	Complete genome
66	Plasma	DENV-1	Genotype I	21.4	5.63	97.46%	1,181	4,693	2,675	99.0	99.1	FJ432734	Complete genome
69	Plasma	DENV-1	Genotype I	20.5	5.85	97.94%	1,207	3,906	1,396	99.0	98.4	FJ432734	Complete genome
73	Plasma	DENV-1	Genotype I	18.4	7.56	98.58%	1,138	6,193	1,501	98.0	99.3	FJ432734	Complete genome
77	Plasma	DENV-4	Genotype I	24.0	4.62	90.03%	2,943	10,204	5,034	90.0	99.8	JX024758	Complete genome
78	Plasma	DENV-4	Genotype I	24.0	4.62	94.38%	3,592	18,034	8,042	91.0	98.4	JX024758	Complete genome
79	Plasma	DENV-4	Genotype I	28.8	2.80	91.33%	2,001	12,023	7,320	90.0	77.2	JX024758	Partial Consensus
82	Plasma	DENV-3	Genotype II	21.9	5.51	90.49%	2,018	8,302	3,220	98.0	98.9	EU660409	Complete genome
83	Plasma	DENV-3	Genotype II	22.6	5.26	95.00%	1,738	8,021	2,993	96.0	98.9	EU660409	Complete

													genome
84	Mosquito	DENV-4	Genotype I	23.9	4.69	97.00%	1,003	5,639	2,012	94.0	98.8	JX024758	Complete genome
85	Mosquito	DENV-4	Genotype I	23.9	4.68	99.32%	3,502	9,012	4,025	92.0	98.9	JX024758	Complete genome
86	Plasma	DENV-1	Genotype I	31.5	1.13	90.78%	1,349	9,102	5,820	99.2	55.7	FJ432734	Partial Consensus
87	Plasma	DENV-1	Genotype I	32.5	0.20	89.74%	2,048	12,945	3,011	97.4	48.0	FJ432734	Partial Consensus
88	Plasma	DENV-4	Genotype I	20.3	5.85	99.12%	2,850	10,384	7,024	99.1	99.1	JX024758	Complete genome
89	Plasma	DENV-4	Genotype I	25.7	3.60	95.13%	1,482	9,021	3,001	97.2	98.3	JX024758	Complete genome
90	Plasma	DENV-4	Genotype I	28.7	2.82	95.12%	2,003	11,234	6,402	96.8	79.8	JX024758	Partial Consensus
91	Plasma	DENV-4	Genotype I	27.7	3.06	96.56%	1,484	9,302	3,005	98.2	99.4	JX024758	Complete genome
92	Plasma	DENV-3	Genotype II	28.3	2.90	97.76%	1,404	12,522	4,021	97.2	98.3	EU660409	Complete genome
93	Plasma	DENV-3	Genotype II	24.7	3.86	95.23%	1,304	11,044	4,343	98.1	98.0	EU660409	Complete genome
94	Plasma	DENV-3	Genotype II	30.0	1.73	97.56%	1,402	14,820	4,562	99.1	98.7	EU660409	Complete genome
95	Plasma	DENV-3	Genotype II	31.2	1.73	90.54%	1,863	14,923	5,002	97.6	38.3	EU660409	Partial Consensus
96	Plasma	DENV-2	Asian I	18.8	7.50	99.56%	2,044	5,811	4,230	99.2	99.5	EU482447	Complete genome
97	Plasma	DENV-2	Asian I	19.5	6.81	98.47%	1,445	9,103	4,285	97.3	99.2	EU482447	Complete genome
98	Plasma	DENV-2	Asian I	31.7	1.02	89.13%	2,421	8,391	3,967	96.3	57.2	EU482447	Partial

													Consensus
99	Plasma	DENV-2	Asian I	30.5	1.82	94.12%	2,001	9,204	6,924	98.2	56.3	EU482447	Partial Consensus
100	Plasma	DENV-2	Asian I	18.6	7.53	99.45%	1,740	7,391	2,137	98.3	99.2	EU482447	Complete genome
101	Plasma	DENV-2	Asian I	17.2	6.33	99.43%	1,012	8,920	8,376	97.4	98.7	EU482447	Complete genome
102	Plasma	DENV-1	Genotype I	27.3	3.17	97.23%	1,094	10,483	5,501	99.1	98.8	FJ432734	Complete genome
103	Plasma	DENV-1	Genotype I	26.2	3.54	94.13%	1,094	8,742	2,862	98.5	98.8	FJ432734	Complete genome
104	Plasma	DENV-1	Genotype I	24.6	3.89	93.55%	1,990	13,005	3,054	99.2	99.1	FJ432734	Complete genome
105	Plasma	DENV-1	Genotype I	24.6	4.07	98.45%	1,404	19,201	4,967	98.4	98.4	FJ432734	Complete genome
106	Plasma	DENV-1	Genotype I	24.4	4.22	96.21%	1,302	12,242	5,307	93.4	98.8	FJ432734	Complete genome
107	Plasma	DENV-1	Genotype I	23.4	4.98	97.40%	1,031	5,922	3,354	96.4	99.1	FJ432734	Complete genome
108	Plasma	DENV-1	Genotype I	19.0	7.03	98.29%	1,032	9,201	3,903	98.4	98.2	FJ432734	Complete genome
109	Plasma	DENV-4	Genotype I	31.3	1.22	99.23%	802	7,470	4,096	96.5	47.0	JX024758	Partial Consensus
110	Plasma	DENV-4	Genotype I	30.8	1.26	98.46%	930	5,759	5,459	97.0	60.3	JX024758	Partial Consensus
111	Plasma	DENV-1	Genotype I	30.1	1.70	98.23%	903	3,950	1,932	98.3	58.7	FJ432734	Partial Consensus
112	Plasma	DENV-2	Asian I	18.3	6.85	97.43%	3,043	8,024	7,237	95.9	98.3	EU482447	Complete genome
113	Plasma	DENV-2	Asian I	17.1	7.97	98.12%	3,405	9,023	3,861	96.1	98.5	EU482447	Complete

													genome
114	Plasma	DENV-2	Asian I	17.8	7.12	95.92%	2,045	6,023	3,269	98.0	98.9	EU482447	Complete genome
115	Plasma	DENV-2	Asian I	16.8	8.16	96.78%	4,021	5,028	2,235	98.3	99.1	EU482447	Complete genome
116	Plasma	DENV-3	Genotype II	17.0	8.06	97.23%	3,042	9,303	5,024	96.3	99.2	EU660409	Complete genome
117	Plasma	DENV-3	Genotype II	19.3	5.85	96.19%	4,013	10,034	6,604	97.1	98.8	EU660409	Complete genome
118	Plasma	DENV-2	Asian I	28.4	2.74	91.23%	894	7,294	3,595	95.6	73.7	EU482447	Partial Consensus
119	Plasma	DENV-2	Asian I	23.9	3.81	99.59%	1,044	6,393	4,024	97.2	99.2	EU482447	Complete genome
120	Plasma	DENV-2	Asian I	29.3	2.68	99.34%	746	5,924	3,035	98.1	98.2	EU482447	Complete genome
121	Plasma	DENV-2	Asian I	27.1	3.13	98.73%	645	7,723	1,349	99.5	98.2	EU482447	Complete genome
122	Plasma	DENV-2	Cosmopolitan	26.0	3.19	96.48%	1,424	5,483	5,035	95.5	99.4	EU482447	Complete genome
123	Plasma	DENV-2	Cosmopolitan	23.1	3.92	97.35%	1,495	8,906	4,070	93.1	98.2	EU482447	Complete genome
124	Plasma	DENV-2	Cosmopolitan	24.3	3.70	95.32%	968	10,455	3,060	95.7	99.6	EU482447	Complete genome
125	Plasma	DENV-2	Cosmopolitan	25.1	3.59	99.53%	2,096	8,924	5,039	96.7	98.5	EU482447	Complete genome
126	Plasma	DENV-2	Cosmopolitan	25.2	3.54	96.43%	1,096	10,481	4,068	95.8	99.4	EU482447	Complete genome
127	Plasma	DENV-2	Cosmopolitan	25.5	4.08	98.32%	895	9,035	2,991	98.4	99.6	EU482447	Complete genome
128	Plasma	DENV-2	Cosmopolita	25.9	3.22	97.56%	1,009	8,035	4,066	98.5	98.5	EU482447	Complete

			n										genome
129	Plasma	DENV-2	Cosmopolitan	26.2	3.17	98.78%	941	10,495	5,068	96.6	98.6	EU482447	Complete genome
130	Plasma	DENV-2	Cosmopolitan	26.5	3.05	94.64%	2,089	12,046	6,036	96.8	98.3	EU482447	Complete genome
131	Plasma	DENV-2	Cosmopolitan	26.9	2.91	95.64%	1,304	9,406	3,031	97.4	99.5	EU482447	Complete genome
132	Plasma	DENV-2	Asian I	30.0	2.90	95.45%	2,068	12,055	4,047	94.7	98.0	EU482447	Complete genome
133	Plasma	DENV-2	Asian I	27.7	3.06	96.45%	984	8,904	4,559	95.4	99.5	EU482447	Complete genome
134	Plasma	DENV-2	Asian I	27.8	2.99	97.86%	1,049	9,331	2,451	95.9	98.2	EU482447	Complete genome
135	Plasma	DENV-2	Asian I	27.8	2.92	98.87%	949	7,841	3,003	96.5	98.5	EU482447	Complete genome

Appendix 4: Consensus length of 66 samples at different read depths (100x, 400x, 1000x)

Sample Number	Serotype	Ct value	Log 10 Copies/ reaction	Depth (x)	Consensus length (bp)	Genome coverage (%)	CDS coverage (%)	Reference for mapping
3	DENV-1	23.66	4.85	100	10194	98.3	100.0	FJ432734
3	DENV-1	23.66	4.85	400	10150	97.9	99.7	FJ432734
3	DENV-1	23.66	4.85	1000	9463	91.2	93.0	FJ432734
4	DENV-1	23.84	4.71	100	10212	98.5	100.0	FJ432734
4	DENV-1	23.84	4.71	400	10168	98.0	99.9	FJ432734
4	DENV-1	23.84	4.71	1000	9443	91.0	92.8	FJ432734
19	DENV-1	23.73	4.83	100	10123	97.6	99.4	FJ432734
19	DENV-1	23.73	4.83	400	10157	97.9	99.8	FJ432734
19	DENV-1	23.73	4.83	1000	9444	91.1	92.8	FJ432734
20	DENV-1	24.89	3.80	100	10193	98.3	100.0	FJ432734
20	DENV-1	24.89	3.80	400	10134	97.7	99.6	FJ432734
20	DENV-1	24.89	3.80	1000	9091	87.6	89.3	FJ432734
21	DENV-1	23.47	4.97	100	10213	98.5	100.0	FJ432734
21	DENV-1	23.47	4.97	400	10174	98.1	100.0	FJ432734
21	DENV-1	23.47	4.97	1000	9643	93.0	94.7	FJ432734
22	DENV-1	24.71	3.85	100	10184	98.2	100.0	FJ432734
22	DENV-1	24.71	3.85	400	10153	97.9	99.7	FJ432734
22	DENV-1	24.71	3.85	1000	9172	88.4	90.1	FJ432734
28	DENV-2	20.74	5.79	100	10213	97.9	100.0	EU482447

28	DENV-2	20.74	5.79	400	10178	97.6	100.0	EU482447
28	DENV-2	20.74	5.79	1000	9685	92.8	95.2	EU482447
29	DENV-2	24.46	4.10	100	10214	97.9	100.0	EU482447
29	DENV-2	24.46	4.10	400	10159	97.4	99.8	EU482447
29	DENV-2	24.46	4.10	1000	9181	88.0	90.2	EU482447
40	DENV-2	23.63	4.88	100	10221	98.0	100.0	EU482447
40	DENV-2	23.63	4.88	400	10159	97.4	99.8	EU482447
40	DENV-2	23.63	4.88	1000	9543	91.5	93.8	EU482447
41	DENV-2	25.18	3.73	100	10239	98.1	100.0	EU482447
41	DENV-2	25.18	3.73	400	10179	97.6	100.0	EU482447
41	DENV-2	25.18	3.73	1000	9244	88.6	90.8	EU482447
42	DENV-2	24.56	4.08	100	10232	98.1	100.0	EU482447
42	DENV-2	24.56	4.08	400	10152	97.3	99.8	EU482447
42	DENV-2	24.56	4.08	1000	9204	88.2	90.4	EU482447
50	DENV-3	23.74	4.82	100	10231	97.8	100.0	EU660409
50	DENV-3	23.74	4.82	400	10178	97.3	100.0	EU660409
50	DENV-3	23.74	4.82	1000	9411	90.0	92.5	EU660409
51	DENV-3	24.31	4.26	100	10241	97.9	100.0	EU660409
51	DENV-3	24.31	4.26	400	10187	97.4	100.0	EU660409
51	DENV-3	24.31	4.26	1000	9201	87.9	90.4	EU660409
52	DENV-3	26.42	3.26	100	10215	97.6	100.0	EU660409
52	DENV-3	26.42	3.26	400	10169	97.2	100.0	EU660409
52	DENV-3	26.42	3.26	1000	8669	82.9	85.2	EU660409
56	DENV-4	24.04	4.62	100	10234	98.7	100.0	JX024758
56	DENV-4	24.04	4.62	400	10225	98.6	100.0	JX024758
56	DENV-4	24.04	4.62	1000	9277	89.5	91.3	JX024758
57	DENV-4	24.91	3.75	100	10200	98.4	100.0	JX024758

57	DENV-4	24.91	3.75	400	10148	97.9	99.8	JX024758
57	DENV-4	24.91	3.75	1000	9214	88.9	90.7	JX024758
65	DENV-4	23.58	4.92	100	10215	98.6	100.0	JX024758
65	DENV-4	23.58	4.92	400	10164	98.1	100.0	JX024758
65	DENV-4	23.58	4.92	1000	9672	93.3	95.2	JX024758
66	DENV-1	21.36	5.63	100	10222	98.6	100.0	FJ432734
66	DENV-1	21.36	5.63	400	10188	98.2	100.0	FJ432734
66	DENV-1	21.36	5.63	1000	9652	93.1	94.8	FJ432734
69	DENV-1	20.54	5.85	100	10200	98.3	100.0	FJ432734
69	DENV-1	20.54	5.85	400	10164	98.0	99.9	FJ432734
69	DENV-1	20.54	5.85	1000	9643	93.0	94.7	FJ432734
73	DENV-1	18.38	7.56	100	10204	98.4	100.0	FJ432734
73	DENV-1	18.38	7.56	400	10169	98.0	99.9	FJ432734
73	DENV-1	18.38	7.56	1000	10009	96.5	98.3	FJ432734
77	DENV-4	24.04	4.62	100	10241	98.8	100.0	JX024758
77	DENV-4	24.04	4.62	400	10141	97.8	99.8	JX024758
77	DENV-4	24.04	4.62	1000	9267	89.4	91.2	JX024758
78	DENV-4	24.04	4.62	100	10197	98.4	100.0	JX024758
78	DENV-4	24.04	4.62	400	10167	98.1	100.0	JX024758
78	DENV-4	24.04	4.62	1000	9192	88.7	90.4	JX024758
82	DENV-3	21.89	5.51	100	10263	98.1	100.0	EU660409
82	DENV-3	21.89	5.51	400	10259	98.1	100.0	EU660409
82	DENV-3	21.89	5.51	1000	9627	92.0	94.6	EU660409
83	DENV-3	22.56	5.26	100	10247	97.9	100.0	EU660409
83	DENV-3	22.56	5.26	400	10230	97.8	100.0	EU660409
83	DENV-3	22.56	5.26	1000	9542	91.2	93.8	EU660409
84	DENV-4	23.89	4.69	100	10219	98.6	100.0	JX024758

84	DENV-4	23.89	4.69	400	10199	98.4	100.0	JX024758
84	DENV-4	23.89	4.69	1000	9345	90.2	91.9	JX024758
85	DENV-4	23.9	4.68	100	10226	98.7	100.0	JX024758
85	DENV-4	23.9	4.68	400	10190	98.3	100.0	JX024758
85	DENV-4	23.9	4.68	1000	9297	89.7	91.5	JX024758
88	DENV-4	20.33	5.85	100	10234	98.7	100.0	JX024758
88	DENV-4	20.33	5.85	400	10221	98.6	100.0	JX024758
88	DENV-4	20.33	5.85	1000	9688	93.5	95.3	JX024758
89	DENV-4	25.69	3.60	100	10147	97.9	100.0	JX024758
89	DENV-4	25.69	3.60	400	10165	98.1	100.0	JX024758
89	DENV-4	25.69	3.60	1000	9168	88.5	90.2	JX024758
91	DENV-4	27.68	3.06	100	10254	98.9	100.0	JX024758
91	DENV-4	27.68	3.06	400	10089	97.3	99.3	JX024758
91	DENV-4	27.68	3.06	1000	8677	83.7	85.4	JX024758
92	DENV-3	28.32	2.90	100	10239	97.9	100.0	EU660409
92	DENV-3	28.32	2.90	400	9991	95.5	98.2	EU660409
92	DENV-3	28.32	2.90	1000	8624	82.4	84.8	EU660409
93	DENV-3	24.7	3.86	100	10229	97.8	100.0	EU660409
93	DENV-3	24.7	3.86	400	10179	97.3	100.0	EU660409
93	DENV-3	24.7	3.86	1000	9153	87.5	90.0	EU660409
94	DENV-3	29.98	1.73	100	10279	98.3	100.0	EU660409
94	DENV-3	29.98	1.73	400	9987	95.5	98.2	EU660409
94	DENV-3	29.98	1.73	1000	8538	81.6	83.9	EU660409
96	DENV-2	18.76	7.50	100	10234	98.1	100.0	EU482447
96	DENV-2	18.76	7.50	400	10243	98.2	100.0	EU482447
96	DENV-2	18.76	7.50	1000	10008	95.9	98.3	EU482447
97	DENV-2	19.5	6.81	100	10216	97.9	100.0	EU482447

97	DENV-2	19.5	6.81	400	10233	98.1	100.0	EU482447
97	DENV-2	19.5	6.81	1000	9642	92.4	94.8	EU482447
100	DENV-2	18.61	7.53	100	10229	98.0	100.0	EU482447
100	DENV-2	18.61	7.53	400	10225	98.0	100.0	EU482447
100	DENV-2	18.61	7.53	1000	10089	96.7	99.1	EU482447
101	DENV-2	17.16	6.33	100	10232	98.1	100.0	EU482447
101	DENV-2	17.16	6.33	400	10209	97.9	100.0	EU482447
101	DENV-2	17.16	6.33	1000	9978	95.6	98.1	EU482447
102	DENV-1	27.28	3.17	100	10207	98.4	100.0	FJ432734
102	DENV-1	27.28	3.17	400	10098	97.4	99.2	FJ432734
102	DENV-1	27.28	3.17	1000	8702	83.9	85.5	FJ432734
103	DENV-1	26.21	3.54	100	10203	98.4	100.0	FJ432734
103	DENV-1	26.21	3.54	400	10140	97.8	99.6	FJ432734
103	DENV-1	26.21	3.54	1000	8766	84.5	86.1	FJ432734
104	DENV-1	24.64	3.89	100	10198	98.3	100.0	FJ432734
104	DENV-1	24.64	3.89	400	10168	98.0	99.9	FJ432734
104	DENV-1	24.64	3.89	1000	9193	88.6	90.3	FJ432734
105	DENV-1	24.57	4.07	100	10194	98.3	100.0	FJ432734
105	DENV-1	24.57	4.07	400	10158	97.9	99.8	FJ432734
105	DENV-1	24.57	4.07	1000	9196	88.7	90.3	FJ432734
106	DENV-1	24.35	4.22	100	10204	98.4	100.0	FJ432734
106	DENV-1	24.35	4.22	400	10149	97.8	99.7	FJ432734
106	DENV-1	24.35	4.22	1000	9234	89.0	90.7	FJ432734
107	DENV-1	23.36	4.98	100	10245	98.8	100.0	FJ432734
107	DENV-1	23.36	4.98	400	10168	98.0	99.9	FJ432734
107	DENV-1	23.36	4.98	1000	9641	93.0	94.7	FJ432734
108	DENV-1	19.02	7.03	100	10189	98.2	100.0	FJ432734

108	DENV-1	19.02	7.03	400	10154	97.9	99.8	FJ432734
108	DENV-1	19.02	7.03	1000	9793	94.4	96.2	FJ432734
112	DENV-2	18.34	6.85	100	10203	97.8	100.0	EU482447
112	DENV-2	18.34	6.85	400	10178	97.6	100.0	EU482447
112	DENV-2	18.34	6.85	1000	9977	95.6	98.0	EU482447
113	DENV-2	17.14	7.97	100	10218	97.9	100.0	EU482447
113	DENV-2	17.14	7.97	400	10184	97.6	100.0	EU482447
113	DENV-2	17.14	7.97	1000	9989	95.7	98.2	EU482447
114	DENV-2	17.78	7.12	100	10297	98.7	100.0	EU482447
114	DENV-2	17.78	7.12	400	10231	98.1	100.0	EU482447
114	DENV-2	17.78	7.12	1000	9998	95.8	98.3	EU482447
115	DENV-2	16.75	8.16	100	10263	98.4	100.0	EU482447
115	DENV-2	16.75	8.16	400	10209	97.9	100.0	EU482447
115	DENV-2	16.75	8.16	1000	9887	94.8	97.2	EU482447
116	DENV-3	17.02	8.06	100	10203	97.5	100.0	EU660409
116	DENV-3	17.02	8.06	400	10252	98.0	100.0	EU660409
116	DENV-3	17.02	8.06	1000	10067	96.2	99.0	EU660409
117	DENV-3	19.34	5.85	100	10244	97.9	100.0	EU660409
117	DENV-3	19.34	5.85	400	10241	97.9	100.0	EU660409
117	DENV-3	19.34	5.85	1000	9808	93.7	96.4	EU660409
119	DENV-2	23.85	3.81	100	10223	98.0	100.0	EU482447
119	DENV-2	23.85	3.81	400	10189	97.7	100.0	EU482447
119	DENV-2	23.85	3.81	1000	9375	89.9	92.1	EU482447
120	DENV-2	29.26	2.68	100	10199	97.8	100.0	EU482447
120	DENV-2	29.26	2.68	400	10047	96.3	98.7	EU482447
120	DENV-2	29.26	2.68	1000	8575	82.2	84.3	EU482447
121	DENV-2	27.11	3.13	100	10199	97.8	100.0	EU482447

121	DENV-2	27.11	3.13	400	10120	97.0	99.4	EU482447
121	DENV-2	27.11	3.13	1000	8785	84.2	86.3	EU482447
122	DENV-2	26.04	3.19	100	10327	99.0	100.0	EU482447
122	DENV-2	26.04	3.19	400	10200	97.8	100.0	EU482447
122	DENV-2	26.04	3.19	1000	9038	86.6	88.8	EU482447
123	DENV-2	23.13	3.92	100	10219	97.9	100.0	EU482447
123	DENV-2	23.13	3.92	400	10178	97.6	100.0	EU482447
123	DENV-2	23.13	3.92	1000	9543	91.5	93.8	EU482447
124	DENV-2	24.33	3.70	100	10193	97.7	100.0	EU482447
124	DENV-2	24.33	3.70	400	10198	97.7	100.0	EU482447
124	DENV-2	24.33	3.70	1000	9188	88.1	90.3	EU482447
125	DENV-2	25.12	3.59	100	10229	98.0	100.0	EU482447
125	DENV-2	25.12	3.59	400	10168	97.5	99.9	EU482447
125	DENV-2	25.12	3.59	1000	9258	88.7	91.0	EU482447
126	DENV-2	25.22	3.54	100	10329	99.0	100.0	EU482447
126	DENV-2	25.22	3.54	400	10189	97.7	100.0	EU482447
126	DENV-2	25.22	3.54	1000	9168	87.9	90.1	EU482447
127	DENV-2	25.53	4.08	100	10348	99.2	100.0	EU482447
127	DENV-2	25.53	4.08	400	10190	97.7	100.0	EU482447
127	DENV-2	25.53	4.08	1000	9085	87.1	89.3	EU482447
128	DENV-2	25.93	3.22	100	10234	98.1	100.0	EU482447
128	DENV-2	25.93	3.22	400	10195	97.7	100.0	EU482447
128	DENV-2	25.93	3.22	1000	9089	87.1	89.3	EU482447
129	DENV-2	26.17	3.17	100	10245	98.2	100.0	EU482447
129	DENV-2	26.17	3.17	400	10145	97.2	99.7	EU482447
129	DENV-2	26.17	3.17	1000	8921	85.5	87.7	EU482447
130	DENV-2	26.46	3.05	100	10209	97.9	100.0	EU482447

130	DENV-2	26.46	3.05	400	10145	97.2	99.7	EU482447
130	DENV-2	26.46	3.05	1000	8754	83.9	86.0	EU482447
131	DENV-2	26.9	2.91	100	10339	99.1	100.0	EU482447
131	DENV-2	26.9	2.91	400	10116	97.0	99.4	EU482447
131	DENV-2	26.9	2.91	1000	8893	85.2	87.4	EU482447
132	DENV-2	29.97	2.90	100	10179	97.6	100.0	EU482447
132	DENV-2	29.97	2.90	400	9989	95.7	98.2	EU482447
132	DENV-2	29.97	2.90	1000	8503	81.5	83.6	EU482447
133	DENV-2	27.68	3.06	100	10337	99.1	100.0	EU482447
133	DENV-2	27.68	3.06	400	10012	96.0	98.4	EU482447
133	DENV-2	27.68	3.06	1000	8636	82.8	84.9	EU482447
134	DENV-2	27.81	2.99	100	10200	97.8	100.0	EU482447
134	DENV-2	27.81	2.99	400	9990	95.8	98.2	EU482447
134	DENV-2	27.81	2.99	1000	8636	82.8	84.9	EU482447
135	DENV-2	27.83	2.92	100	10228	98.0	100.0	EU482447
135	DENV-2	27.83	2.92	400	10056	96.4	98.8	EU482447
135	DENV-2	27.83	2.92	1000	8642	82.8	84.9	EU482447

Appendix 5: DENV-1 Genome Copies: Six infectious blood meals, between-passage material from different patients and Passage numbers, and *w*Mel density in *w*Mel-infected *Ae. aegypti* passages

NA: Not Applicable

Patient Number	Passage Number	Pool Number	Mosquito line	DENV-1 Copies/tissue	Log10 viral load	*Ae.aegypti*-Ct value	*w*Mel-Ct value	*w*Mel density
172	0	C1	Wt	5.09E+07	7.71	19.45	NA	NA
172	0	C2	wMel	2.65E+07	7.42	18.75	18.29	1.38
172	0	C3	Wt	5.94E+07	7.77	19.87	NA	NA
172	0	C4	wMel	1.81E+07	7.26	18.68	18.32	1.28
172	0	C5	Wt	3.24E+07	7.51	19.82	NA	NA
172	0	C6	wMel	2.18E+07	7.34	18.52	18.08	1.36
172	10	C1	Wt	3.45E+07	7.54	19.51	NA	NA
172	10	C2	wMel	2.05E+07	7.31	19.86	19.43	1.35
172	10	C3	Wt	2.26E+07	7.35	19.75	NA	NA
172	10	C4	wMel	1.56E+07	7.19	19.98	19.75	1.17
172	10	C5	Wt	2.93E+07	7.47	19.55	NA	NA
172	10	C6	wMel	5.94E+06	6.77	18.06	17.78	1.21
172	15	C1	Wt	2.69E+07	7.43	19.25	NA	NA
172	15	C3	Wt	2.64E+07	7.42	19.03	NA	NA
172	15	C4	wMel	1.32E+07	7.12	18.42	18.28	1.10
172	15	C5	Wt	3.46E+07	7.54	19.29	NA	NA
172	15	C6	wMel	2.10E+07	7.32	18.9	18.47	1.35
172	20	C1	Wt	4.02E+07	7.60	19.27	NA	NA
172	20	C3	Wt	6.76E+07	7.83	19.73	NA	NA
172	20	C4	wMel	3.83E+07	7.58	18.82	18.56	1.20
172	20	C5	Wt	5.07E+07	7.71	19.67	NA	NA
172	**Plasma**	NA	NA	1.31E+10	10.12	NA	NA	NA
176	0	C1	Wt	1.24E+07	7.09	19.03	NA	NA
176	0	C2	wMel	1.59E+07	7.20	18.98	18.75	1.17
176	0	C3	Wt	5.30E+06	6.72	19.88	NA	NA
176	0	C4	wMel	1.30E+07	7.11	18.8	18.51	1.22
176	0	C5	Wt	1.09E+07	7.04	19.02	NA	NA
176	0	C6	wMel	2.42E+07	7.38	19.15	18.93	1.16

176	10	C1	Wt	6.36E+07	7.80	19.74	NA	NA
176	10	C3	Wt	3.77E+07	7.58	19.49	NA	NA
176	10	C4	wMel	5.99E+07	7.78	18.76	18.55	1.16
176	10	C5	Wt	7.36E+07	7.87	19.19	NA	NA
176	10	C6	wMel	7.50E+07	7.88	19.07	18.89	1.13
176	15	C1	Wt	2.52E+07	7.40	19.91	NA	NA
176	15	C3	Wt	4.96E+07	7.70	19.34	NA	NA
176	15	C4	wMel	2.55E+07	7.41	18.72	18.55	1.13
176	15	C5	Wt	2.01E+07	7.30	19.62	NA	NA
176	15	C6	wMel	6.47E+06	6.81	18.75	18.15	1.52
176	20	C1	Wt	2.26E+07	7.35	19.48	NA	NA
176	20	C3	Wt	6.90E+07	7.84	19.8	NA	NA
176	20	C5	Wt	6.86E+07	7.84	19.07	NA	NA
176	20	C6	wMel	4.16E+07	7.62	19.7	19.51	1.14
176	Plasma	NA	NA	5.17E+07	7.71	NA	NA	NA
180	0	C1	Wt	1.06E+07	7.03	19.49	NA	NA
180	0	C2	wMel	5.41E+06	6.73	19.48	18.98	1.41
180	0	C3	Wt	1.17E+07	7.07	19.1	NA	NA
180	0	C4	wMel	1.56E+07	7.19	19.84	19.52	1.25
180	0	C5	Wt	1.47E+07	7.17	19.04	NA	NA
180	0	C6	wMel	1.74E+07	7.24	19.64	19.47	1.13
180	10	C1	Wt	2.19E+07	7.34	19.1	NA	NA
180	10	C2	wMel	2.63E+06	6.42	19.01	18.45	1.47
180	10	C3	Wt	2.16E+07	7.34	18.47	NA	NA
180	10	C4	wMel	7.29E+06	6.86	19.69	19.5	1.14
180	10	C5	Wt	1.57E+07	7.20	18.72	NA	NA
180	10	C6	wMel	1.29E+07	7.11	19.57	19.22	1.27
180	15	C1	Wt	8.36E+07	7.92	19.01	NA	NA
180	15	C2	wMel	2.46E+07	7.39	19.13	18.49	1.56
180	15	C3	Wt	6.04E+07	7.78	19.54	NA	NA
180	15	C4	wMel	2.86E+07	7.46	19.74	19.56	1.13
180	15	C5	Wt	1.24E+07	7.09	19.47	NA	NA
180	15	C6	wMel	2.66E+07	7.43	19.43	19.11	1.25
180	20	C1	Wt	6.08E+07	7.78	19.73	NA	NA
180	20	C2	wMel	1.72E+08	8.24	19.06	18.57	1.40
180	20	C3	Wt	3.31E+07	7.52	19.26	NA	NA
180	20	C4	wMel	2.81E+08	8.45	19.18	18.79	1.31
180	20	C5	Wt	3.35E+08	8.53	19.5	NA	NA
180	20	C6	wMel	2.72E+08	8.43	19.96	19.76	1.15
180	Plasma	NA	NA	2.53E+08	8.40	NA	NA	NA
185	0	C1	Wt	7.57E+06	6.88	19.99	NA	NA
185	0	C2	wMel	2.05E+07	7.31	19.44	19.05	1.31
185	0	C3	Wt	5.51E+06	6.74	19.16	NA	NA
185	0	C4	wMel	4.89E+06	6.69	19.32	18.58	1.67
185	0	C5	Wt	1.00E+07	7.00	19.04	NA	NA
185	0	C6	wMel	8.57E+05	5.93	18.91	18.31	1.52

185	10	C1	Wt	1.68E+07	7.22	19.8	NA	NA
185	10	C2	wMel	7.43E+06	6.87	19.43	19.23	1.15
185	10	C3	Wt	1.18E+09	9.07	19.56	NA	NA
185	10	C4	wMel	2.13E+07	7.33	19.83	19.33	1.41
185	10	C5	Wt	5.84E+07	7.77	19.95	NA	NA
185	10	C6	wMel	3.26E+07	7.51	19.82	19.67	1.11
185	15	C1	Wt	6.11E+07	7.79	18.7	NA	NA
185	15	C2	wMel	5.31E+07	7.72	19.92	19.49	1.35
185	15	C3	Wt	6.26E+07	7.80	18.78	NA	NA
185	15	C4	wMel	1.20E+08	8.08	19.45	19.25	1.15
185	15	C5	Wt	9.50E+07	7.98	18.71	NA	NA
185	15	C6	wMel	1.82E+08	8.26	19.54	19.33	1.16
185	20	C1	Wt	5.29E+08	8.72	19.44	NA	NA
185	20	C2	wMel	5.24E+08	8.72	18.76	18.54	1.16
185	20	C3	Wt	9.36E+08	8.97	19.56	NA	NA
185	20	C4	wMel	4.11E+08	8.61	18.46	18.23	1.17
185	20	C5	Wt	5.94E+08	8.77	18.75	NA	NA
185	20	C6	wMel	1.11E+08	8.05	19.73	19.15	1.49
185	Plasma	NA	NA	1.52E+08	8.18	NA	NA	NA
193	0	C1	Wt	7.05E+07	7.85	19.43	NA	NA
193	0	C2	wMel	4.30E+07	7.63	19.66	18.95	1.64
193	0	C3	Wt	7.12E+07	7.85	19.75	NA	NA
193	0	C4	wMel	5.65E+07	7.75	19.47	18.79	1.60
193	0	C5	Wt	1.27E+08	8.10	19.74	NA	NA
193	0	C6	wMel	1.04E+08	8.02	19.64	18.97	1.59
193	10	C1	Wt	1.32E+08	8.12	19.73	NA	NA
193	10	C2	wMel	1.74E+08	8.24	18.62	18.29	1.26
193	10	C4	wMel	2.14E+08	8.33	19.51	19.01	1.41
193	10	C5	Wt	1.70E+08	8.23	19.75	NA	NA
193	10	C6	wMel	3.86E+08	8.59	19.63	19.28	1.27
193	15	C1	Wt	2.75E+08	8.44	19.24	NA	NA
193	15	C2	wMel	1.88E+09	9.27	19.87	19.56	1.24
193	15	C4	wMel	5.63E+04	4.75	18.79	18.31	1.39
193	15	C5	Wt	3.26E+08	8.51	19.29	NA	NA
193	15	C6	wMel	6.44E+08	8.81	19.39	19.17	1.16
193	20	C1	Wt	1.61E+08	8.21	19.99	NA	NA
193	20	C2	wMel	6.19E+07	7.79	19.57	19.43	1.10
193	20	C4	wMel	4.11E+08	8.61	19.33	19.06	1.21
193	20	C5	Wt	1.05E+08	8.02	18.81	NA	NA
193	20	C6	wMel	2.47E+07	7.39	19.79	19.58	1.16
193	Plasma	NA	NA	1.01E+08	8.00	NA	NA	NA
197	0	C1	Wt	3.30E+07	7.52	19.91	NA	NA
197	0	C2	wMel	4.22E+07	7.63	18.15	17.9	1.19
197	0	C3	Wt	8.71E+07	7.94	19.24	NA	NA
197	0	C4	wMel	3.91E+07	7.59	18.94	18.59	1.27
197	0	C5	Wt	6.10E+07	7.79	19.22	NA	NA

197	0	C6	wMel	2.59E+07	7.41	18.95	18.67	1.21
197	10	C1	Wt	8.79E+08	8.94	19.8	NA	NA
197	10	C2	wMel	5.71E+08	8.76	18.17	17.89	1.21
197	10	C3	Wt	3.92E+08	8.59	19.53	NA	NA
197	10	C4	wMel	4.05E+08	8.61	18.3	17.89	1.33
197	10	C5	Wt	1.70E+08	8.23	19.65	NA	NA
197	10	C6	wMel	9.36E+08	8.97	18.76	18.28	1.39
197	15	C1	Wt	1.61E+08	8.21	19.37	NA	NA
197	15	C2	wMel	9.00E+09	9.95	18.25	17.78	1.39
197	15	C3	Wt	2.94E+08	8.47	18.71	NA	NA
197	15	C4	wMel	3.78E+08	8.58	19.97	19.72	1.19
197	15	C5	Wt	3.32E+08	8.52	18.81	NA	NA
197	15	C6	wMel	1.18E+08	8.07	19.34	18.73	1.53
197	20	C1	Wt	1.77E+08	8.25	19.22	NA	NA
197	20	C2	wMel	6.39E+07	7.81	19.61	19.2	1.33
197	20	C3	Wt	2.69E+08	8.43	19.63	NA	NA
197	20	C4	wMel	4.54E+07	7.66	19.39	19.08	1.24
197	20	C5	Wt	4.77E+08	8.68	19.15	NA	NA
197	20	C6	wMel	8.54E+07	7.93	18.85	18.55	1.23
197	Plasma	NA	NA	1.38E+08	8.14	NA	NA	NA

Appendix 6: Realtime RT-PCR results and Quality control metrics for DENV-1 sequencing of patient-derived passage material and mosquito controls

NA (* Not Applicable) NRD (*No reads detected) ND (*Not detected)

Patient Number	Passage Number	Pool Number/ Control type	Mosquito line	Total read count	Average coverage	% Reads with Q score > 30	Sequencing batch ID (YY-NN)	DENV-Ct value	*w*Mel-Ct value	*Ae.aegypti*-Ct value
172	0	1	Wt	453,461	6,879	91.62	17-04	20.55	ND	19.45
172	0	2	wMel	378,041	5,680	90.50	17-04	21.47	18.29	18.75
172	0	3	Wt	516,110	7,743	93.96	17-04	20.34	ND	19.87
172	0	4	wMel	509,583	7,486	95.21	17-04	21.99	18.32	18.68
172	0	5	Wt	513,624	7,705	94.12	17-04	21.19	ND	19.82
172	0	6	wMel	569,206	8,268	95.70	17-04	21.74	18.08	18.52
172	10	1	Wt	1,346,649	14,803	94.73	18-02	21.1	ND	19.51
172	10	2	wMel	808,574	9,053	93.22	18-02	21.82	19.43	19.86
172	10	3	Wt	708,740	7,765	95.19	18-02	21.69	ND	19.75
172	10	4	wMel	596,852	6,754	95.25	18-02	22.2	19.75	19.98
172	10	5	Wt	531,028	6,212	95.55	18-02	21.32	ND	19.55
172	10	6	wMel	1,707,798	18,689	95.18	18-02	23.55	17.78	18.06
172	15	1	Wt	366,204	5,184	95.65	18-04	21.44	ND	19.25
172	15	3	Wt	1,760,496	20,197	94.07	18-03	21.47	ND	19.03

172	15	4	wMel	1,340,567	15,667	93.10	18-03	22.44	18.28	18.42
172	15	5	Wt	206,835	3,015	93.63	18-04	21.09	ND	19.29
172	15	6	wMel	351,619	4,959	92.95	18-04	21.79	18.47	18.9
172	20	1	Wt	356,592	5,108	94.09	17-04	20.88	ND	19.27
172	20	3	Wt	306,204	3,842	91.81	19-02	20.16	ND	19.73
172	20	4	wMel	483,853	6,193	95.63	19-02	17.74	18.56	18.82
172	20	5	Wt	502,259	7,355	95.20	17-04	20.56	ND	19.67
172	Plasma	NA	NA	444,200	6,613	92.78	17-04	16.05	ND	ND
176	0	1	Wt	470,988	6,830	95.35	17-04	22.52	ND	19.03
176	0	2	wMel	574,128	8,440	95.61	17-04	22.18	18.75	18.98
176	0	3	Wt	479,496	6,955	92.23	17-04	23.71	ND	19.88
176	0	4	wMel	481,179	7,283	94.34	17-04	22.46	18.51	18.8
176	0	5	Wt	440,486	6,558	93.50	17-04	22.71	ND	19.02
176	0	6	wMel	440,277	6,518	91.43	17-04	21.59	18.93	19.15
176	10	1	Wt	831,720	9,432	95.20	18-02	20.24	ND	19.74
176	10	3	Wt	781,378	8,857	95.39	18-02	20.97	ND	19.49
176	10	4	wMel	638,808	7,151	95.49	18-02	20.33	18.55	18.76
176	10	5	Wt	324,228	3,767	94.59	18-02	20.03	ND	19.19
176	10	6	wMel	489,410	5,822	93.22	18-02	20.02	18.89	19.07
176	15	1	Wt	353,489	5,222	95.45	18-04	21.54	ND	19.91
176	15	3	Wt	212,957	3,177	95.22	18-04	20.59	ND	19.34

176	15	4	wMel	308,397	4,320	95.90	18-04	21.52	18.55	18.72
176	15	5	Wt	308,397	4,320	90.66	18-03	21.85	ND	19.62
176	15	6	wMel	1,534,286	17,441	94.19	18-03	23.43	18.15	18.75
176	20	1	Wt	658,845	8,905	97.46	19-02	21.69	ND	19.48
176	20	3	Wt	1,017,601	9,057	93.83	17-04	20.13	ND	19.8
176	20	5	Wt	376,383	5,492	95.82	17-04	20.14	ND	19.07
176	20	6	wMel	652,660	8,180	98.12	19-02	20.83	19.51	19.7
176	Plasma	NA	NA	429,212	5,884	97.81	17-04	20.19	ND	ND
180	0	1	Wt	473,347	7,153	93.46	17-04	22.74	ND	19.49
180	0	2	wMel	433,149	6,354	94.58	17-04	23.68	18.98	19.48
180	0	3	Wt	420,473	6,317	94.17	17-04	22.61	ND	19.1
180	0	4	wMel	385,134	5,489	96.08	17-04	22.21	19.52	19.84
180	0	5	Wt	394,266	5,889	94.39	17-04	22.28	ND	19.04
180	0	6	wMel	429,086	6,280	96.08	17-04	22.05	19.47	19.64
180	10	1	Wt	432,278	5,076	95.82	18-02	21.73	ND	19.1
180	10	2	wMel	456,219	5,111	95.58	18-02	24.69	18.45	19.01
180	10	3	Wt	545,944	6,091	95.22	18-02	21.75	ND	18.47
180	10	4	wMel	358,472	4,087	94.06	18-02	23.27	19.5	19.69
180	10	5	Wt	471,055	5,416	95.26	18-02	22.2	ND	18.72
180	10	6	wMel	830,737	9,207	94.91	18-02	22.47	19.22	19.57
180	15	1	Wt	389,084	5,800	93.98	18-04	19.86	ND	19.01

180	15	2	wMel	1,732,506	19,761	94.39	18-03	21.57	18.49	19.13
180	15	3	Wt	356,393	5,343	94.12	18-04	20.32	ND	19.54
180	15	4	wMel	1,587,674	18,170	94.70	18-03	21.36	19.56	19.74
180	15	5	Wt	1,767,881	19,693	91.60	18-03	22.53	ND	19.47
180	15	6	wMel	303,617	4,471	95.40	18-04	21.46	19.11	19.43
180	20	1	Wt	486,889	7,243	92.87	17-04	20.31	ND	19.73
180	20	2	wMel	1,767,105	20,797	97.12	19-02	18.85	18.57	19.06
180	20	3	Wt	483,739	7,027	96.34	17-04	21.16	ND	19.26
180	20	4	wMel	1,833,971	21,923	96.52	19-02	18.17	18.79	19.18
180	20	5	Wt	1,885,743	21,772	97.52	19-02	17.92	ND	19.5
180	20	6	wMel	1,595,578	18,722	97.88	19-02	18.21	19.76	19.96
180	Plasma	NA	NA	356,298	5,110	96.75	17-04	19.45	ND	ND
185	0	1	Wt	445,693	6,459	96.53	17-04	23.22	ND	19.99
185	0	2	wMel	455,326	6,689	95.18	17-04	21.78	19.05	19.44
185	0	3	Wt	424,407	6,254	91.45	17-04	23.66	ND	19.16
185	0	4	wMel	378,056	5,555	94.82	17-04	23.31	18.58	19.32
185	0	5	Wt	481,547	7,147	95.14	17-04	22.83	ND	19.04
185	0	6	wMel	391,414	5,711	95.02	17-04	25.17	18.31	18.91
185	10	1	Wt	662,708	7,684	95.33	18-02	22.11	ND	19.8
185	10	2	wMel	891,365	9,734	94.91	18-02	22.86	19.23	19.43
185	10	3	Wt	967,202	11,110	92.30	18-02	17.45	ND	19.56

185	10	4	wMel	1,066,385	11,703	94.80	18-02	21.74	19.33	19.83
185	10	5	Wt	845,541	9,245	94.92	18-02	20.66	ND	19.95
185	10	6	wMel	661,876	7,422	94.73	18-02	21.28	19.67	19.82
185	15	1	Wt	175,239	2,613	93.87	18-04	20.61	ND	18.7
185	15	2	wMel	1,582,272	18,348	95.03	18-03	20.76	19.49	19.92
185	15	3	Wt	2,270,472	25,822	92.41	18-04	20.58	ND	18.78
185	15	4	wMel	1,155,460	13,456	92.63	18-03	19.89	19.25	19.45
185	15	5	Wt	253,467	3,772	94.54	18-04	20.14	ND	18.71
185	15	6	wMel	236,590	3,495	94.83	18-04	19.45	19.33	19.54
185	20	1	Wt	402,483	6,281	91.20	17-04	18.3	ND	19.44
185	20	2	wMel	1,774,564	20,853	97.86	19-02	18.31	18.54	18.76
185	20	3	Wt	2,005,113	21,795	97.41	19-02	15.24	ND	19.56
185	20	4	wMel	2,083,731	23,322	98.05	19-02	18.57	18.23	18.46
185	20	5	Wt	386,929	5,665	94.56	17-04	18.18	ND	18.75
185	20	6	wMel	1,848,505	20,773	97.48	19-02	19.97	19.15	19.73
185	Plasma	NA	NA	365,049	5,090	94.00	17-04	19.28	ND	ND
193	0	1	Wt	167,774	2,503	94.71	18-04	20.46	ND	19.43
193	0	2	wMel	347,554	5,083	93.98	18-04	20.99	18.95	19.66
193	0	3	Wt	234,215	3,568	95.11	18-04	20.45	ND	19.75
193	0	4	wMel	275,930	3,954	95.07	18-04	20.7	18.79	19.47
193	0	5	Wt	271,925	4,116	95.11	18-04	19.83	ND	19.74

193	0	6	wMel	261,624	3,764	95.18	18-04	20.04	18.97	19.64
193	10	1	Wt	180,643	2,699	94.51	18-04	19.78	ND	19.73
193	10	2	wMel	261,122	3,595	95.28	18-04	19.49	18.29	18.62
193	10	4	wMel	413,891	5,575	95.39	18-04	19.28	19.01	19.51
193	10	5	Wt	149,975	2,139	94.74	18-04	19.52	ND	19.75
193	10	6	wMel	385,522	5,171	94.05	18-04	18.64	19.28	19.63
193	15	1	Wt	349,700	5,364	90.10	18-05	19	ND	19.24
193	15	2	wMel	695,990	10,021	90.06	18-05	16.95	19.56	19.87
193	15	4	wMel	441,796	6,842	92.81	18-05	28.08	18.31	18.79
193	15	5	Wt	478,377	7,295	93.56	18-05	18.82	ND	19.29
193	15	6	wMel	540,397	8,603	90.53	18-05	18.09	19.17	19.39
193	20	1	Wt	1,576,991	18,338	97.12	19-02	19.58	ND	19.99
193	20	2	wMel	1,604,645	19,182	98.01	19-02	20.6	19.43	19.57
193	20	4	wMel	910,489	12,956	95.13	19-02	18.58	19.06	19.33
193	20	5	Wt	910,489	12,956	96.13	18-05	20.03	ND	18.81
193	20	6	wMel	1,884,481	21,749	97.85	19-02	21.58	19.58	19.79
193	Plasma	NA	NA	330,356	4,494	95.47	18-04	19.38	ND	ND
197	0	1	Wt	268,106	3,866	94.54	18-04	21.27	ND	19.91
197	0	2	wMel	191,326	2,812	95.03	18-04	21.01	17.9	18.15
197	0	3	Wt	253,423	3,590	95.05	18-04	20.23	ND	19.24
197	0	4	wMel	259,481	3,687	94.38	18-04	21.09	18.59	18.94

197	0	5	Wt	173,621	2,440	94.65	18-04	20.61	ND	19.22
197	0	6	wMel	302,715	4,255	94.80	18-04	21.53	18.67	18.95
197	10	1	Wt	302,215	4,280	94.61	18-04	17.76	ND	19.8
197	10	2	wMel	274,073	4,160	93.29	18-04	18.22	17.89	18.17
197	10	3	Wt	805,884	10,928	93.28	18-04	18.63	ND	19.53
197	10	4	wMel	374,543	5,625	93.77	18-04	18.59	17.89	18.3
197	10	5	Wt	631,249	9,323	94.00	18-04	19.52	ND	19.65
197	10	6	wMel	334,577	5,069	93.87	18-04	17.69	18.28	18.76
197	15	1	Wt	477,702	7,844	98.31	18-05	19.58	ND	19.37
197	15	2	wMel	579,342	8,972	92.49	18-05	12.81	17.78	18.25
197	15	3	Wt	221,193	3,682	95.51	18-05	18.94	ND	18.71
197	15	4	wMel	716,832	10,740	93.98	18-05	18.66	19.72	19.97
197	15	5	Wt	350,764	5,639	97.18	18-05	18.8	ND	18.81
197	15	6	wMel	698,954	10,099	97.90	18-05	19.91	18.73	19.34
197	20	1	Wt	576,992	8,716	95.39	18-05	19.47	ND	19.22
197	20	2	wMel	2,130,395	24,823	97.56	19-02	20.56	19.2	19.61
197	20	3	Wt	1,503,965	17,543	97.89	19-02	19.03	ND	19.63
197	20	4	wMel	1,854,683	21,451	98.78	19-02	20.93	19.08	19.39
197	20	5	Wt	556,627	8,542	93.96	18-05	18.42	ND	19.15
197	20	6	wMel	1,604,486	18,219	97.77	19-02	20.01	18.55	18.85
197	Plasma	NA	NA	327,964	4,304	94.89	18-04	19.24	ND	ND

172	0	Positive	Wt	705,698	6,013	90.40	17-04	21.88	ND	19.37
172	0	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	19.64
172	0	Positive	wMel	695,802	4,102	90.35	17-04	23.15	19.59	19.99
172	0	Negative	wMel	NRD	NRD	NRD	17-04	ND	19.75	20.14
172	5	Positive	Wt	700,495	3,899	94.25	17-04	25.68	ND	19.37
172	5	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	19.64
172	5	Positive	wMel	688,075	4,509	96.12	17-04	25.15	19.59	20.29
172	5	Negative	wMel	NRD	NRD	NRD	17-04	ND	19.15	19.71
172	10	Positive	Wt	477,505	7,829	90.70	18-02	24.93	ND	19.73
172	10	Negative	Wt	NRD	NRD	NRD	18-02	ND	ND	19.51
172	10	Positive	wMel	457,862	3,726	93.17	18-02	24.36	19.22	19.46
172	10	Negative	wMel	NRD	NRD	NRD	18-02	ND	20.52	20.7
172	15	Positive	Wt	423,461	6,857	90.13	18-04	25.92	ND	19.27
172	15	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	18.01
172	15	Positive	wMel	368,232	3,779	95.15	18-03	24.55	19.15	19.63
172	15	Negative	wMel	NRD	NRD	NRD	18-03	ND	19.08	19.1
172	20	Positive	Wt	605,168	7,869	94.36	17-04	24.65	ND	20.68
172	20	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.18
172	20	Positive	wMel	453,559	2,387	93.04	19-02	24.79	20.57	21.16
172	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	18.78	18.99
176	0	Positive	wMel	702,356	5,001	90.28	17-04	25.22	18.48	18.28
176	0	Negative	wMel	NRD	NRD	NRD	17-04	ND	19.78	19.87
176	0	Positive	Wt	805,564	4,402	92.15	17-04	23.07	ND	19.44
176	0	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	18.78
176	5	Positive	wMel	707,456	4,195	92.89	17-04	25.22	19.77	19.65
176	5	Negative	wMel	NRD	NRD	NRD	17-04	ND	18.64	18.32
176	5	Positive	Wt	587,035	3,870	93.59	17-04	22.07	ND	20.35

176	5	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	21.44
176	10	Positive	Wt	478,377	7,295	93.56	18-02	26.16	ND	22.55
176	10	Negative	Wt	NRD	NRD	NRD	18-02	ND	ND	21.48
176	10	Positive	wMel	855,610	11,766	94.97	18-02	22.98	18.55	18.26
176	10	Negative	wMel	NRD	NRD	NRD	18-02	ND	18.77	18.58
176	15	Positive	Wt	521,925	8,437	96.94	18-04	22.96	ND	19.78
176	15	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	20.55
176	15	Positive	wMel	739,256	11,135	92.92	18-03	21.14	17.96	17.55
176	15	Negative	wMel	NRD	NRD	NRD	18-03	ND	21.99	21.82
176	20	Positive	Wt	605,168	7,869	94.36	17-04	24.62	ND	21.87
176	20	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.87
176	20	Positive	wMel	639,964	9,707	92.30	19-02	24.88	21.86	21.45
176	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	19.48	19.07
180	0	Positive	Wt	711,456	5,023	89.79	17-04	24.57	ND	18.68
180	0	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.76
180	0	Positive	wMel	800,234	6,752	93.58	17-04	22.14	19.78	19.88
180	0	Negative	wMel	NRD	NRD	NRD	17-04	ND	18.79	18.74
180	5	Positive	Wt	588,025	4,489	95.89	17-04	25.17	ND	18.68
180	5	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.76
180	5	Positive	wMel	660,389	5,012	94.12	17-04	22.14	18.07	18.35
180	5	Negative	wMel	NRD	NRD	NRD	17-04	ND	20.25	20.63
180	10	Positive	Wt	675,763	10,651	96.63	18-02	26.13	ND	20.82
180	10	Negative	Wt	NRD	NRD	NRD	18-02	ND	ND	19.7
180	10	Positive	wMel	623,475	8,933	95.74	18-02	26.63	20.12	20.86
180	10	Negative	wMel	NRD	NRD	NRD	18-02	ND	19.21	19.57
180	15	Positive	Wt	644,892	10,029	92.94	18-03	24.79	ND	20.18
180	15	Negative	Wt	NRD	NRD	NRD	18-03	ND	ND	21

180	15	Positive	wMel	797,066	3,309	91.76	18-04	23.9	20.29	20.58
180	15	Negative	wMel	NRD	NRD	NRD	18-04	ND	20.58	20.66
180	20	Positive	Wt	497,988	8,209	90.94	17-04	24.53	ND	19.48
180	20	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	19.49
180	20	Positive	wMel	488,479	7,239	91.32	19-02	25.7	20.9	20.87
180	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	20.16	20.22
185	0	Positive	Wt	854,296	6,003	92.13	17-04	25.6	ND	18.57
185	0	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	19.81
185	0	Positive	wMel	744,502	5,112	99.34	17-04	24.54	19.34	19.74
185	0	Negative	wMel	NRD	NRD	NRD	17-04	ND	18.05	18.17
185	5	Positive	Wt	662,898	6,028	98.15	17-04	22.3	ND	17.49
185	5	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.17
185	5	Positive	wMel	701,896	5,236	97.89	17-04	23.11	20.28	20.53
185	5	Negative	wMel	NRD	NRD	NRD	17-04	ND	17.15	17.72
185	10	Positive	Wt	489,562	6,901	91.33	18-02	26.02	ND	19.65
185	10	Negative	Wt	NRD	NRD	NRD	18-02	ND	ND	20.2
185	10	Positive	wMel	499,187	7,967	90.62	18-02	23.85	18.13	18.6
185	10	Negative	wMel	NRD	NRD	NRD	18-02	ND	20.33	20.8
185	15	Positive	Wt	396,014	6,611	89.90	18-03	26.36	ND	20.92
185	15	Negative	Wt	NRD	NRD	NRD	18-03	ND	ND	19.62
185	15	Positive	wMel	688,514	5,730	92.76	18-04	22.57	20.15	20.08
185	15	Negative	wMel	NRD	NRD	NRD	18-04	ND	20.08	20.49
185	20	Positive	Wt	638,318	9,638	95.58	17-04	24.55	ND	18.59
185	20	Negative	Wt	NRD	NRD	NRD	17-04	ND	ND	20.25
185	20	Positive	wMel	557,081	8,742	87.04	19-02	26.63	19.45	19.98
185	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	17.99	18.01
193	0	Positive	Wt	700,589	5,879	90.15	18-04	26.43	ND	18.86

193	0	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	19.26
193	0	Positive	wMel	745,628	6,001	93.85	18-04	23.79	18.59	19.04
193	0	Negative	wMel	NRD	NRD	NRD	18-04	ND	18.42	18.97
193	5	Positive	Wt	688,792	7,015	98.02	18-04	23.31	ND	18.71
193	5	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	18.01
193	5	Positive	wMel	705,896	6,358	97.58	18-04	21.18	17.86	18.69
193	5	Negative	wMel	NRD	NRD	NRD	18-04	ND	19.15	19.3
193	10	Positive	Wt	480,886	8,846	95.25	18-04	23.36	ND	18.77
193	10	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	20.28
193	10	Negative	wMel	NRD	NRD	NRD	18-04	ND	19.97	19.71
193	10	Positive	wMel	537,247	8,629	95.20	18-04	24.76	20.25	20.15
193	10	Negative	wMel	NRD	NRD	NRD	18-04	ND	18.79	19.59
193	15	Positive	Wt	429,212	6,529	94.59	18-05	23.9	ND	20.03
193	15	Negative	Wt	NRD	NRD	NRD	18-05	ND	ND	19.67
193	15	Negative	wMel	NRD	NRD	NRD	18-05	ND	19.78	19.11
193	15	Positive	wMel	440,778	7,138	95.49	18-05	25.23	20.02	19.68
193	15	Negative	wMel	NRD	NRD	NRD	18-05	ND	19.15	18.52
193	20	Positive	Wt	437,973	6,378	95.82	19-02	25.47	ND	20.8
193	20	Negative	Wt	NRD	NRD	NRD	19-02	ND	ND	19.72
193	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	19.08	18.63
193	20	Positive	wMel	475,196	7,494	95.26	19-02	24.77	20.78	19.62
193	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	19.12	18.66
197	0	Positive	Wt	601,853	7,001	92.15	18-04	23.04	ND	18.46
197	0	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	20.69
197	0	Positive	wMel	508,891	6,885	94.16	18-04	22.68	19.88	19.73
197	0	Negative	wMel	NRD	NRD	NRD	18-04	ND	18.78	18.92
197	5	Positive	Wt	66,892	5,023	98.48	18-04	21.76	ND	19.31

197	5	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	18.32
197	5	Positive	wMel	70,825	4,995	97.78	18-04	23.26	18.16	18.55
197	5	Negative	wMel	NRD	NRD	NRD	18-04	ND	17.1	17.07
197	10	Positive	Wt	581,712	9,845	90.45	18-04	23.28	ND	19.51
197	10	Negative	Wt	NRD	NRD	NRD	18-04	ND	ND	18.72
197	10	Negative	wMel	NRD	NRD	NRD	18-04	ND	18.78	18.29
197	10	Positive	wMel	506,867	7,321	98.52	18-04	23.94	18.8	18.6
197	10	Negative	wMel	NRD	NRD	NRD	18-04	ND	18.01	17.82
197	15	Positive	Wt	641,475	8,795	90.08	18-05	23.66	ND	18.73
197	15	Negative	Wt	NRD	NRD	NRD	18-05	ND	ND	19.98
197	15	Negative	wMel	NRD	NRD	NRD	18-05	ND	19.78	19.66
197	15	Positive	wMel	677,866	10,196	97.87	18-05	24.47	20.12	19.9
197	15	Negative	wMel	NRD	NRD	NRD	18-05	ND	18.45	18.1
197	20	Positive	Wt	478,400	7,498	93.45	18-05	25.5	ND	19.83
197	20	Negative	Wt	NRD	NRD	NRD	18-05	ND	ND	19.93
197	20	Negative	wMel	NRD	NRD	NRD	18-05	ND	17.84	17.59
197	20	Positive	wMel	416,240	6,095	97.04	19-02	25.53	19.45	19.13
197	20	Negative	wMel	NRD	NRD	NRD	19-02	ND	20.05	19.78

Appendix 7: Single Nucleotide Variants (SNVs) detected at greater than 50% frequency during serial passage of patient-derived DENV-1 in mosquitoes

Mosquito line	Patient number	Passage Number	Pool Number	CDS Position	Nucleotide change	SNV frequency (%)	Coverage	Quality	CDS Codon Number	Protein - Codon Number	Amino Acid Change
wMel	172	10	2	1447	G > A	61.13	9,053	30	483	E-203	E -> K
wMel	172	10	4	1447	G > A	52.56	6,754	31	483	E-203	E -> K
wMel	172	10	4	2708	T > C	52.23	6,754	31	903	NS1-128	V -> A
wMel	172	10	6	1447	G > A	60.47	18,689	33	483	E-203	E -> K
wMel	172	15	4	1447	G > A	59.39	15,667	37	483	E-203	E -> K
wMel	172	15	4	2708	T > C	61.98	15,667	33	903	NS1-128	V -> A
wMel	172	15	6	1447	G > A	48.98	2,985	37	483	E-203	E -> K
wMel	172	20	4	1447	G > A	50.33	6,193	29	483	E-203	E -> K
wMel	176	10	4	1447	G > A	1.93	1,917	37	483	E-203	E -> K
wMel	176	10	4	1990	G > A	57.6	7,151	38	664	E-384	E -> K
wMel	176	10	6	1447	G > A	74.88	5,822	29	483	E-203	E -> K
wMel	176	10	6	1990	G > A	12.17	6,172	36	664	E-384	E -> K
wMel	176	15	4	1447	G > A	2.94	1,022	37	483	E-203	E -> K
wMel	176	15	4	1990	G > A	72.82	4,320	30	664	E-384	E -> K
wMel	176	15	6	1447	G > A	52.81	17,441	28	483	E-203	E -> K
wMel	176	15	6	1990	G > A	37.73	25,656	36	664	E-384	E -> K
wMel	176	20	6	1447	G > A	27.88	4,520	37	483	E-203	E -> K
wMel	176	20	6	1990	G > A	64.65	8,180	34	664	E-384	E -> K
wMel	176	20	6	3062	T > C	52.41	8,180	35	1021	NS1-245	I -> T
wMel	180	10	2	1447	G > A	40.18	1,558	37	483	E-203	E -> K
wMel	180	10	2	1990	G > A	88.4	5,111	33	664	E-384	E -> K

wMel	180	10	4	1447	G > A	64.24	4,087	37	483	E-203	E -> K
wMel	180	10	4	9142	A > G	75.75	4,087	38	3048	NS5-555	I -> V
wMel	180	10	6	1990	G > A	73.87	9,207	37	664	E-384	E -> K
wMel	180	15	2	1447	G > A	66.5	19,761	36	483	E-203	E -> K
wMel	180	15	4	595	G > A	63.59	18,170	36	199	PrM-85	E -> K
wMel	180	15	4	1447	G > A	73.08	18,170	37	483	E-203	E -> K
wMel	180	15	4	9142	A > G	85.06	18,170	38	3048	NS5-555	I -> V
wMel	180	15	6	1990	G > A	93	4,471	34	664	E-384	E -> K
wMel	180	20	4	595	G > A	96.21	21,923	38	199	PrM-85	E -> K
wMel	180	20	4	1447	G > A	67.88	21,923	38	483	E-203	E -> K
wMel	180	20	4	1990	G > A	10.35	16,654	36	664	E-384	E -> K
wMel	180	20	4	9142	A > G	73.59	21,923	38	3048	NS5-555	I -> V
wMel	180	20	6	1990	G > A	97.59	18,722	32	664	E-384	E -> K
wMel	185	10	6	1447	G > A	84.71	7,422	38	483	E-203	E -> K
wMel	185	15	4	1447	G > C	89.46	13,456	36	483	E-203	G -> R
wMel	185	15	6	1447	G > A	98.97	3,495	30	483	E-203	E -> K
wMel	185	20	2	1990	G > A	75.95	20,853	38	664	E-384	E -> K
wMel	185	20	2	3497	T > C	78.83	20,853	37	1166	NS2A-39	V -> A
wMel	185	20	4	1447	G > C	70.74	23,322	38	483	E-203	G -> R
wMel	185	20	6	1447	G > A	99.02	20,773	32	483	E-203	E -> K
wMel	193	10	2	1990	G > A	60.78	3,595	34	664	E-384	E -> K
wMel	193	15	2	1990	G > A	76.34	10,021	34	664	E-384	E -> K
wMel	193	20	2	1990	G > A	71.88	19,182	34	664	E-384	E -> K
wMel	193	20	4	1447	G > A	20.46	9,895	36	483	E-203	E -> K
wMel	193	20	4	1990	G > A	49.66	27,238	37	664	E-384	E -> K
wMel	197	15	2	1990	G > A	79.64	8,972	38	664	E-384	E -> K
wMel	197	15	2	2540	C > T	67.78	8,972	37	847	NS1-72	S -> L

wMel	197	15	6	1990	G > A	60.2	10,099	37	664	E-384	E -> K
wMel	197	20	2	1990	G > A	91.29	24,823	37	664	E-384	E -> K
wMel	197	20	2	2540	C > T	81.47	24,823	38	847	NS1-72	S -> L
wMel	197	20	4	1990	G > A	61.01	21,451	38	664	E-384	E -> K
wMel	197	20	6	1990	G > A	63.64	18,219	37	664	E-384	E -> K
wMel	197	20	6	5489	T > C	64.91	18,219	33	1830	NS3-355	F -> S
Wt	172	10	5	1447	G > A	20.84	1,963	36	483	E-203	E -> K
Wt	172	10	5	1990	G > A	69.19	6,212	34	664	E-384	E -> K
Wt	172	15	3	214	T > C	58.35	20,197	32	72	C-72	F -> L
Wt	172	15	3	1990	G > A	55.5	20,197	31	664	E-384	E -> K
Wt	172	15	5	1447	G > A	20.52	775	37	483	E-203	E -> K
Wt	172	15	5	1990	G > A	70.55	3,015	33	664	E-384	E -> K
Wt	172	20	3	214	T > C	60.55	3,842	34	72	C-72	F -> L
Wt	172	20	3	1990	G > A	61.02	3,842	31	664	E-384	E -> K
Wt	172	20	5	1447	G > A	10.53	2,052	36	483	E-203	E -> K
Wt	172	20	5	1990	G > A	74.23	7,355	31	664	E-384	E -> K
Wt	176	10	1	1990	G > A	74.6	9,432	31	664	E-384	E -> K
Wt	176	10	3	1447	G > A	30.86	4,245	37	483	E-203	E -> K
Wt	176	10	3	1990	G > A	54.42	8,857	36	664	E-384	E -> K
Wt	176	15	1	1447	G > A	1.88	1,492	37	483	E-203	E -> K
Wt	176	15	1	1990	G > A	72.5	5,222	30	664	E-384	E -> K
Wt	176	15	3	1447	G > A	23.81	1,117	37	483	E-203	E -> K
Wt	176	15	3	1990	G > A	69.57	3,177	35	664	E-384	E -> K
Wt	176	15	5	1447	G > A	22.21	9,274	36	483	E-203	E -> K
Wt	176	15	5	1990	G > A	67.38	4,320	30	664	E-384	E -> K
Wt	176	20	1	1990	G > A	67.7	81,905	33	664	E-384	E -> K
Wt	176	20	3	1447	G > A	14.14	3,026	36	483	E-203	E -> K

Wt	176	20	3	1990	G > A	72.09	9,057	32	664	E-384	E -> K
Wt	176	20	5	1447	G > A	11.63	1,324	36	483	E-203	E -> K
Wt	176	20	5	1990	G > A	72.97	5,492	31	664	E-384	E -> K
Wt	180	10	1	1447	G > A	35.84	1,892	37	483	E-203	E -> K
Wt	180	10	1	1990	G > A	50.38	5,076	31	664	E-384	E -> K
Wt	180	10	3	1447	G > A	20.95	2,048	37	483	E-203	E -> K
Wt	180	10	3	1990	G > A	51.7	6,091	28	664	E-384	E -> K
Wt	180	15	1	1447	G > A	25.2	1,393	37	483	E-203	E -> K
Wt	180	15	1	1990	G > A	67.22	5,800	30	664	E-384	E -> K
Wt	180	15	3	1447	G > A	14.79	1,298	37	483	E-203	E -> K
Wt	180	15	3	1990	G > A	70.24	5,343	33	664	E-384	E -> K
Wt	180	15	5	1990	G > A	93.5	19,693	37	664	E-384	E -> K
Wt	180	20	1	1447	G > A	17.23	2,217	35	483	E-203	E -> K
Wt	180	20	1	1990	G > A	71.63	7,243	29	664	E-384	E -> K
Wt	180	20	3	1447	G > A	8.74	1,957	36	483	E-203	E -> K
Wt	180	20	3	1990	G > A	80.65	7,027	37	664	E-384	E -> K
Wt	180	20	5	1990	G > A	96.57	21,772	38	664	E-384	E -> K
Wt	185	10	1	1990	G > A	88.2	7,684	38	664	E-384	E -> K
Wt	185	10	1	5419	G > A	85.58	7,684	37	1807	NS3-332	V -> I
Wt	185	10	3	770	C > T	98.85	11,110	36	257	PrM-143	A -> V
Wt	185	10	3	1990	G > A	97.46	11,110	36	664	E-384	E -> K
Wt	185	10	5	1990	G > A	94.53	9,245	35	664	E-384	E -> K
Wt	185	15	1	1990	G > A	89.25	2,613	36	664	E-384	E -> K
Wt	185	15	1	5419	G > A	89.99	2,613	38	1807	NS3-332	V -> I
Wt	185	15	3	770	C > T	99.25	25,822	37	257	PrM-143	A -> V
Wt	185	15	3	1990	G > A	97.55	25,822	37	664	E-384	E -> K
Wt	185	15	5	1990	G > A	93.63	3,772	35	664	E-384	E -> K

Wt	185	20	1	1990	G > A	83.57	6,281	38	664	E-384	E -> K
Wt	185	20	1	5419	G > A	82.01	6,281	36	1807	NS3-332	V -> I
Wt	185	20	3	770	C > T	99.72	21,795	37	257	PrM-143	A -> V
Wt	185	20	3	1990	G > A	98.81	21,795	35	664	E-384	E -> K
Wt	185	20	3	3007	T > A	71.98	21,795	33	1003	NS1-228	S -> T
Wt	185	20	5	1990	G > A	96.43	5,665	33	664	E-384	E -> K
Wt	193	15	1	1447	G > A	49.76	2,060	36	483	E-203	E -> K
Wt	193	20	5	1447	G > A	29.61	4,965	36	483	E-203	E -> K
Wt	193	20	5	1990	G > A	49.93	12,062	35	664	E-384	E -> K
Wt	197	10	1	1990	G > A	63.9	4,280	36	664	E-384	E -> K
Wt	197	10	3	1990	G > A	70.97	10,928	30	664	E-384	E -> K
Wt	197	15	1	1447	G > A	11.5	1,765	37	483	E-203	E -> K
Wt	197	15	1	1990	G > A	63.15	7,844	36	664	E-384	E -> K
Wt	197	15	3	1990	G > A	55.81	3,682	30	664	E-384	E -> K
Wt	197	20	1	1447	G > A	3.69	2,224	36	483	E-203	E -> K
Wt	197	20	1	1990	G > A	78.73	8,716	33	664	E-384	E -> K
Wt	197	20	3	1990	G > A	64.55	17,543	33	664	E-384	E -> K