EFFECTS OF A SINGLE MUTATION IN CHIKUNGUNYA VIRUS NON-STRUCTURAL PROTEIN ON VIRUS INFECTION IN *AEDES ALBOPICTUS* AND MAMMALIAN CELL LINES

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ABSTRACT

Chikungunya virus (CHIKV) has caused large-scale epidemics of fever, rash and arthritis since 2004. This unprecedented re-emergence has been mainly associated with mutations in genes encoding structural envelope proteins, providing increased fitness in the secondary vector *Aedes* (*Ae.*) *albopictus*. In the 2008-2013 CHIKV outbreaks across Southeast Asia, an R82S mutation in non-structural protein 4 (nsP4) emerged early in Malaysia or Singapore and quickly became predominant. To determine whether this nsP4-R82S mutation provides a selective advantage in host cells which may have contributed to the epidemic, the fitness of infectious clone-derived CHIKV with nsP4-82R and nsP4-82S were compared in *Ae. albopictus* and mammalian cell lines. Viral infectivity, dissemination and transmission in *Ae. albopictus* were not affected by the mutation when the two variants were tested separately. In competition, the nsP4-82R variant showed an advantage over nsP4-82S in dissemination to the salivary glands, but only in late infection (10 days). In human rhabdomyosarcoma (RD) and embryonic kidney (HEK-293T) cell lines coinfected at a 1:1 ratio, wild-type nsP4-82R virus was rapidly outcompeted by nsP4-82S virus as early as one passage (3 days). However, this fitness advantage was not observed in Vero cells. In conclusion, the nsP4-R82S mutation provides a greater selective advantage in human cells than in *Ae. albopictus*, which may explain its apparent natural selection during CHIKV spread in Southeast Asia. This is an unusual example of a naturally occurring mutation in a non-structural protein which may have facilitated epidemic transmission of CHIKV.

Keywords: chikungunya virus, nsP4, *Aedes albopictus*, human cell lines, Southeast Asia

ABSTRAK

Infeksi virus chikungunya (CHIKV) boleh menyebabkan demam, ruam dan artritis. Adaptasi virus CHIKV kepada vektor sekunder *Aedes* (*Ae.*) *albopictus* telah dikaitkan dengan beberapa wabak berskala besar sejak tahun 2004. Adaptasi ini disebabkan oleh penggantian asid amino E1-A226V yang memberikan infeksi dan penyebaran yang lebih baik di *Ae. albopictus*. Dalam wabak CHIKV 2008-2013 di seluruh Asia Tenggara, mutasi R82S dalam protein bukan struktural 4 (nsP4) CHIKV muncul awal di Malaysia atau Singapura dan cepat menjadi dominan. Untuk menentukan sama ada penggantian asid amino ini memberikan pemilihan yang berfaedah yang mungkin menyumbang kepada wabak, kecergasan klon jangkitan CHIKV nsP4-82R dan nsP4-82S dibandingkan dalam *Ae. albopictus*, spesies nyamuk yang terlibat dalam penyebaran wabak, dan dalam sel mamalia. Infeksi dan penyebaran virus di *Ae. albopictus* tidak terjejas oleh mutasi ini apabila virus diuji secara berasingan. Dalam persaingan, virus nsP4-82R menunjukkan kelebihan dalam penyebaran kepada kelenjar air liur, tetapi hanya di akhir jangkitan (10 hari). Dalam sel 'rhabdomyosarcoma' (RD) dan sel embrio pinggang (HEK-293T) manusia yang dijangkiti virus nsP4-82R dan nsP4-82S dalam nisbah 1:1, virus nsP4-82S mampu mengatasi virus nsP4-82R dalam populasi virus dalam jangka masa 3 hari. Walau bagaimanapun, kelebihan selektif ini tidak diperhatikan dalam sel Vero. Secara kesimpulan, mutasi nsP4-R82S memberikan kelebihan selektif yang lebih besar dalam sel manusia berbanding *Ae. albopictus*, dan ini boleh menerangkan pemilihan semulajadi mutasi ini semasa penyebaran CHIKV di Asia Tenggara. Ini adalah contoh yang luar biasa untuk mutasi yang muncul secara semulajadi dalam protein bukan struktural yang mungkin memudahkan penyebaran epidemik CHIKV.

Kata kunci: virus chikungunya, nsP4, *Aedes albopictus*, sel manusia, Asia Tenggara

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LIST OF SYMBOLS AND ABBREVIATIONS

Ae.	:	*Aedes*
BHK-21	:	Baby hamster kidney cells
bp	:	Base pair
CHIKV	:	chikungunya virus
CMC	:	Carboxymethylcellulose sodium salt
CPE	:	Cytopathic effect
Cx.	:	*Culex*
DMEM	:	Dulbecco's minimal essential medium
dpi	:	Days post-infection
ECSA	:	East/Central/South African
EEEV	:	Eastern equine encephalitis virus
FBS	:	Fetal bovine serum
GMEM	:	Glasgow's minimal essential medium
HEK-293T	:	Human embryonic kidney cells
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	:	Hours post-infection
IFN	:	Interferon
IL	:	Indian lineage
IOL	:	Indian Ocean lineage
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
LOD	:	Limit of detection
LOQ	:	Limit of quantification
MEB	:	Midgut escape barrier

MEM	:	Minimum essential media
MIB	:	Midgut infection barrier
MOI	:	Multiplicity of infection
nsP	:	Non-structural protein
ORF	:	Open reading frame
P/S	:	Penicillin/streptomycin
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
pfu/ml	:	Plaque forming unit per millilitre
PNK	:	Polynucleotide kinase
RD	:	Rhabdomyosarcoma cells
RdRp	:	RNA-dependent RNA polymerase
RRV	:	Ross River virus
SAL	:	Southeast Asian lineage
SARS	:	Severe acute respiratory syndrome
SFV	:	Semliki Forest virus
SGEB	:	Salivary gland escape barrier
SGIB	:	Salivary gland infection barrier
SINV	:	Sindbis virus
TPB	:	Tryptose phosphate broth
UTR	:	Untranslated region
VEEV	:	Venezuelan equine encephalitis virus
Vero	:	African green monkey kidney cells
WNV	:	West Nile virus
X-Gal	:	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Chikungunya virus (CHIKV) is from the family *Togaviridae* and genus *Alphavirus*. It is transmitted primarily by *Aedes* (*Ae.*) *aegypti* and *Ae*. *albopictus* mosquitoes, and causes outbreaks of fever, rash and debilitating joint pain (1). The CHIKV genome consists of a single-stranded, linear and positive-sense RNA of approximately 11.8 kb. The genome encodes for two polyproteins flanked by the 5' and 3' untranslated region (UTR); the first polyprotein contains non-structural proteins (nsP1-nsP4) essential for CHIKV replication, and the second contains structural proteins (E3 and 6K), capsid (C) and the envelope glycoproteins (E2 and E1) that make up the virion coat (2-4). The 5' UTR could alter immune restriction and viral pathogenesis (5) while the 3' UTR consist of stem-loop structures and direct repeats that enhanced viral adaptation to mosquitoes (6). CHIKV is classified into three genotypes: West African, East/Central/South African (ECSA) and Asian (7).

Since 2005, CHIKV of the ECSA genotype has caused several large outbreaks, notably in the Indian Ocean and India (8). Virus mutations leading to enhanced vectorial capacity have been one of the factors contributing to the re-emergence and global spread of CHIKV (9, 10). The initial acquisition of E1-A226V mutation is believed to have facilitated the epidemics in La Réunion and the Indian Ocean islands by increasing CHIKV adaptability, fitness and transmission efficiency in *Ae. albopictus* (11). Subsequently, the E1-A226V mutation independently emerged in three different geographical regions (12), followed by emergence of region-specific second-step adaptive mutations (E2-K252Q, E2-L210Q and E3-S18F/E2-R198Q), all of which further increase CHIKV infectivity in *Ae. albopictus* (13, 14).

Malaysia experienced a nationwide CHIKV (ECSA genotype) outbreak in 2008-2009, affecting tens of thousands of patients (15). *Ae. albopictus* was suspected to be the main vector (16). The outbreak strains carried the *Ae. albopictus*-adaptive E1-A226V mutation and were shown to replicate more efficiently in *Ae. albopictus* compared to *Ae. aegypti* (15, 17). During analysis of CHIKV clinical strains obtained during this outbreak, a substitution from arginine to serine at position 82 of nsP4 (nsP4-R82S) was found to have increased in frequency from the beginning of the outbreak in 2008 to fully replace the wild-type strain in 2009. This nsP4-R82S mutation was similarly reported in a contemporaneous outbreak in neighbouring Singapore (18), but is absent in all other CHIKV sublineages.

Mutations in the nsP4 gene, which encodes RNA-dependent RNA polymerase (RdRp), have been experimentally shown to affect polymerase fidelity and viral fitness of alphaviruses such as CHIKV and Sindbis virus (SINV) in mosquitoes and mice (19-22). However, naturally occurring genetic changes that increase CHIKV adaptation to hosts have been described only in the envelope proteins (23, 24) and 3'UTR (25), but not in non-structural proteins (26, 27).

In this study, phylogenetic analysis showed that the nsP4-R82S mutation, which arose during the Malaysia and Singapore outbreak in early 2008, became predominant in the main CHIKV sublineage in Southeast Asia. It is hypothesized that this nsP4-R82S mutation emerged and was selected as it enhances CHIKV fitness in either the *Ae. albopictus* vector or in the vertebrate host. The effect of this mutation was tested on *Ae. albopictus* midgut infectivity, dissemination to salivary glands and transmission to saliva, as well as assessing fitness in mammalian cell lines.

1.2 OBJECTIVES

The overall objective of this study is to identify the specific host (*Ae. albopictus* vector or mammalian host) in which the nsP4-R82S mutation was selected during the widespread CHIKV outbreak across Southeast Asia. Hence, the specific objectives are as follows:

1. To determine the replication kinetics of nsP4-82S and nsP4-82R CHIKV variants in *Ae. albopictus* mosquito (C6/36 and U4.4) and mammalian (RD, HEK-293T and Vero) cell lines.
2. To investigate the effect of the nsP4-R82S mutation on CHIKV fitness in *Ae. albopictus*.
3. To investigate the selection of the nsP4-R82S mutation in mammalian cell lines using competition assays.

CHAPTER 2: LITERATURE REVIEW

2.1 CHIKUNGUNYA VIRUS GENOME ORGANIZATION

The CHIKV genome has a single-stranded, linear and positive-sense RNA of about 11.8 kb (Figure 2.1). The genome has two open reading frames (ORF) flanked by a 5' and 3' UTR. The first ORF is located immediately after the 5' 7-methylguanosine cap and encodes for nsP1, nsP2, nsP3 and nsP4, while the second ORF, located downstream of the non-structural genes, has a subgenomic promoter that encodes for structural proteins (C, E3, E2, 6K and E1) followed by a 3' poly (A) tail (6). CHIKV RNA is packed into a 60-70 nm diameter capsid surrounded by a phospholipid envelope that is susceptible to desiccation and high temperatures above 58ºC. The cell adhesion molecule Mxra8 has recently been identified as the cell receptor that mediates the entry of CHIKV (28) while the internalization of CHIKV is mediated by clathrin-mediated endocytosis (29).

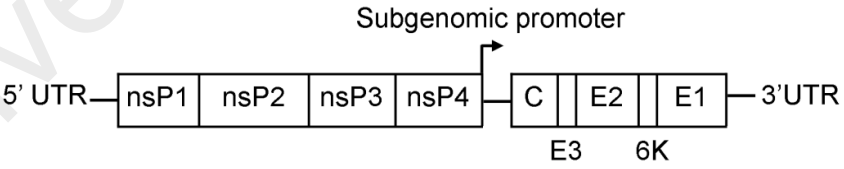


Figure 2.1: The CHIKV genomic structure. Figure is adapted from Powers and Logue (9).

2.2 CHIKV VIRAL PROTEINS: STRUCTURES AND FUNCTIONS

The non-structural proteins (nsPs) are directly translated from the first ORF of CHIKV genomic RNA. The nsP1 protein plays an important role in membrane binding and directing the alphaviral capping reaction as the protein catalyzes methyltransferase and guanylyltransferase activities during viral infection (30, 31). The nsP2 protein acts as a helicase, triphosphatase and protease during viral replication as well as shutting down host macromolecular synthesis and inducing cytopathic effects (CPE) in infected cells (32-34). Although the precise role of nsP3 protein during viral replication is unknown, the protein is essential for RNA synthesis (35). In addition, nsP3 is also involved in mediating virus-host interaction and could be a significant determinant of pathogenesis (36). The nsP4 protein has the core polymerase domain and motifs, and is mainly responsible for RNA synthesis activity although it requires the other three nsPs (nsP1-nsP3) to form the viral replicase complex (37, 38).

RNA synthesis is regulated by the nsPs through the production of non-structural polyprotein precursors. During translation, the majority (~90%) of precursors produced is P123 polyprotein, with a minimal amount of P1234 polyprotein produced from the read through of the opal stop codon between the nsP3 and nsP4 junction (39). The P123 polyprotein by itself cannot synthesise RNA without the presence of RdRp (the nsP4 protein). Minus-strand RNA is synthesised during early infection when the P123 polyprotein and RdRp form a replication complex (P123/nsP4), which is short-lived, before the complex is further processed. The nsP1 protein is cleaved from the P123/nsP4 complex to form the nsP1/P23/nsP4 replicase complex that functions to synthesise positive-sense RNA. The replicase complex is further subjected to proteolytic cleavage to yield nsP1/nsP2/nsP3/nsP4, which represents the whole replication complex and functions to synthesise genomic and subgenomic RNAs (40). The nsP4 protein is produced in significantly lower amounts compared to other nsPs

and is targeted for degradation unless it forms a replication complex (41). Although proteolytic processing of the nsPs is well studied, the functional arrangement of the replicase complex remains poorly understood. However, the interaction between nsP2 (helicase) and nsP4 (RdRp) has been reported to be essential in maintaining replication complex fidelity (42, 43).

The structural proteins are essential components for the formation of virions. Upon infection, the subgenomic mRNAs are actively translated while the translation of cellular mRNAs is shut down (44). The capsid protein forms the nucleocapsid that produces nuclear export and import signals to facilitate entry and exit of the protein from the cell nucleus (45). The E1 and E2 of CHIKV form the transmembrane glycoprotein that mediates cell entry. The E1 protein acts as a fusion protein while the E2 protein mediates cell attachment (46). The virion genome and structural proteins are subsequently assembled on the plasma membrane of infected mammalian cells and mosquito cells, where budding of the virions occurs (47).

2.3 CHIKV CLINICAL SYMPTOMS

Prior to the recent high profile outbreaks, CHIKV was likely to have been grossly underreported over the years due to the overlapping of signs and symptoms with dengue (which is spread by the same mosquito vectors) and unavailability of diagnostic assays (48). The incubation period of the disease upon a mosquito bite varies from 3 to 12 days. The symptoms typically begin with a rapid onset of high fever (>38ºC), arthralgia and myalgia, and rashes. The fever and rashes typically subside within a few days while arthralgia may be very intense, affecting the extremities (phalanges, ankles, wrists) and large joints, where the pain could incapacitate one within a short period of time (49-51). In some cases, apyrexial recurrence of joint pains may persist for months to years after initial infection and the same patient could experience pain at different joints at different

times. The pain is usually more severe in the morning and may cause loss of mobility. In severe cases, patients may be forced to lie on their sides with all their joints in flexion position to provide slight relief from pain (51, 52).

CHIKV mainly targets joints, muscle, skin, and less commonly, the liver, kidneys and central nervous system (53). In skin, symptoms such as maculopapular rash, bullous rash and petechiae (mainly in children) occur in about 40-50% of cases. As CHIKV-induced symptoms are generally self-limiting, the natural course of healing involves gradual improvement until the symptoms are fully resolved (54).

Although CHIKV is not life-threatening, severe symptoms such as encephalitis, encephalopathy, myocarditis and hepatitis have been observed in increased frequency in the past 15 years. The risks of developing these severe symptoms are intensified in neonates, elderly people (>65 years old) suffering from primary medical conditions, and immunosuppressed patients (55). The major outbreak in La Réunion island in 2004 also led to recognition of new pathological manifestations such as encephalopathy and hemorrhagic fever (56), and the first case of peripartum mother-to-child transmission (57).

2.4 CHIKV ORIGINS AND EPIDEMIOLOGY

Chikungunya disease was first described in an epidemic that struck the Makonde Plateau in the Southern Province of Tanganyika (now Tanzania), Africa in 1953. As the severe onset of joint pains distinguished the disease, it came to be known as *chikungunya*, meaning 'that which bends up' (51, 58). CHIKV was first isolated from a patient during the 1953 epidemic (52). Although the outbreak was suspected to be dengue virus (DENV), but serological and antigenic relatedness of the isolates indicated

the virus was an alphavirus closely related to Mayaro virus and Semliki Forest viruses (SFV) (59).

2.4.1 CHIKV IN AFRICA

CHIKV is believed to have originated from Africa between 1950s and 1990s, and was repeatedly isolated from various countries in East, Central and Southern and Western Africa (9). Genetic analysis of CHIKV strains has identified three genotypes (60). The two major genotypes West African and East/Central/South African (ECSA) first circulated in Africa. The ECSA genotype subsequently spread to Asia and initiated the Asian genotype (7).

Epidemic strains from the ECSA genotype emerged from Kenya in 2004 and spread to the Indian Ocean islands, causing an outbreak of unprecedented magnitude (61). The epidemic strain of CHIKV circulating in the Indian Ocean islands make up the Indian Ocean lineage (IOL) and was probably transmitted by *Ae. albopictus* (62), due to the E1-A226V mutation which increased CHIKV dissemination and transmission in *Ae. albopictus* (11, 63).

Travellers returning from affected areas in Indian Ocean islands were associated with the spread of CHIKV to Europe, Canada, Hong Kong, Taiwan and Sri Lanka (56). In recent years, CHIKV outbreaks were continuously reported around the world including Yemen (64), Bhutan (65), Saudi Arabia (66), Brazil (67), India (68), French Polynesia (69), Colombia (70) and the Americas (71). Currently, CHIKV has spread to almost 40 countries worldwide (72).

2.4.2 CHIKV IN ASIA

CHIKV was believed to have spread to southern and southeast Asia via shipping from Africa, establishing the Asian genotype (73). Sporadic outbreaks (Figure 2.2a) caused by viruses of the Asian genotype were reported from late 1950s to 2005 in India, Sri Lanka, Philippines, Vietnam, Myanmar, Malaysia, China, Thailand, Cambodia and Indonesia. Although the Asian genotype still remains in circulation (Figure 2.2b), from 2005 onwards the majority of the large outbreaks in Southeast Asia have been caused by the ECSA genotype (74, 75).

In India, explosive CHIKV outbreaks were reported from 1963-1965 (76-78) and remained dormant until CHIKV re-emerged after a 32 year gap, and caused a recorded 1.3 million cases in 13 states (79). The India outbreak strains (E1-226A) belonged to a distinct Indian lineage (IL) of ECSA genotype (55). The E1-A226V mutation was first detected in 2006 (80) but it was not until 2007 that India experienced a resurgence of CHIKV outbreaks caused by the E1-226V strains (81). Phylogenetic analysis showed that the emergence of E1-A226V mutation occur independently in India and was not introduced from the Indian Ocean epidemics (82). Since then, both E1-226A and E1-226V strains continue to co-circulate in India. This unique situation was likely due to the vast spectrum of urban to rural settings in India which may have contributed to the delineation of circulating CHIKV according to the relative populations of *Ae. albopictus* and *Ae. aegypti* (83).

Seroprevalence studies revealed that CHIKV may have been circulating in Sri Lanka from as early as 1955 (84). CHIKV re-emerged in 2006 (85), likely due to high population susceptibility after four decades of absence (75). The Sri Lankan isolates further diverged into 3 subclades. The first subclade consisted of 2006-2007 Sri Lankan isolates, carrying the E1-226A amino acid. All later Sri Lankan CHIKV isolates contain

nsP1-Q488R and nsP3-V331A substitutions. The second subclade consisted of 2008 Sri Lankan isolates with unique genetic signatures of nsP3-Y38H, nsP3-M394I and E3-S18F. The third subclade also consisted of Sri Lankan isolates from 2008, with a unique signature of nsP3-T444M, E2-V222I and E1-K211N substitutions. However, these Sri Lankan isolates do not contain the E1-M269V substitution found in the isolates of both IL and IOL as they cluster separately from these lineages, suggesting independent evolution within Sri Lanka (18, 86).

(a)

(b)

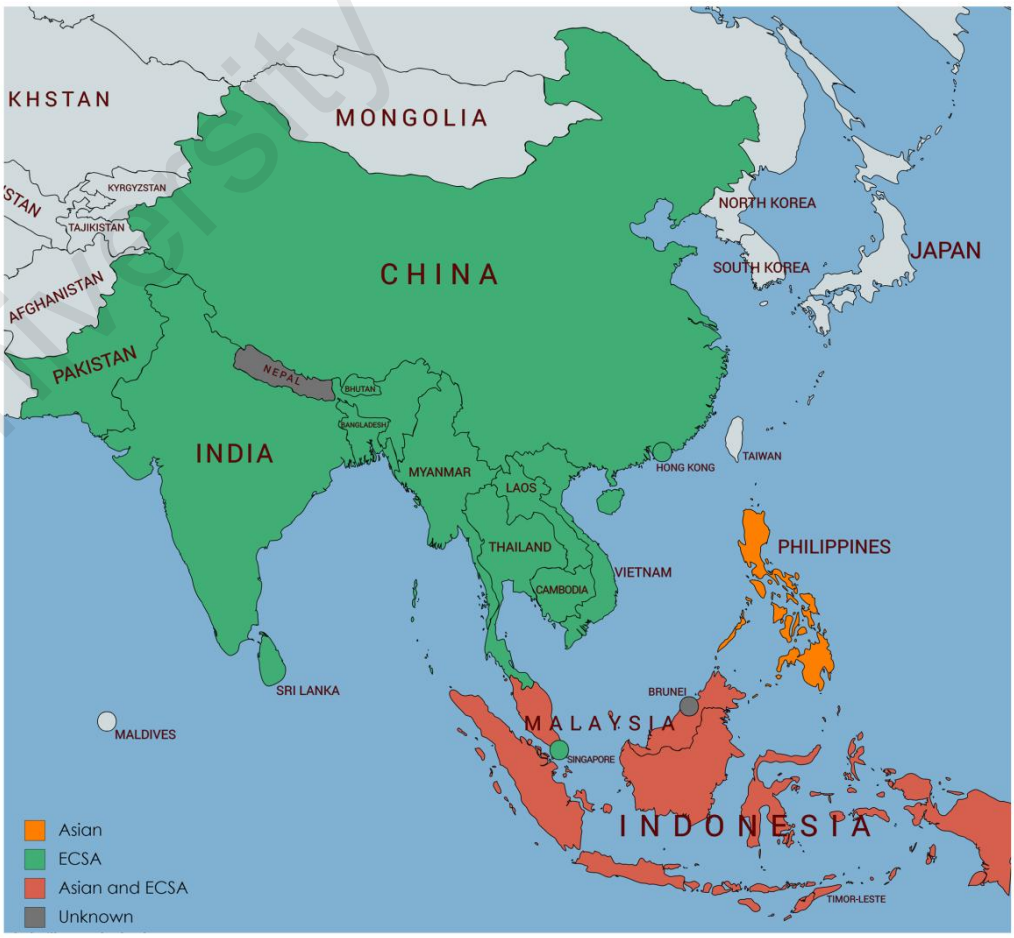


Figure 2.2: Asian countries with locally transmitted CHIKV or exported CHIKV cases that were reported by another country. CHIKV of Asian genotype isolated from 1958-2005 (a) and CHIKV isolated from 2005-2016, where countries are colour coded to represent CHIKV genotypes (b). Figures were adapted from Sam (75) and drawn using online website (https://mapchart.net/world.html).

Singapore is a small city state located south of Malaysia and has frequently experienced imported cases of CHIKV. There was no reported autochthonous transmission until January 2008 when the first local transmission was detected (87). All sequenced isolates belonged to E1-226A strain of the ECSA genotype related to Indian Ocean isolates from 2005, with *Ae. aegypti* serving as the primary vector during the outbreak (18). In May 2008, Singapore experienced a large-scale outbreak, this time due to CHIKV E1-226V strain in *Ae. albopictus*-dominant areas (74, 88). The isolates from this outbreak, including three isolates imported from neighbouring Malaysia, had the E1-A226V, nsP2-L539S, nsP4-R82S and E2-K252Q substitutions (18). The two unique substitutions, nsP2-L539S and E2-K252Q were also present in isolates from Kerala, India dating from 2007 onward (89), and have since been identified in isolates sequenced from Southeast and East Asian countries, including Thailand (90), China (91), Indonesia (92), Myanmar (93) and Cambodia (94). CHIKV then remain undetected until Singapore experienced another outbreak in 2013, caused by CHIKV E1-226V strain where *Ae. albopictus* was identified as the primary vector (72, 95).

Thailand experienced several significant CHIKV outbreaks of Asian genotype from the 1960s to 1995 (96-98). In 2008-2009, the southern provinces of Thailand suffered major outbreaks caused by CHIKV of ECSA genotype, which was believed to be mainly transmitted by *Ae. albopictus*, as CHIKV was isolated from wild mosquitoes caught from outbreak areas (99). From the initial outbreak, CHIKV then spread to 43 of the 75 provinces of Thailand (90). Phylogenetic analysis of Thailand isolates during the 2008-2009 outbreaks showed that all the isolates shared common signature mutations with Malaysian and Singapore isolates, indicating that they are closely related phylogenetically. This is supported by the temporal association between CHIKV cases occurring first in northern Malaysian states followed by southern Thai provinces across the border. All the Thailand isolates contain the E1-A226V substitution (100) and

several novel substitutions in the structural and non-structural regions (90). The presence of unique signature mutations in each of the countries isolates further suggests geographic microevolution of CHIKV (18, 90), though the function of these mutations remained unknown.

In China, CHIKV was first isolated in 1987 in Yunnan Province (101). Although several small outbreaks were reported in 2008, they were all imported cases (102). In 2010, a large scale outbreak struck Guangdong Province, China and phylogenetic analysis showed the isolates carried E1-226V and clustered within the IOL, and were highly similar to Thailand strains isolated from 2009 (103, 104). Since then, there has been no official report of further CHIKV transmission in China.

Myanmar reported high prevalence of CHIKV in 1973 and 1984 (105). In 2010, 6% of patients diagnosed with DENV infection had co-infection of CHIKV. The CHIKV isolates carried E1-226V and are closely related to isolates from China, Thailand and Malaysia, indicating the CHIKV isolates were introduced from neighbouring countries (93, 106).

In Cambodia, CHIKV was first detected in 1961, where the Asian genotype was most likely circulating in the area. Since 2000, CHIKV remained undetected in Cambodia. In 2011-2012, CHIKV of ECSA genotype carrying the E1-A226V mutation was detected in several locations within the country and phylogenetic analysis revealed that the CHIKV strain originated from Thailand (94, 107).

In Vietnam, CHIKV transmission was widespread and affected a geographically diverse population prior to 1985 (108). CHIKV re-emerged in southern Vietnam in 2012 and the sequences were highly similar to ECSA strain (E1-A226V) that was

circulating in Cambodia. This indicated that CHIKV was circulating in several regions of Vietnam during the time (109).

In Laos, although CHIKV outbreaks were not reported, CHIKV seropositivity rate was up to 30% in the 1960s and 1970s (110, 111). In 2012, a CHIKV outbreak was detected for the first time in villages in southern Laos (112). In 2013, Laos experienced a large-scale dengue outbreak and a study done in the affected area showed that CHIKV was still circulating, and the strains showed high similarity to ECSA E1-A226V strains from Cambodia (113).

In Indonesia, CHIKV was first identified in 1972 (111) and sporadic CHIKV outbreaks were reported throughout Indonesia up until 1985 (114). After almost 16 years of quiescence, CHIKV re-emerged to cause outbreaks from 2001 to 2003 (115). Although there were no published work on the genotype of isolated CHIKV, it was highly likely that these outbreaks were caused by Asian genotype given the time frame (97). In 2008, phylogenetic analysis identified Indonesian isolates of ECSA genotype imported into Singapore in the Malaysia/Singapore/India cluster, suggesting a common origin between the strains (88). However, CHIKV outbreaks detected in 2005-2009 (116) and 2010-2011 (117) in DENV cluster studies showed that all isolates has Asian genotype. Sporadic CHIKV outbreaks caused by Asian genotype have since been reported in Indonesia from 2012 to 2017 (118).

CHIKV was first reported in the Philippines in 1965, followed by detection of sporadic cases in the islands of Mindanao, Cebu and Masbate (119). Until the early 2010s, only the Asian genotype circulated in the Philippines (13). In 2011-2013, the Philippines experienced a nationwide outbreak involving CHIKV of Asian genotype. Phylogenetic analysis indicated that the circulating Asian genotype originated from Indonesia in 2010. CHIKV of ECSA genotype (E1-226V strain) was also reported to

co-circulate with the Asian genotype, although the distribution of the ECSA genotype was limited to the southern province of Mindanao (120). Given the prevalence of the ECSA genotype in causing major CHIKV outbreaks in other Asian countries during the time, the reason for the explosive outbreaks caused by the Asian genotype remain unclear.

Although CHIKV of ECSA genotype has become endemic in many of the Asian countries since the epidemiological shift from Asian genotype, the latter continued to remain in circulation, especially in Indonesia and Philippines. The Asian genotype unexpectedly continued to spread to Pacific islands including New Caledonia, Papua New Guinea and Yap State (Federated States of Micronesia) in 2011-2013 (121). The CHIKV then moved on to cause large outbreaks in the Caribbean and Americas in late 2013 (73). In 2014-2015, CHIKV spread from the Caribbean and caused a large scale-outbreak in French Polynesia, where the disease continued to spread to other islands, such as Cook Island in the 2015 (122).

2.4.3 CHIKV IN MALAYSIA

In Malaysia, CHIKV outbreak was first reported in Klang, Selangor in 1998 (49), and re-emerged in Bagan Panchor, Perak in 2006 after a 7 year hiatus (123). Although the outbreak in 2006 coincided with the peak of 2005-2006 outbreaks in La Réunion and with the start of the Indian subcontinent outbreak, phylogenetic analysis confirmed that all the Malaysian outbreaks were caused by the Asian genotype. The first outbreak involving the ECSA genotype (E1-226A strain) occurred in Ipoh, Perak in 2006, and was most likely imported from India. However, the outbreak was brief and contained (124).

In 2008-2009, Malaysia experienced a large-scale outbreak affecting 14 out of 15 states and federal territories. The epidemic first started in Johor in the early 2008, where Singapore also experienced cluster of CHIKV cases (125), and subsequently spread to different parts of the country including Sarawak in East Malaysia (126, 127). Sequenced isolates showed the outbreak was caused by CHIKV E1-226V strain that is similar to Indian strains from Kerala, India, from 2007 (15, 124). Epidemiological studies showed that rural area such as rubber or palm oil plantations tend to have higher risk of CHIKV infection (128, 129). *Ae. albopictus* was suspected to be the vector facilitating the outbreak due to its abundance in rural and affected area (16).

2.5 CHIKV TRANSMISSION CYCLE

There are two types of epidemiologic transmission cycles of CHIKV: the sylvatic wild primates-mosquito cycle and the urban human-mosquito cycle. In West and Central Africa, CHIKV circulates in a sylvatic cycle between wild primates and forest-dwelling *Aedes* species mosquitoes. The urban epidemic emergence of CHIKV is due to transition from an enzootic, sylvatic cycle to an urban peridomestic cycle involving spillage to humans (130). This is supported by the detection of significant levels of antibody against CHIKV in both human and wild primates throughout Africa (131). In 2004, CHIKV spread out from Africa into Indian Ocean islands and subsequently initiated global expansion into new geographic regions (132). Although CHIKV infections have been reported in long-tailed/cynomolgus macaques (*Macaca fascicularis*) (110, 133-137), naturally occurring sylvatic cycles have not been clearly shown in Asia (132).

The following sections described the interactions between CHIKV, vectors and vertebrates in establishing transmission, as well as research conducted to improve understanding on these interactions.

2.5.1 VECTOR COMPETENCE FOR CHIKV

The ability of the mosquito to acquire, maintain and transmit arboviruses, otherwise known as vector competence, depends on the genetic makeup and intrinsic mechanisms of the mosquito (138). Although *Ae. aegypti* plays a significant role as the primary vector for CHIKV in urban areas as the peridomestic mosquitoes favor humans as their host (61), *Ae. albopictus* has long been identified as a potential competitor (139, 140) through larval competitive advantage for resources (62, 140).

The *Ae. albopictus* mosquito, which originated from Asia, is an aggressive, day-time biting insect that was originally zoophilic but has progressively adapted to urbanized environments that provide alternative blood sources (domestic animals and human) and optimal larval habitats (141). In the last few decades, *Ae. albopictus* has spread to Africa, the Middle East, Europe and the Americas, thus becoming the most invasive mosquito in the world (140, 142, 143).

In the last decade, *Ae. albopictus* has been reported to be the primary vector that facilitated the spread of DENV and CHIKV outbreaks in the Indian Ocean islands, Central Africa and China (144). The versatile and invasive *Ae. albopictus* feed on a range of vertebrate species (145), and coupled with its susceptibility to infection by multiple viruses (146) may have further contribute to the widespread CHIKV epidemic.

The availability of competent vectors also plays an important role in the spread and establishment of CHIKV in new endemic regions (55). For example, although *Ae. polynesiensis* is a less efficient vector for CHIKV than *Ae. aegypti*, it mainly facilitates CHIKV outbreaks in the Pacific islands such as the Cook Islands and Samoa, as the predominant vector in these areas (147).

Besides the variation in susceptibility of different mosquito species towards CHIKV infection, studies of mosquitoes collected from geographically different locations confirmed that there is also susceptibility variation within a species. The intraspecific variation between mosquito populations could result in, for example, *Ae. aegypti* from one location being highly susceptible to arbovirus infection, while those from other being relatively resistant (132). One plausible explanation for this is that the susceptibility of *Ae. aegypti* could be influenced by variation in genetic factors of different mosquito populations (148). This intraspecific variation was also demonstrated when the CHIKV E1-226V La Réunion strain was shown to have increased fitness in *Ae. albopictus* populations from La Réunion, but not in *Ae. albopictus* from Congo (149).

2.5.2 HOST PREFERENCE

Host preference refers to whether a mosquito will feed on a viremic host and be subsequently infected by the arbovirus. Some mosquito species are quite selective in host selection. For instance, *Ae. aegypti* is anthropophilic, and they prefer to feed on humans and often feed on several individuals within a short period of time (61). Although *Ae. albopictus* also has similar feeding tendencies, the mosquito tend to be more opportunistic as they feed on a broad range of vertebrate species (chicken, dog and cow) depending on host availability (150).

Humans are generally identified as the primary vector in the urban transmission. All major CHIKV outbreaks reported from 1959 to 2016 were mainly facilitated by human, *Ae. aegypti* and *Ae. albopictus* in tropical and sub-tropical regions of the world. Excluding vaccination studies, most of the CHIKV studies in humans are observational involving surveillance for CHIKV virus and seropositivity during epidemics (151). A recent study conducted on CHIKV infected patients in La Réunion showed that CHIKV

titres could reach as high as 1.2×10^9 RNA copies/mL, more than sufficient to infect many susceptible mosquitos' species upon feeding (152). Human cells that are particularly susceptible to infection include endothelial cells, primary fibroblasts and monocyte-derived macrophages (53). The innate immune response is triggered by infection and high levels of interferon (IFN) alpha are produced (153).

Many experimental studies have been conducted on non-human vertebrate hosts to better understand the capacity of these hosts in sustaining CHIKV circulation. Non-human primates have been widely studied for their role as the primary host for CHIKV infection in sylvatic cycles. Several studies had showed that infected non-human primates such as the vervet monkey (154), rhesus macaque (155), cynomolgus monkey (156) and baboon (157) could be involved in CHIKV transmission cycle as they develop sufficient viraemia to infect mosquitoes upon feeding. However, urban or periurban non-human primates were demonstrated to be an unlikely host in establishing sylvatic cycle in Latin America due to the host resistance to CHIKV infection, highlighting the relevance of targeted epidemiological investigations of non-human primates for CHIKV infection (158).

Besides non-human primates, a study conducted by Bosco-Lauth, Nemeth, Kohler and Bowen (159) showed that the big brown bat (*Eptesicus fuscus*) developed detectable viraemia upon infection, while avian birds and other mammalian species which represents the domestic and wild animals in North America developed neutralizing antibody responses to CHIKV without the presence of viraemia.

2.5.3 MOSQUITO ANATOMY AND VIRUS INFECTION

Most arboviruses are horizontally transmitted during blood feeding between the vertebrates that serve as amplification hosts and the arthropod vectors. To ensure

successful transmission, the female mosquito must feed on a viremic vertebrate host that produces a high enough viral titre for the mosquitoes to become infected (160). The key steps for an arbovirus to successfully establish infection (Figure 2.3) in a mosquito vector are: (1) initial infection of midgut after a blood meal; (2) spread of infection across midgut epithelium; (3) dissemination into secondary sites; (4) establishment of infection in secondary sites; (5) infection of salivary gland; (6) release of virus into saliva; and (7) transmission to an uninfected vertebrate host upon feeding (161). Vector competence is affected by existing tissue barriers in mosquitoes including the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection barrier (SGIB) and salivary gland escape barrier (SGEB) (162). The differences in overall vector competence depends on the strengths of barriers to resist viral infection (163).

After feeding, the infectious blood reaches the mosquito midgut. The ability of the virus to infect or replicate in the midgut epithelial cells defines the MIB in the mosquito vector (164, 165). While the degree of infection varies depending on the virus-mosquito species combination, the MIB is associated with receptor recognition as a requirement for successful entry of virus into midgut. Even in a situation where virus and mosquito vector are compatible, only a small amount of midgut epithelial cells may be susceptible to virus infection (161).

Following midgut infection, the arbovirus needs to escape the midgut and disseminate to the salivary glands. The MEB is associated with the presence of basal lamina surrounding the midgut epithelial cells. The viral dissemination from the midgut has been described via the trachea complex or intussuscepted foregut where the basal lamina is more permeable. The presence of MEB in the mosquito midgut has been demonstrated by the inability of *Culex* (*Cx.*) *taeniopus* and *Cx. pipiens* to disseminate

Venezuelan equine encephalitic virus (VEEV) (166) and Rift Valley fever virus (167), respectively, despite having infected midguts.

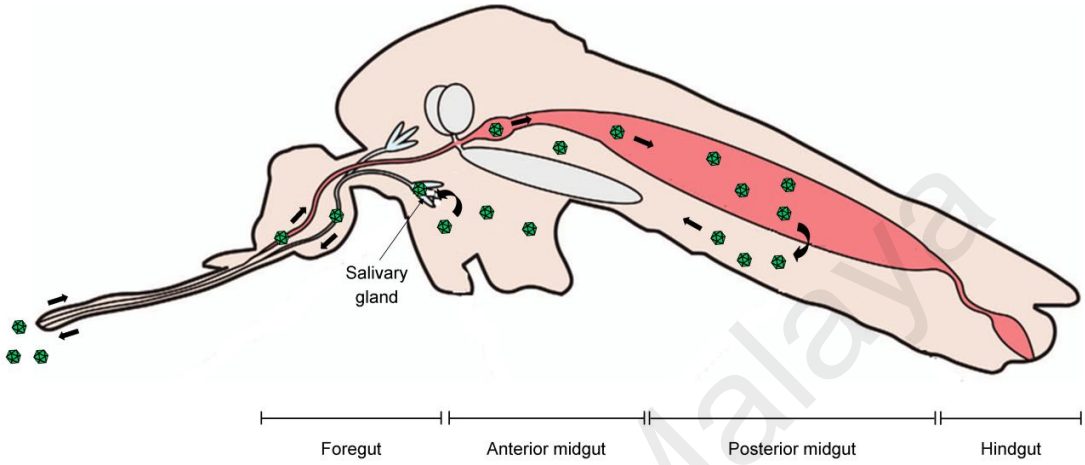


Figure 2.3: Viral transmission route in a mosquito upon a blood meal. Upon feeding, the infectious blood reaches the midgut where infection first takes place. After escaping from the midgut, the virus disseminates to secondary sites including salivary gland. Successful escape from the salivary gland indicates the virus can be transmitted into the saliva. Figure is adapted from Vogels, Goertz, Pijlman and Koenraadt (168) .

Although arbovirus may successfully disseminate from the midgut to secondary sites such as salivary gland, the presence of the SGIB may render the virus unable to infect or replicate in the organ. This was demonstrated by the failure of Eastern equine encephalitis virus (EEEV) and West Nile virus (WNV) to establish infection in the salivary glands of *Ae. albopictus* (169) and *Cx. pipiens* (170), respectively, even though dissemination had occurred.

Once the salivary gland is infected, the arbovirus is then released into the saliva. However, the presence of SGEB may prevent the escape of virus into the saliva, as

demonstrated by the inability of *Cx. thieleri* to transmit SINV into the saliva despite having infected salivary gland (171). Should the virus successfully overcome the SGEB, the virus could then be transmitted to another susceptible host upon feeding. Positive transmission is commonly confirmed through detection of viral RNA in mosquito saliva (172).

2.5.4 LABORATORY STUDIES

Early studies mainly focused on field investigations to identify the mosquito species responsible for CHIKV transmission. In the 1970s, entomologic surveillance in Senegal, Côte d'Ivoire and South Africa showed that CHIKV could be isolated from sylvatic *Aedes* vectors such as *Ae. africanus*, *Ae. neoafricanus*, *Ae. furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. dalzieli* and *Ae. aegypti* (173, 174). Other peridomestic mosquito species such as *Ae. vittatus* that are predominant in CHIKV-endemic areas have been demonstrated to have competent vectorial capacity, especially for Asian and West African genotypes compared to *Ae. aegypti* (175-177).

Mosquito susceptibility to CHIKV has been continually studied in order to determine the potential risk of CHIKV causing an epidemic in a given region. Studies conducted in Malaysia (17), Taiwan (178), India (179), Florida (180, 181) and Brazil (180) showed that the native mosquitoes vectors such as *Ae. aegypti* and *Ae. albopictus* were susceptible to CHIKV. Furthermore, although imported vectors in New Zealand such as *Ae. notoscriptus* and *Ae. australis* have not yet been incriminated as vectors in the wild, their high competency for CHIKV could potentially result in CHIKV epidemics (182).

Experimental study of potential CHIKV vectors revealed that *Ae. aegypti formosus*, the sylvan form of *Ae. aegypti* thought to be refractory to arboviral infection, is a competent vector for CHIKV (183), and has since developed an increasing preference for human (184). This indicated that should CHIKV be introduced into *Ae. aegypti*

formosus-rich areas, CHIKV could establish transmission among people (185). In addition, CHIKV could potentially establish a sylvatic transmission cycle in the Americas where the sylvatic primatophilic mosquito species such as *Haemagogus leucocelaenus* and *Ae. terrens* are shown to be susceptible to CHIKV infection. This could trigger an immediate public health consequences as there are currently no efficient methods of controlling the enzootic stages of any arboviruses (186).

2.6 ADAPTATION OF ARBOVIRUS DUE TO GENETIC CHANGES

RNA viruses have an inherent ability to adapt to new environments as their error-prone polymerase results in rapid mutations and generation of a heterogenous population of genetically related variants (187). Although these mutations could be lethal for the virus during selection pressure (bottleneck effect), this mechanism could also be beneficial for the virus at some point of their life cycle including escape from the host immune response, adaptation to different environments, and enhanced transmission (188).

Genetic adaptation to a new host is one of the most crucial factors in determining the ability of an arbovirus to emerge and adapt to a new environment. The selection of mutants is complicated as the transmission of arbovirus involves both arthropod vectors and vertebrate hosts, therefore the mutant arbovirus must be able to adapt to different physiological environments (189). The history and mechanisms of the emergence of arboviruses have demonstrated that simple point mutations in the viral genome could alter host range and/or infection efficiency in host or vectors (26).

2.6.1 BOTTLENECK EFFECT

The bottleneck effect is a random event that decreases the genetic diversity of the population. In mosquitoes, the bottleneck effect mainly occurs during infection of

midgut, escape from the midgut into hemocoel and infection of the salivary gland (190, 191) due to the presence of basal lamina that limits viral penetration. Although the population size could recover during intra-tissue population expansion, the genetic diversity of the population will remain diminished compared to the input virus (192). In addition, the recovered population could contain potentially deleterious mutations, depending on the vector, ultimately resulting in lower fitness in a vertebrate host (163). On the contrary, infection in vertebrate hosts such as humans involves the evasion of both innate and adaptive immune responses as well as replication to a sufficiently high viral titre to facilitate next round of transmission to arthropod vector (192).

The bottleneck effect shapes arbovirus evolution during the natural transmission cycle (193). Transmission cycle involving more than one host typically constrains the ability of the arbovirus to adapt to new hosts, most likely due to fitness trade-offs during alternation between mosquitoes and vertebrates (194). Releasing the arboviruses from host alternation cycles via serial passages in a single host could potentially facilitate adaptive evolution as demonstrated in St. Louis encephalitis virus (195) and WNV (196, 197). Another example of this is that CHIKV serially passaged in single host cells demonstrated increased genetic diversity and fitness in a novel cell line, which correlated with greater adaptability. On the other hand, CHIKV passaged alternately between two different hosts cell lines were associated with increased fitness but restricted genetic diversity, where mutations that are neutral or beneficial to both hosts were retained in order to maintain fitness in each alternate cycle (198). These data suggest an evolutionary trade-off event where viral fitness and genetic diversity are maintained at an optimal level to ensure maximum adaptability (192).

2.6.2 GENETIC CHANGES IN UNTRANSLATED REGIONS (UTR)

The UTR at the 5' and 3' ends of the alphavirus genome regulate the expression, replication and translation of viral genes, and facilitate interaction between virus and host. These factors have significant implications on viral evolution as sequence and structural changes in the alphavirus 5'UTR could alter immune restriction and viral pathogenesis (5). For example, a single point mutation in the 5'UTR attenuated VEEV and resulted in avirulence in mice. The mutation also reduced viral fitness in cell culture through increased sensitivity to alpha/beta interferon (IFN) (199, 200). Recently, it was demonstrated that the RNA structural elements in the 5'UTR of VEEV and SINV help the virus to avoid immune restriction in mammalian host cells (201). Similarly, sequence deletions and point mutations in the 5'UTR of SINV and SFV could affect pathogenicity and neurovirulence in rodents (202, 203).

Although the 3'UTRs of alphaviruses differ in length and may exhibit extensive substitutions, insertions and deletions (6), the 3'UTR is essential in directly or indirectly facilitating viral genome replication in both mosquito and mammalian cells (204, 205). In CHIKV, the evolution of 3'UTR is shaped by the fitness trade-offs observed between mosquito and mammalian hosts (5). The CHIKV 3'UTR contains several direct repeats (DRs 1, 2 and 3), which vary in different lineages, and deletion of DR may lead to fitness loss in mosquitoes but increased fitness in mammalian models (6). A study identified a novel 3'UTR duplication structure that was not observed in nature previously in CHIKV clinical samples isolated during the recent Caribbean and American outbreaks. This 3'UTR duplication structure was shown to increase CHIKV replication in insect cells, thus emphasizing the potential importance of the 3'UTR in adaptation and evolution of CHIKV in facilitating epidemics (25). Another example showed that mutation in the SINV 3'UTR conserved sequence element region reduced the efficiency of negative strand synthesis (206). In addition, short deletions in various

parts of the SINV 3'UTR have been demonstrated to affect host-dependent fitness in chicken and mosquito cells, implying that the 3'UTR may interact with different host-specific cellular factors (202).

2.6.3 ADAPTIVE MUTATIONS IN STRUCTURAL PROTEINS

To date, adaptive mutations in alphaviruses associated with cross-species jumps and increased viral fitness have mostly been described in the structural proteins E2 and E1 (207). A fine example of naturally adaptive mutation is the emergence of VEEV epidemics. The VEEV enzootic strain is relatively avirulent for equines and do not cause viraemia. However, the emergence of a single mutation in the E2 gene increases VEEV replication efficiency, viremia induction and virulence in equines, as well as increased adaptation to the vector *Ae. taeniorhynchus*. This adaptation in vertebrate hosts and vectors facilitated virus spillover to humans who live in close association with infected equines, triggering an epidemic disease (208).

There are several notable naturally acquired adaptive mutations of CHIKV to *Aedes* mosquitoes. During the La Réunion outbreak in 2005, a mutation in the E1 glycoprotein (E1-A226V) of CHIKV was identified as the primary cause that drove the spread of CHIKV in the island and across Indian Ocean islands (11). The E1-A226V mutation then emerged independently in three different geographical regions, suggesting an evolutionary convergence of CHIKV in nature (12). The mutation was shown to enhance infection in the midgut, dissemination to mosquito secondary organs and transmission efficiency of *Ae. albopictus*, but had little effect on *Ae. aegypti* infectivity (11). Dissemination and transmission efficiency of CHIKV E1-A226V strain are controlled at the midgut barrier level, where direct competition between both E1-226A and E1-226V strains via blood meal led to the selection of E1-226V while intra-thoracic inoculation failed to produce the same effect (209).

Apart from primary adaptive mutation, complex epistatic interactions could constrain the adaptation of alphaviruses to new vectors, thus restricting the emergence of epidemics. For example, despite the abundance of *Ae. albopictus* in Asia, the adaptation of Asian genotype to the mosquitoes was restricted by E1-98T, even when the E1-A226V mutation was present. On the contrary, the E1-T98A mutation carried by the IOL CHIKV strains does not inhibit the effect of E1-A226V, which explains how the IOL CHIKV strains could rapidly replace the Asian genotype in *Ae. albopictus*-predominant regions in Asia (210). Similarly, E2-I211T shows an epistatic interaction with the E1-A226V mutation that further promotes adaptation of the ECSA genotype to *Ae. albopictus* (211).

Multiple second-step adaptive mutations emerged after CHIKV acquires the initial E1-A226V mutation, resulting in rapid diversification of lineages in nature. Among the prominent second-step adaptive mutations are E2-L210Q and E2-K252Q, and two mutations E2-R198Q and E3-S18F with epistatic relation which significantly increase viral fitness for *Ae. albopictus*. As most of the second-step adaptive mutations share a common replacement of an amino acid by glutamine or glutamic acid in the acid-sensitive region of the E2 glycoprotein, the discovery of the key substitution region in the E2 region enabled the prediction of potential second-step adaptive mutations, such as the E2-K233Q mutation that was experimentally proven to increase CHIKV dissemination efficiency in *Ae. albopictus* (212).

Interestingly, sequenced CHIKV genomes isolated from a major outbreak in Kenya in 2016 revealed two novel mutations (E1-K211E and E2-V264A) in the background of E1-226A. Both mutations increased CHIKV infectivity, dissemination and transmission in *Ae. aegypti*. This *Ae. aegypti*-adaptive strain emerged within the ECSA genotype between 2005 and 2008, most likely in India, and has since replaced the less adapted

wild-type CHIKV in *Ae. aegypti*-dominant regions. This strain then caused large outbreaks in New Delhi, India in 2010 and re-emerged in Pakistan and Kenya in 2016, and Bangladesh in 2017 (23, 213).

As the emergence of adaptive variants in nature may take years to occur, experimental study has successfully identified and predicted potential adaptive mutations in a shorter period. The selection of E1-V80I and E1-A129V mutations in laboratory simulations of natural transmission from mosquitoes to mammals demonstrated that both mutations increased CHIKV stability and fusogenic activity in cell-free environments such as in the hemocoel and saliva of mosquitoes or bloodstream of mammals, thus increasing viral fitness and infectivity in both hosts. This simulation of transmission model allows the prediction and study of virus evolution trajectories before current circulating strains are replaced by new variants (214).

2.6.4 ADAPTIVE MUTATIONS IN NON-STRUCTURAL PROTEINS

The replication of alphavirus requires specific interaction between the nsPs protein and host factors to successfully form replication complexes (36). Although mutations in nsPs have been experimentally demonstrated to affect CHIKV replication in vertebrates and mosquitoes, to date there are no confirmed examples of naturally occurring adaptive mutations in CHIKV nsPs,

The nsP1 protein is essential in recruiting other non-structural proteins to form a functional replication complex (215) and for minus-strand RNA synthesis (216). Mutations in the nsP1 of Ross River virus (RRV) increase virulence in mice through regulation of RRV sensitivity to type I IFN (217). In SINV, the nsP1-A348T mutation has been demonstrated to impair minus-strand RNA synthesis at a restrictive temperature (218). Similarly, the nsP1-D119N and nsP1-E529D mutations in SFV also

rendered the virus defective in minus-strand RNA synthesis (30). The replacement of cysteine with alanine amino acid or their deletion in the nsP1 of SFV has been shown to decrease viral fitness in mammalian cell lines, possibly due to the disruption of nsP1-nsP4 interaction, a catalytic subunit of the replicase (219).

The nsP2 protein is multifunctional as it serves as an important co-factor for the maturation of the viral replication complex (220). Studies have demonstrated the importance of nsP2 in establishing persistent replication in mammalian cells. The nsP2-P718S and nsP2-P726S mutations in CHIKV and SINV, respectively, were shown to affect viral pathogenesis by inhibiting the IFN-induced JAK-STAT signaling pathway in BHK-21 cells (221, 222). In addition, the nsP2-C478A mutation in CHIKV was shown to inactivate protease and completely impair viral replication in BHK-21 cells (223).

Although the precise role of the alphavirus nsP3 in the replication complex is less clear, the protein plays an important role in regulation of viral RNA transcription. The nsP3 protein exists in multiple phosphorylated states during infection (224). The nsP3-A68G mutation in SINV alters the degree of phosphorylation of nsP3, which subsequently reduced efficiency of minus-strand RNA synthesis (225). The deletion of two major threonine phosphorylation sites in nsP3 of SFV decreased the rate of RNA synthesis as well as greatly reduced pathogenicity in mouse models (226). A mutation that changed the phosphorylation state of nsP3 protein of VEEV was demonstrated to increase viral fitness in mosquito cells but did not affect virus replication in mammalian cells (227). Interestingly, the substitution of the opal termination codon with arginine in the nsP3 of CHIKV decreased virulence in mice but produced the opposite effect in SFV (228), highlighting the differential effects conferred by a mutation in different alphaviruses.

The alphavirus polymerase encoded by nsP4 is a highly conserved protein (193). Various studies have identified several functional sites in the nsP4 that could affect viral virulence and fitness in the host. For example, nsP4-C483 and nsP4-C482 were shown to be a fidelity determinant of CHIKV (19, 20) and SINV (20), respectively, and amino acid changes at these sites alter viral fitness in mosquitoes and mice. The nsP4-R183 of SINV was demonstrated to contribute to the initiation of minus strand synthesis and the formation of its replicase (21). In SINV, the tyrosine residues in the N-terminal in the nsP4 interacts with the nsP1 for minus-strand RNA synthesis and replacement of the tyrosine residue has been linked to defects in RNA synthesis (229). In addition, the nsP4-G83L mutation located in the N-terminal region of polymerase could affect negative strand RNA synthesis as well as inhibit host cell gene expression following virus infection. In addition, the identification of second-site mutations in nsP1, nsP2 and nsP3 were shown to be able to correct the initial RNA synthesis defects directly, suggesting close interaction between nsP4 and the other nsPs (22).

CHAPTER 3: MATERIALS AND METHODS

3.1 PHYLOGENETIC ANALYSIS

All CHIKV complete genome sequences (excluding vaccine and cloning vector strains) available as of March 2018 were downloaded from the GenBank database (www.ncbi.nlm.nih.gov/genbank/), assembled and trimmed using Geneious Pro version 8.0 (Biomatters, New Zealand), and manually adjusted according to amino acid sequence alignments to preserve codon homology. Only the ORFs comprising of an alignment of 11,238 nucleotides were used for phylogenetic tree construction. Sequences with missing isolation date, origin or strain name were omitted from the analysis. A total of 115 CHIKV whole genome sequences (88 ECSA strains, 16 Asian strains and 11 West African strains) were aligned using ClustalX version 2 (230) and the best substitution model for the alignment was determined using jModelTest version 2.1.1 (231, 232). A phylogenetic tree was generated with BEAST version 2.4.7 (233), using the settings of a relaxed molecular clock, Bayesian skyline coalescent tree and 100,000,000 chain length, to achieve effective sample sizes above 200 for all parameters. Traces were viewed with Tracer version 1.6. The tree was viewed and analysed using FigTree version 1.4.2 (234).

3.2 CELL CULTURE

Baby hamster kidney cells (BHK-21; ATCC no. CCL-10) were grown in Glasgow's minimal essential medium (GMEM; Gibco, USA) with 5% foetal bovine serum (FBS; Bovogen, Australia), 1% penicillin/streptomycin (P/S; Gibco, USA), 2 mM L-glutamine (Gibco, USA), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco, USA) and 10% of tryptose phosphate broth (TPB; Sigma, USA) at 37ºC with 5% CO_2. African green monkey kidney cells (Vero; ATCC no. CCL-81) and

rhabdomyosarcoma cells (RD; ATCC no. CCL-136) were grown in Dulbecco's minimal essential medium (DMEM; Sigma, USA) with 10% FBS, 1% P/S and 2 mM L-glutamine at 37ºC with 5% CO_2. Human embryonic kidney cells (HEK-293T; ATCC no. CRL-1573) was maintained in similar media as Vero and RD cells with the addition of 1% sodium pyruvate (Thermo Fisher Scientific, US). *Ae. albopictus* C6/36 cells (ATCC no. CRL-1660) were grown in Leibovitz's L-15 medium (Gibco, USA) with 10% FBS, 1% P/S and 1% TPB at 28ºC without CO_2. *Ae. albopictus* U4.4 cells (235), provided by Professor John Fazakerley of the University of Edinburgh, were maintained in a similar condition as C6/36 cells.

3.3 *AEDES ALBOPICTUS* MOSQUITOES

The mosquito species used in this study was *Ae. albopictus* (Seputeh strain), collected in the field in Taman Seputeh, Kuala Lumpur and maintained in the insectarium in the Department of Parasitology, Faculty of Medicine, University of Malaya by Prof. Dr. Indra Vythilingam and Associate Professor Dr. Wan Yusoff Wan Sulaiman. Adult mosquitoes were maintained at 28ºC ± 1ºC and 80 ± 10% humidity with a 12 hr:12 hr photoperiod and fed with 10% sucrose solution supplemented with vitamin B complex.

3.4 CONSTRUCTION OF CHIKV INFECTIOUS CLONES

The CHIKV strain (SGP011) used in this study contains nsP4-82S and was isolated in Singapore during an outbreak in 2008 (236). The strain was from the Indian lineage of the ECSA genotype, and clusters with contemporaneous Malaysian isolates. The infectious clones (Figure 3.1) encoding full-length CHIKV (nsP4-82S), and those expressing ZsGreen and mCherry fluorescent markers (nsP4-82S-ZsG and nsP4-82S-mCh) were constructed by Aleksei Lulla from the University of Tartu, Estonia as

previously described (237). The ZsGreen and mCherry fluorescent genes are expressed 5' to the structural genes from the viral subgenomic promoter while the structural proteins are expressed from a duplicate subgenomic promoter (238). The wild-type infectious clones (nsP4-82R, nsP4-82R-ZsG and nsP4-82R-mCh) containing the amino acid arginine at position 82 of nsP4 was constructed for this study.

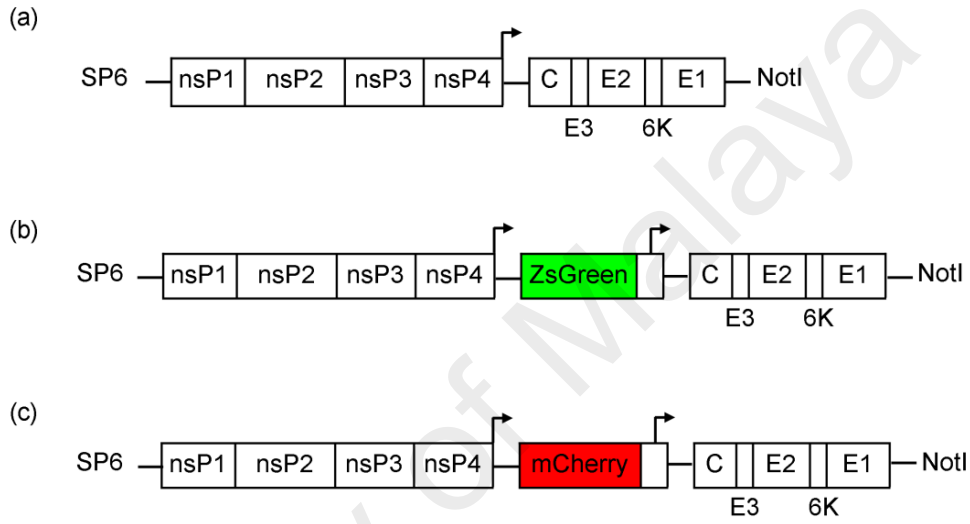


Figure 3.1: Genome structures of CHIKV infectious clones. The infectious clones encoding full-length CHIKV (a), and those expressing ZsGreen (b) and mCherry (c) fluorescent markers were presented.

3.4.1 CHIKV NSP4 AMPLIFICATION

Due to the large size of CHIKV infectious clones (approximately 14 kbp), the region surrounding nsP4-82 was cloned into a smaller cloning vector pGEM-T (Promega, USA) for ease of downstream site-directed mutagenesis work. Conventional PCR was used to amplify a 3,908 base pair (bp) region corresponding to nucleotides 5,644-9,551 of the nsP4-82S infectious clone. The PCR mixture contained 0.5 µM forward primer (5'-ATGACTAGACAGGGCAGGTGG-3'), 0.5 µM reverse primer (5'-TTATACGGCTCGTTGTTGCCC-3'), 200 µM dNTP (Thermo Fisher, USA), 0.01 U

Q5 High-Fidelity DNA polymerase (NEB, USA), 1X Q5 Reaction buffer (NEB, USA), 1 ng of nsP4-82S infectious clone template and nuclease-free water to a final volume of 50 µl. Applied Biosystems Veriti Thermal Cycler was used to perform the polymerase chain reaction (PCR) reaction with initial denaturation at 98ºC for 30 seconds, 20 cycles of 98ºC for 10 seconds, 69ºC for 30 seconds and 72ºC for 4 minutes, followed by final extension at 72ºC for 2 minutes. For nsP4-82S-ZsG and nsP4-82S-mCh infectious clones, 4,768 bp and 4,783 bp fragments corresponding to nucleotides 5,644-10,411 and 5,644-10,426, respectively, were amplified in a similar manner using the same set of primers.

3.4.2 GEL PURIFICATION

Each DNA amplicon was gel purified using Expin Combo GP kit (GeneAll, Korea) according to the manufacturer's protocol. Briefly, the PCR products were electrophoresed in a 0.8% agarose gel and the desired DNA fragment was excised from the gel using a scalpel and placed into a 1.5 ml tube. The gel was weighed and GB buffer was added to a 3:1 ratio. The gel mixture was incubated at 50ºC for 10 minutes to dissolve the gel and the mixture was transferred to the provided SV column and centrifuged for 1 minute at 17,000 × g. NW buffer was added to wash the column and the purified DNA was eluted out from the column using 40 µl of nuclease-free water and its concentration was measured using Epoch Microplate Spectrophotometer (BioTek, USA). The amplicons were ready to be cloned into pGEM-T vector.

3.4.3 LIGATION INTO pGEM-T VECTOR

An A overhang was first added to the 3' blunt end of the DNA amplicon to facilitate TA cloning into the pGEM-T vector which contains a T overhang at the insertion site. The amplicon was mixed with 5 U of MyTaq polymerase and 20 U of reaction buffer

(Bioline, UK), and incubated at 70ºC for 5 minutes. The mixture was then purified using DNA Clean & Concentrator-5 kit (Zymo, USA) according to the protocol provided. In short, 200 µl of DNA Binding Buffer was mixed with 40 µl of amplicon mixture and transferred to a Zymo-Spin column and centrifuged at 11,000 × g for 30 seconds. The flow-through was discarded and DNA Wash Buffer was added to the column which was then centrifuged for 30 seconds. To elute the amplicon, 10 µl of nuclease-free water was added into the column which was then centrifuged for 30 seconds.

To insert the amplicon with A overhang into the pGEM-T vector, the purified amplicon was mixed with 50 ng of pGEM-T vector, 400 U of T4 DNA ligase and 1X T4 DNA ligase buffer (NEB, USA), and incubated at room temperature for 1 hour. The ligation mixture was then transformed into competent bacteria for plasmid amplification.

3.4.4 TRANSFORMATION (BLUE-WHITE SCREENING)

Blue-white screening was used to identify plasmids (termed as nsP4-pGEM-T) with positive amplicon insertion through colour selection. XL10-Gold (Agilent, Australia) was mixed with 5 µl of ligation mixture and incubated on ice for 30 minutes. The mixture was heat-pulsed for 30 seconds at 42ºC and placed on ice for at least 2 minutes. The mixture was supplemented with 200 µl of LB broth (Fisher Scientific, US) and incubated in a growth chamber at 37ºC with shaking at 220 rpm. After 1 hour of incubation, the mixture was spread on an agar plate containing 5 mg of ampicillin (Merck Millipore, USA), 10 µM of isopropyl β-D-1-thiogalactopyranoside (IPTG; Vivantis, USA) and 2.5 mg of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal; Vivantis, USA). The agar plate was then incubated for 16 hours at 37ºC. White colonies

were selected over blue colonies and grown in 5 ml of LB medium containing 0.5 mg of ampicillin for 16 hours at 37ºC with shaking at 220 rpm.

3.4.5 PLASMID EXTRACTION

The nsP4-pGEM-T plasmid was extracted from the bacteria culture using Hybrid-Q Plasmid Rapidprep (GeneAll, Korea) according to the standard protocol. Briefly, the bacteria culture was centrifuged at 10,000 × g for 10 minutes and the supernatant was discarded. S1, S2 and G3 buffer were added to the pellet and the mixture centrifuged at 14,000 × g for 10 minutes. The supernatant was transferred to a spin column and centrifuged for 30 seconds. PW buffer was added to wash the column. The plasmid was eluted out from the column using 50 µl of nuclease-free water and its concentration was measured. The plasmid was sequenced using SP6 promoter primer (5'-ATTTAGGTGACACTATAG-3') to ensure the correct plasmid was selected for downstream site-directed mutagenesis work.

3.4.6 SITE-DIRECTED MUTAGENESIS BY PCR

Point mutation T→A at nucleotide position 3,284 (amino acid S→R at nsP4-82) was introduced into nsP4-pGEM-T plasmid by PCR using 0.5 µM forward primer (5'-AGGCTGTAGACTATACTTAAGTCAGAGACCC-'3), 0.5 µM reverse primer (5'-CTCTTTAGTCTCTGGATGATTGCTGC-3'), 200 µM dNTP, 0.01 U Q5 High-Fidelity DNA polymerase, 1X Q5 Reaction buffer, 1 ng of nsP4-pGEM-T plasmid and nuclease-free water to a final volume of 25 µl. The PCR parameters were as follows: 98ºC for 30 seconds, 20 cycles of 98ºC for 10 seconds, 69ºC for 30 seconds and 72ºC for 4 minutes, followed by final extension at 72ºC for 2 minutes.

The PCR product containing the nsP4-82R was mixed with 5 U T4 polynucleotide kinase, 400 U T4 DNA ligase, 1X T4 DNA ligase buffer and 10 U *DpnI* for rapid

recircularization of the linear PCR product and removal of the template DNA. The mixture was incubated at room temperature for 1 hour and 10 µl of the mixture was transformed into XL10-Gold following the protocols stated in section 3.4.4. The transformed mixture was spread on an agar plate containing 5 mg of ampicillin. Positive colonies were picked and grown in LB broth containing 5 mg of ampicillin overnight at 37ºC, and plasmid extraction was carried out using Hybrid-Q Plasmid Rapidprep following the protocols stated in section 3.4.5.

3.4.7 CLONING OF CHIKV NSP4-82R INTO INFECTIOUS CLONES

The fragment encompassing the nsP4-82R in the nsP4-pGEM-T plasmid was cloned back into the CHIKV infectious clone using RE digestion and ligation. One microliter of FastDigest *AgeI*, FastDigest *XhoI* and 10X FastDigest buffer (Thermo Scientific, USA) was added to 1 µg of nsP4-pGEM-T plasmid (Figure 3.2a) and CHIKV nsP4-82S infectious clone (Figure 3.2b), which was then incubated for 20 minutes at 37ºC. The RE-digested nsP4-82S infectious clone (termed CHIKV infectious clone backbone) was additionally incubated with 1 U FastAP thermosensitive alkaline phosphatase (Thermo Scientific, USA) for another 10 minutes at 37ºC to prevent recircularization of plasmid during downstream ligation. The nsP4-82R fragment and CHIKV infectious clone backbone were gel purified following protocols mentioned in section 3.4.2. A ligation mixture containing 5 U T4 PNK, 400 U T4 DNA ligase, 1X T4 DNA ligase buffer, 100 ng of nsP4-82R fragment and 10 ng of CHIKV infectious clone backbone was prepared and incubated at room temperature for 2 hours (Figure 3.2c).

As high yields of plasmids were needed for downstream plasmid linearization work, the ligation mixtures (nsP4-82R, nsP4-82R-sG and nsP4-82R-mCh) and existing infectious clones (nsP4-82S, nsP4-82S-ZsG and nsP4-82S-mCh) were transformed into XL10-Gold according to the protocol stated in section 3.4.6. The PureLink HiPure

Plasmid Miniprep Kit (ThermoFisher Scientific, USA) was used to extract the plasmids according to the manufacturer's manual. To ensure no new mutation was introduced during PCR, the nsP4-82 fragment was fully sequenced using the primers shown in Table 3.1.

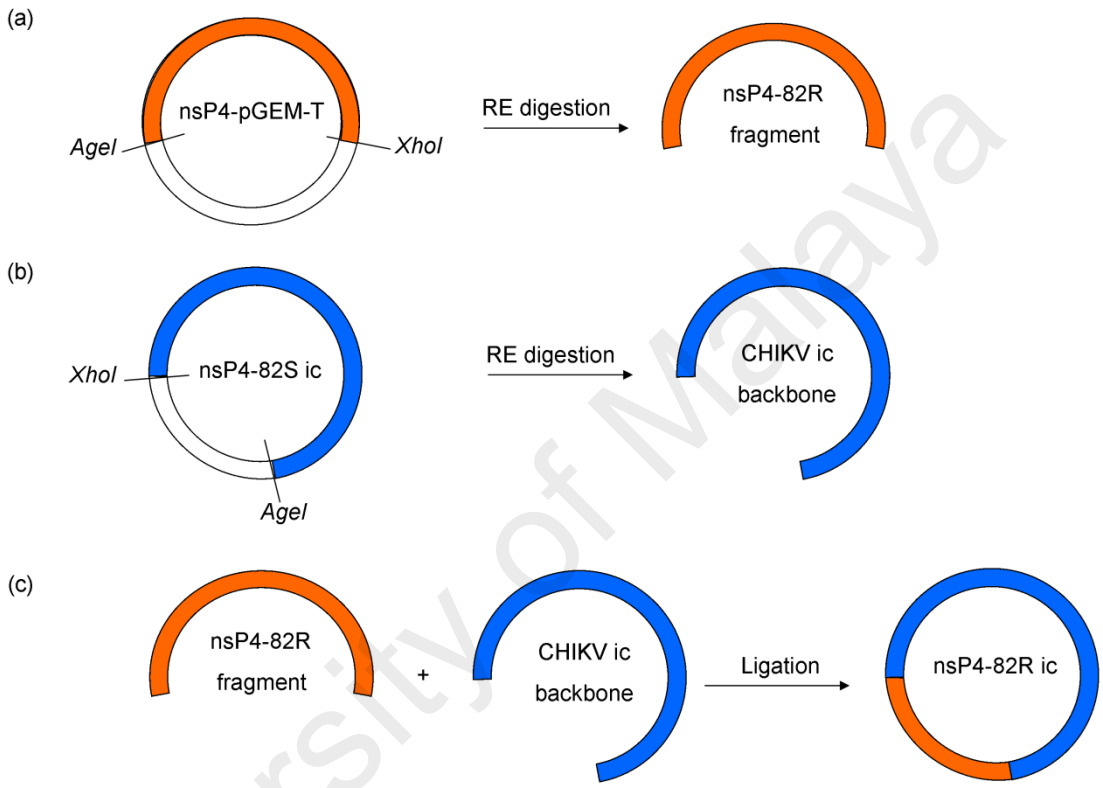


Figure 3.2: Ligation of CHIKV nsP4-82R fragment into the CHIKV infectious clone backbone. The nsP4-pGEM-T plasmid containing the nsP4-82R was RE digested to yield the nsP4-82R fragment (a), while the nsP4-82S infectious clone was similarly RE digested and gel purified to obtain the CHIKV infectious clone backbone without the nsP4-82S region (b). The nsP4-82R gene fragment was ligated into the CHIKV infectious clone backbone to obtain the nsP4-82R infectious clone (c).

Table 3.1: Primer list for sequencing of nsP4-82 fragment.

Primers	Primer sequence (5' to 3')
ECSA 12F	ATCAGAAGCGCTGTACCGTC
ECSA 13F	TCATAGCCGCACACTTTAAGC
ECSA 14F	AGATGGCAACGAACAGGGC
ECSA 15F	TCGAAGTCAAGCACGAAGG
ECSA 16F	TGCTTGAGGACAACGTCATGAG
ECSA 17F	AGTCCGGCAACGTAAAGATCAC
ZsGreen F	AAGGGCGACGTGAGCATGTACC
mCherry F	AAGGGCGAGATCAAGCAGAGG

3.5 GENERATION OF LIVE VIRUS STOCK

3.5.1 PLASMID LINEARIZATION AND *IN VITRO* TRANSCRIPTION

Approximately 6 µg of infectious clone plasmids were incubated with 1 µl of FastDigest *NotI* and 10X FastDigest buffer (Thermo Fisher Scientific) at 37ºC for 2 hours. The linearized plasmids were purified using DNA Clean & Concentrator-5 kit (Zymo, USA) following protocols stated in section 3.4.3 and quantitated. The complete linearization of plasmids was confirmed by gel electrophoresis.

The linearized plasmids were subjected to *in vitro* synthesis of RNA using mMESSAGE mMACHINE Kits (Ambion, USA). The transcription reaction consisted of 2X NTP/CAP, 1 µl of enzyme mix, 10X Reaction buffer and 300 ng of linearized plasmids and incubated at room temperature for 2 hours. One microliter of DNase was added into the mixture and incubated for another 15 minutes at 37ºC to remove any traces of template DNA. The integrity and concentration of RNA were determined using

Epoch Microplate Spectrophotometer before proceeding to electroporation of CHIKV RNA into BHK-21 cells.

3.5.2 ELECTRPORATION

BHK-21 cells were seeded in a 75 cm^2 tissue culture flask at a density of 4×10^6 cells and incubated at 37ºC with 5% CO_2 for 24 hours prior to electroporation. The BHK-21 cells were first detached from the flask surface using 1.5 ml of 0.05% trypsin and incubated for 5 minutes at 37ºC. The cell suspension was neutralised with 5 ml of 5% FBS GMEM and centrifuged at 400 × g for 5 minutes. The cell pellet was then washed and resuspended in 800 µl of ice-cold Dulbecco's phosphate buffer saline (DPBS; Gibco, USA). The cell suspension was transferred to a pre-chilled 4mm gap electroporation cuvette (Bio-Rad, USA) and 5 µg of RNA was added. The cuvette was placed in the electroporation chamber of GenePulser-Xcell (Bio-Rad, USA) and pulsed twice with a square wave protocol with the following settings: 3 seconds pulse interval at 240V and 25 msec time constant after each pulse. After electroporation, 800 µl of 5% FBS GMEM was immediately added into the cuvette and incubated for 5 minutes at room temperature. The cells were then transferred to a 75 cm^2 cell culture flask filled with 5% FBS GMEM and incubated at 37ºC with 5% CO_2. The virus-cell supernatant was collected at 24 hours post-electroporation, centrifuged at 4,500 × g for 10 minutes to remove cell debris and the supernatant was split into several aliquots to be stored at -80ºC for downstream experiments. Pictures were taken for BHK-21 cells electroporated with fluorescent infectious clones using an Eclipse TE2000 Inverted Microscope (Nikon, USA) to confirm positive expression of fluorescent proteins.

3.6 PLAQUE ASSAY

Plaque assay is used to quantitate the electroporated virus titres. Approximately 6×10^5 BHK-21 cells were seeded onto a 6-well plate and incubated for 24 hours at 37ºC with 5% CO_2. The virus stock was fast-thawed at 37ºC and serially diluted 10-fold in minimum essential media (MEM; Gibco, USA). Growth media was first removed from the BHK-21 cells and replaced with 0.4 ml of the diluted virus. The plate was rocked gently at room temperature for 1 hour to allow virus attachment onto cell surface. The virus inoculum was then removed and replenished with 2 ml of plaque medium made from 3 parts 2% FBS GMEM and 2 parts carboxymethylcellulose sodium salt (CMC; Sigma, USA) dissolved in Milli-Q water. The plate was incubated at 37ºC with 5% CO_2 for 48 hours before the plaque medium was removed. The cells were then fixed with 3.7% paraformaldehyde and stained with crystal violet (Merck, Germany). Plaques were enumerated and used to calculate the plaque forming units per millilitre (pfu/ml) of the virus sample using the formula:

$$\frac{\text{Number of plaques}}{\text{Volume of virus inoculum}} \times \text{Dilution factor} = \text{pfu/ml}$$

3.7 CHIKV REPLICATION KINETIC IN CELL LINES

Since all four fluorescent viruses produced similar viral titre post-electroporation, the nsP4-82S-mCh and nsP4-82R-ZsG were selected for downstream experiments. Replication kinetics of the nsP4-82S, nsP4-82R, nsP4-82S-mCh and nsP4-82R-ZsG viruses were compared in C6/36, U4.4, RD, HEK-293T and Vero cells. Approximately 1×10^5 C6/36 and U4.4 cells, and 5×10^5 RD, HEK293T and Vero cells per well were grown in 24-well plates overnight before infection. The cell monolayers were infected with the viruses at multiplicity of infection (MOI) of 0.1 for C6/36, U4.4 cells, RD, HEK293T and Vero cells, and incubated for 1 hour at room temperature with gentle

rocking. The cells were washed twice with DPBS and replenished with 2% L-15 for C6/36 and U4.4 cells, and 2% FBS DMEM for RD, HEK-293T and Vero cells. Virus supernatant was collected from individual wells at 0, 8, 12, 24, 48, 72 and 96 hours post-infection (hpi) and titrated by plaque assay as described in section 3.6. Two biological replicates were performed for each series. Viral growth curves were generated for each cell line.

3.8 CHIKV GROWTH KINETICS IN *AE. ALBOPICTUS*

The nsP4-82S and nsP4-82R viruses were evaluated for their ability to infect, disseminate and transmit in *Ae. albopictus*. The mosquitoes were exposed to infectious blood meal and incubated until selected time points when they were sacrificed. Midguts, salivary glands and saliva were collected and analysed to determine the infection rate, dissemination rate and transmission rate, respectively.

3.8.1 INFECTIOUS BLOOD MEAL

Fifty female mosquitoes aged 5-7 days old were first segregated into each paper cup specifically designed to feed and keep the mosquitoes. The viruses were diluted to a working titre of 10^6 pfu/ml and mixed 1:10 with fresh blood obtained from one of the research members who is seronegative for CHIKV antibodies. The virus-blood mixture was pre-warmed and maintained at 37ºC during the whole feeding duration, and placed on top of the netting of the cup to allow access by the mosquitoes. The blood meal was fed to the mosquitoes in the dark for 1 hour using an artificial collagen membrane attached to a Hemotek meal reservoir (Discovery Workshops, Accrington, UK).

After feeding, the mosquitoes were snap frozen for 30 seconds and ten engorged mosquitoes were transferred to each new cup. The cups housing the infected mosquitoes were stored in a translucent plastic container and subsequently placed in a secured

environmental chamber under the conditions previously mentioned in section 3.3. For the first 24 hours of incubation, a cotton ball dampened with distilled water was placed on top of the net to keep the mosquitoes hydrated. The cotton ball was replaced with a new cotton ball dampened with sucrose supplemented with vitamin B daily. Dead mosquitoes were removed from the cup daily to prevent contamination.

3.8.2 ORGAN HARVESTING

The nsP4-82S- and nsP4-82R-infected mosquitoes were collected at 3, 5 and 7 days post-infection (dpi). At each time-point, 20 mosquitoes were immobilized by snap freezing and had their legs and wings removed to prevent them from escaping. The mosquito's proboscis was inserted into a 10 µl pipette tip containing 10 µl of FBS with 10% sucrose and the mosquito was allowed to salivate for 1 hour. The saliva was then transferred into a PCR tube filled with 40 µl of MEM. Mosquitoes were dissected using dissecting needles under a stereoscopic microscope. Midguts and salivary glands were harvested and placed individually into a 1.5 ml zirconium beads tube (Benchmark Scientific, USA) prefilled with 500 µl of MEM supplemented with 2% amphotericin B. The organs were then homogenized at 4,000 rpm for 15 seconds using a microtube homogenizer (Benchmark Scientific, USA). The homogenate was transferred to a new microcentrifuge tube and stored at -80ºC for further analysis.

3.8.3 CYTOPATHIC EFFECT ASSAY

Cytopathic effect (CPE) assay was used to determine the presence of infectious virus in the mosquito midguts and salivary glands. BHK-21 cells were seeded into a 96-well plate the day before infection. At 80% confluence, 100 µl of mosquito homogenates were added to the cells in duplicate and incubated at 37ºC for 1 hour. The homogenates were then removed and the cells were washed twice with DPBS. Then, 2% FBS GMEM

supplemented with 2% amphotericin B was added to the cells, which were then incubated at 37ºC with 5% CO_2. The plate was observed daily for 3 days and cells with 80% CPE were considered positive for the presence of infectious virus. The saliva samples were titrated on BHK-21 cells using the plaque assay mentioned in section 3.6.

The infection rate was calculated based on number of positive midguts over total number of midguts tested. The dissemination rate was determined based on the number of positive salivary glands over the number of positive midguts. Finally, the transmission rate was determined based on the number of positive saliva over the number of positive salivary glands.

3.9 NEXT-GENERATION SEQUENCING

Mosquito homogenates at 7 dpi obtained from the growth kinetic assay (section 3.8.2) were deep sequenced to estimate population diversity of variants in *Ae. albopictus*. Three groups of samples were included in the process: electroporated virus stocks (as controls), midgut homogenates and salivary gland homogenates. Five samples were pooled together to increase sample number and two biological replicates were included for each group except control.

3.9.1 RNA EXTRACTION

Viral RNA was extracted from the homogenates using QiAmp Viral RNA Mini kit (QIAGEN, Germany) according to manufacturer's instructions. Briefly, 140 µl of homogenate was added to AVL buffer with carrier RNA and incubated for 10 minutes. Ethanol was added and the mixture loaded into a QIAamp Mini column and centrifuged at 6,000 × g for 1 minute. The column was rinsed with AW1 and AW2 buffer, followed by elution using 40 µl of nuclease-free water. The RNA was stored at -80ºC.

3.9.2 REVERSE TRANSCRIPTION AND PCR AMPLIFICATION

RNA pools were generated by mixing 2 µl of each sample RNA (5 midguts/pool and 5 salivary glands/pool) into a new tube. Five sets of primers (Table 3.2) were designed to amplify 5 overlapping fragments of the CHIKV full genome (excluding 5' and 3' terminal) with each fragment size ranging between 2,482 to 3,047 bp with 505-833 bp of overlapping regions. The RNA was reverse transcribed using 0.2 µM reverse primer (Table 3.2), 1 mM dNTP, 1 µl of pooled RNA and nuclease-free water to a final volume of 13 µl. The mixture was incubated at 65ºC for 5 minutes and placed on ice for 5 minutes. Then, 200 U Superscript III, 40 U RNAse OUT, 5 mM DTT and 5X First Strand Buffer (Invitrogen, USA) was added to the reaction mixture to a total volume of 20 µl. The mixture was incubated at 25ºC for 5 minutes, 50ºC for 60 minutes and 70ºC for 15 minutes.

The synthesised cDNA was subsequently PCR-amplified by adding 0.5 µM of forward and reverse primer (Table 3.2), 200 µM dNTP, 0.01 U Q5 High-Fidelity DNA polymerase, 1X Q5 Reaction buffer, 2.5 µl of cDNA and nuclease-free water in a 25 µl mixture. The PCR reaction was performed at 98ºC for 30 seconds, followed by 35 cycles of 98ºC for 10 seconds, annealing temperature (Table 3.2) for 30 seconds, 72ºC for 90 seconds, followed by final extension at 72ºC for 2 minutes. The PCR product was gel purified using Expin Mini Spin Purification Kit according to the protocol described in section 3.4.2. The amplified PCR amplicons was used for downstream deep sequencing.

Table 3.2: Primer sequences used to amplify CHIKV genome for deep sequencing.

CHIKV fragment	Primers	Primer sequence (5' – 3')	Annealing Temperature (ºC)
1	FP1	ATGGATCCTGTGTACGTGG	65
	RP1	TTGGGATGCGGCTGC	
2	FP2	TTGCGATGCACGGACC	62
	RP2	TTCCCTTGGACTTACGC	
3	FP3	ATCTGAGGCCATACAGATGCG	62
	RP3	TATCGTGCAGTATAAACCCTCC	
4	FP4	AGAGAGCTGGTTAGGAGGC	62
	RP4	TTACATTTGCCAGCGGAAACGG	
5	FP5	ATGGGACACTTCATCCTGGC	62
	RP5	TTAGTGCCTGCTGAACG	

3.9.3 NORMALIZATION OF AMPLICONS

The integrity and concentration of the amplicons were measured using Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, USA) following the protocols provided. The amplicon was diluted 10-fold and 2 µl of the diluted DNA was added to 198 µl of working solution. The tube was incubated at room temperature for 2 min. Standard A and Standard B were used to calibrate the Qubit 2.0 fluorometer (Thermo Fisher, USA) before the amplicon was measured. The concentration of the amplicon was determined using the fluorometer and the following formula:

$$\text{Concentration of sample} = \text{QF value} \times \frac{200}{x}$$

Where QF value = the value given by the Qubit 2.0 fluorometer

x = the number of microliters of sample added to the assay tube

All amplicons were subsequently standardized to 1 ng/µl using nuclease-free water. Four microliters of each amplicon were added to a single tube and the pooled amplicon concentration was measured using the Qubit 2.0 fluorometer. Using nuclease-free water, the pooled amplicon was diluted to a final concentration of 0.2 ng/µl.

3.9.4 TAGMENTATION OF AMPLICON

Nextera XT DNA Library Preparation Kit (Illumina, USA) was used to perform tagmentation of amplicons, amplification and cleaning up of libraries according to manufacturer's instructions. During tagmentation, transposase and adapter sequences were added to the amplicons to prepare the DNA for amplification. Briefly, 5 µl of amplicon tagment mix (ATM), 10 µl of tagment DNA buffer (TD) and 5 µl of amplicon (0.2 ng/µl) were added into a tube, and centrifuged at 280 × g at 20ºC for 1 minute. The mixture was incubated at 55ºC for 5 minutes and maintained at 10ºC until 5 µl of neutralise tagment buffer (NT) was added. The mixture was then centrifuged at 280 × g at 20ºC for 1 minute, and incubated at room temperature for 5 minutes.

The tagmented amplicon was amplified using Index 1 (i7), Index 2 (i5), and full adapter sequences. The indexes were arranged such that i7 was placed horizontally (column) while i5 was placed vertically (row). All positions were recorded and inserted into the lab tracking record. Then, 5 µl i7, 5 µl i5, and 15 µl Nextera PCR mastermix (NPM) was added to the tube with tagmented DNA. The mixture was centrifuged at 280 × g at 20ºC for 1 minute and then placed in a thermal cycler with the following parameters: 72ºC for 3 minutes, 95ºC for 30 seconds, 12 cycles of 95ºC for 10 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds, followed by 72ºC for 5 minutes and hold at 10ºC.

3.9.5 LIBRARY CLEAN UP

The PCR product was transferred into a new tube and the library was purified by adding 30 µl of AMPure XP beads into the tube and incubated at room temperature for 5 minutes before being placed onto a magnetic stand for approximately 2 minutes until the liquid turned clear. The supernatant was discarded while the beads were washed twice with 80% ethanol. The beads were air-dried for 15 minutes and resuspended in 52.5 µl of Resuspension Buffer (RSB). After 2 minutes of incubation at room temperature, the tube was placed onto the magnetic stand again until the liquid turned clear. The supernatant containing the purified library were transferred into a new tube and stored at -20ºC.

The size and quality of the purified libraries were determined using High Sensitivity DNA Analysis Kit (Agilent, USA) outsourced to Science Vision Sdn. Bhd. The average fragment size should be within 300 bp to 1,000 bp. The concentration of the libraries was checked with Qubit dsDNA High Sensitivity Assay Kit following protocols mentioned in section 3.9.3.

All libraries were adjusted to 2 nM using nuclease-free water and 5 µl of each library were pooled together in a single tube. The 2 nM pooled library was confirmed using Qubit dsDNA High Sensitivity Assay Kit. To denature the pooled library, 1 µl of 1N sodium hydroxide was incubated with 9 µl of the library for 5 minutes, followed by the addition of pre-chilled HT1 reagent to produce an 18 pM denatured library. The library was further diluted to 10 pM using HT1 to a final volume of 700 µl. The denatured library was spiked with PhiX Control at 5% total volume and then added to a 300-cycles cartridge of MiSeq Reagent Kit v2 (Illumina).

Illumina Experiment Manager Software version 1.12.0 was used to produce the sample sheet to control the Illumina Miseq run settings. CLC Genomics Workbench

version 8.0 (QIAGEN Bioinformatics, USA) was used to analyse the sequence reads. A quality score of Q30 was set as the cut-off to trim the sequence reads and each sample run sequence was mapped against the reference genes of nsP4-82S and nsP4-82R. Once the alignments were done, the emergence of new non-synonymous amino acid was recorded and changes in variants frequency at each nucleotide position against the virus stocks were calculated by chi-square analysis. Total non-synonymous amino acids variants excluding deletions and insertions were also calculated to determine the mutation frequencies.

3.10 COMPETITION ASSAYS IN *AE. ALBOPICTUS*

To determine whether the nsP4-R82S mutation provides a competitive advantage in *Ae. albopictus*, competition assays were designed such that the nsP4-82S and nsP4-82R viruses were competed against each other in mosquitoes. The midgut and salivary gland (also represented as head) were sampled to determine the infection and dissemination competency of the viruses. The relative fitness of the viruses was analysed using two methods, a real-time PCR assay for nsP4-82S:nsP4-82R infection and fluorescence detection for nsP4-82S-mCherry:nsP4-82R-ZsGreen infection.

3.10.1 REAL-TIME PCR ASSAY STANDARD CURVE

A multi-probe real-time PCR assay was designed to detect the infection status of nsP4-82S and nsP4-82R in a co-infected mosquito. A short RNA template was prepared to generate standard curves for nsP4-82S and nsP4-82R. Both nsP4-82S and nsP4-82R viral RNA were first reverse transcribed into cDNA according to protocols stated in section 3.9.2. Instead of specific reverse primer, 129 ng of random primers (Promega, USA) and 1 µl of RNA were used. The nsP4 fragment was amplified using 0.5 µM CV forward primer (5'-GCCAACAGAAGCAGGTATCA-3'), 0.5 µM CV reverse primer

(5'-TAGTCCGGTAAGTAGGGACTTT-3'), 200 µM dNTP, 0.01 U Q5 High-Fidelity DNA, 1X Q5 Reaction buffer, 5 µl cDNA and nuclease-free water. The PCR was performed with initial denaturation of 30 seconds at 98ºC, 20 cycles of 98ºC for 10 seconds, 65ºC for 30 seconds and 72ºC for 30 seconds, followed by final extension at 72ºC for 2 minutes. The PCR products were confirmed by gel electrophoresis.

The nsP4 fragment was incorporated into pJET1.2/blunt vector (Thermo Scientific, USA) downstream of a T7 promoter, which is necessary for *in vitro* transcription. The ligation mixture consisted of 0.5 µl non-purified PCR product, 0.5 µl T4 DNA ligase, 2X reaction buffer, 25 ng/µl of pJET1.2/blunt cloning vector and nuclease-free water. The mixture was incubated at room temperature for 5 minutes and transformed into XL10-Gold following protocols stated in section 3.4.6. The plasmid was then sequenced using T7 promoter primer (5´-TAATACGACTCACTATAGGG-3´) to confirm correct orientation of the inserted fragment.

The nsP4 fragment with the T7 promoter was PCR amplified using 0.5 µM T7 promoter primer, 0.5 µM CV reverse primer, 200 µM dNTP, 0.01 U Q5 High-Fidelity DNA polymerase, 1X Q5 Reaction buffer, 10 ng of plasmid and nuclease-free water in a 50 µl reaction volume. The PCR was performed with initial denaturation of 30 seconds at 98ºC, 20 cycles of 98ºC for 10 seconds, 54ºC for 30 seconds and 72ºC for 30 seconds, followed by final extension at 72ºC for 2 minutes. The nsP4 fragment was confirmed by gel electrophoresis.

The nsP4 fragment was *in vitro* transcribed using MEGAshortscript Kit (Ambion, USA) and purified using MEGAclear Kit (Ambion, USA) following the standard protocol provided. To maximize RNA recovery, the elution procedure was repeated with a second pre-heated 50 µl of Elution Solution and the eluate was collected in the same elution tube. The integrity of the RNA was confirmed by adding Gel Loading

Buffer II (Ambion, USA) to RNA eluate in a ratio of 2:1 and performing gel electrophoresis. The RNA concentration was quantitated and converted to viral copy number using the NEBioCalculator tool provided online (https://nebiocalculator.neb.com/#!/ssrnaamt). The RNA was diluted to 10^{11} copies/µl using nuclease-free water and divided into small aliquots of 10 µl to be stored in -80ºC for downstream work.

3.10.2 REAL-TIME PCR ASSAY VALIDATION

Single-probe and double-probe real-time PCR assay were performed on ABI Step One Plus (ABI, USA). Primers and mutation-specific probes were designed and synthesised by IDT, Singapore. Two PrimeTime Locked Nucleic Acid (LNA) probes were designed such that the probes hybridised specifically with either nsP4-82S or nsP4-82R, thus allowing a competitive analysis. The probes were labeled with two different fluorescent dyes: HEX for nsP4-82S and FAM for nsP4-82R. The same set of primers (CV forward and CV reverse) were used to amplify the 127 bp of nsP4 so that discrimination of the mutations could be achieved using the probes. To generate standard curves, cDNA was generated from the RNA template using protocol mentioned in section 3.1.10.1. The limit of detection (LOD), limit of quantification (LOQ), amplification efficiency, assay specificity and sensitivity of nsP4-82S-HEX and nsP4-82R-FAM probes were determined.

For single-probe assay, the cDNA was serially diluted 10-fold ranging from 10^7 copies/µl to 1 copy/µl using nuclease-free water to generate a standard curve. The 10 µl mixtures contained 0.9 µM CV forward and CV reverse primer, 0.25 µM 82S-HEX probe (5'-5HEX/AGAGTGGCTG/3IABkFQ-3') or 82R-FAM probe (5'-56-FAM/AGAGAGGCTG/3IABkFQ-3'), 1 µl cDNA template, 2X Taqman Genotyping Master Mix (Thermo Fisher Scientific, USA) and nuclease-free water. To generate

double-probe standard curve, the 10 µl mixtures contain 0.9 µM CV forward and CV reverse primer, 0.25 µM 82S-HEX probe and 82R-FAM probe, 1 µl nsP4-82S and nsP4-82R cDNA template at equal copy number (ranging from 10^7 copies/µl to 10^2 copy/µl), 2X Taqman Genotyping Master Mix and nuclease-free water. Thermal cycling conditions were as follows: 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Standard curves for both nsP4-82S and nsP4-82R were plotted and LOD and LOQ were determined. A Ct-value of 40 was chosen as the cut-off value for standard positivity. The standard curve efficiency was considered acceptable at 100% ± 10% with an R^2 value of 1 ± 0.02. Each sample was run in triplicate.

The cross-reactivity test was designed to determine the specificity of each probe in detecting the one nucleotide difference in a mixed population. The assay was carried out using a series of diluted templates; individual nsP4-82S or nsP4-82R cDNA at 3 log, 5 log and 7 log cDNA copy numbers/reaction were amplified with both nsP4-82S-HEX and nsP4-82R-FAM probes following double-probes assay protocols stated above.

The sensitivity test was designed to determine the minimum difference between nsP4-82S and nsP4-82R cDNA in which the assay could accurately detect. Mixtures of nsP4-82S:nsP4-82R cDNA at different ratios (1:1, 1:4, 1:8, 1:10, 1:100, 1:1000 and vice versa) were prepared and tested using the double-probes assay protocols stated above. Specifically, a 1:1 variants mixture contained 1×10^5 nsP4-82S and 1×10^5 nsP4-82R cDNA copies, while a 1:4 variants mixture contained 1×10^5 nsP4-82S and 4×10^5 nsP4-82R cDNA copies.

3.10.3 MOSQUITO INFECTION

Seven- to eight-day-old female *Ae. albopictus* were orally exposed to a blood meal containing an equal ratio of nsP4-82S:nsP4-82R or nsP4-82S-mCherry:nsP4-82R-ZsGreen (10^5 pfu/ml). The sorting of mosquitoes and feeding procedure followed protocols mentioned in section 3.8.1. Mosquitoes were processed at least 72 hours after blood feeding to ensure minimal damage to midgut due to blood-caused distension and for clearer viewing of midgut epithelia. Mosquitoes were harvested at 3, 7 and 10 dpi.

For nsP4-82S:nsP4-82R infection, the mosquito midgut, head and saliva were collected. The midgut and head were homogenized and viral RNA was extracted following protocols mentioned in section 3.9.1. The viral RNA was reverse transcribed to cDNA and used to run real-time PCR assay. Briefly, the 10 µl mixtures contain 0.9 µM CV forward and CV reverse primer, 0.25 µM 82S-HEX probe and 82R-FAM probe, 1 µl cDNA template, 2X Taqman Genotyping Master Mix and nuclease-free water. Thermal cycling conditions were as follows: 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Each sample was run in triplicates.

For nsP4-82S-mCherry:nsP4-82R-ZsGreen infection, mosquito midguts and salivary glands were dissected and imaged with an Eclipse TE2000 Inverted Microscope (Nikon, USA). Infection of midgut with nsP4-82S-mCherry leads to the expression of red fluorescence while nsP4-82R-ZsGreen yields green fluorescence. At least 2 images were taken to capture the expression of nsP4-82S-mCherry and nsP4-82R-ZsGreen in the same field of vision. The fluorescence intensity was measured and analysed using ImageJ version 1.49 (NIH). The fitness ratio was calculated by dividing the fluorescence intensity of nsP4-82S-mCh with nsP4-82R-ZsG of the same mosquito.

3.11 COMPETITION ASSAYS IN MAMMALIAN CELL LINES

Mixtures of nsP4-82S:nsP4-82R at known ratios (1:1, 10:1 and 1:10) were serially passaged in RD, HEK-293T and Vero cells for a total of 3 passages. An approximate of 1×10^5 of RD cells/well, 2×10^5 of HEK-293T cells/well and 5×10^4 of Vero cells/well were seeded into 24-well plate 24 hours prior to infection. To initiate the first passage series, the cells were infected with mixtures of the variants, in duplicates, at total MOI 0.5 for 1 hour at 37ºC. The cells were rinsed twice with DPBS and supplemented with 2% FBS medium. The cells were then incubated at 37ºC and observed daily for CPE. The virus supernatant from each passage was harvested when 80-100% CPE was observed or at 72 hpi (when CPE was not observed for RD cells), and centrifuged at 4,500 × g for 5 minutes to remove the cell debris. The supernatant was titrated in BHK-21 cells using plaque assay as described in section 3.6. Subsequent passages were initiated by infecting the cells with the previously passaged viruses using the same protocols mentioned above.

Viral RNA was extracted from the virus supernatant and a fragment flanking the nsP4-82 was amplified by RT-PCR. First, the viral cDNA was reverse transcribed by incubating 50 ng of random hexamer, 10 mM of dNTP mix, 2 µl of viral RNA and nuclease-free water at 65ºC for 5 minutes and on ice for 5 minutes. Then, 5X SSIV buffer, 100 mM DTT, 1µl of Ribonuclease Inhibitor and 200 U of Superscript IV (Thermo Fisher, US) was added to the mixture. The cycling parameters were as follows: 23ºC for 10 minutes, 50ºC for 10 minutes and 80ºC for 10 minutes.

The cDNA was then amplified by PCR using a mixture of 10 µM of forward primer (5'-ATCGCGCGTAAGTCCAAGG-3') and reverse primer (5'-TTCCCAGTATTCTTGGTTGCATG-3'), 2 µl of cDNA, 2X Mytaq Mix (Bioline, UK), and nuclease-free water. The cycling parameters included initial denaturation at 95ºC

for 1 minute followed by 30 cycles of 95ºC for 15 seconds, 66ºC for 15 seconds and 72ºC for 10 seconds. The PCR fragment was confirmed by gel analysis and sequenced. Based on the sequencing chromatograms, the codon at nsP4-82 was used to determine the proportion of each of the two competing viruses. Two replicates were performed for each passage series.

3.12 STATISTICAL ANALYSIS

The Mann-Whitney test was used to compare electroporation efficiency of CHIKV infectious clones into BHK-21 cells. CHIKV replication kinetics in cell lines were analysed using two-way ANOVA with Bonferroni post hoc test while Fisher's exact test (two-tailed) was used to analyse CHIKV growth kinetics in *Ae. albopictus*. The chi-square test was used to determine the significance of non-synonymous amino acid frequency change in mosquito samples. For the CHIKV competition assay in *Ae. albopictus*, the Fisher's exact test was performed to compare infection and dissemination rates between nsP4-82S and nsP4-82R. The two-tailed Wilcoxon matched-pairs signed rank test was used to compare the fluorescence intensity of nsP4-82S-mCherry and nsP4-82R-ZsGreen in co-infected mosquitoes. P-values$\leq$0.05 was considered statistically significant and all statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, USA).

CHAPTER 4: RESULTS

4.1 EMERGENCE OF NSP4-82S INDEPENDENT CLADE

A total of 115 CHIKV whole genomes available in GenBank (Appendix A) were included in the phylogenetic analysis. The phylogenetic tree (Figure 4.1) displayed three distinct CHIKV clades: ECSA (88 sequences), Asian (16 sequences) and West African (11 sequences). In 2004, two new ECSA sublineages carrying E1-226A emerged independently from Kenya; the IOL spread into the Indian Ocean basin, while the IL spread into India. The tree shows the E1-A226V mutation then arose independently in each of the two epidemic ECSA sublineages, in La Réunion (2005) and India (2007). From La Réunion, the E1-226V strain spread to France, Mauritius, USA, Madagascar and Mayotte in 2006. India (represented by the basal sequence FJ000069) appears to be the origin of CHIKV that spread to Southeast Asia, resulting in the Southeast Asian sublineage (SAL). The initial strains of the SAL sublineage in Malaysia carried nsP4-82R, before the emergence of the nsP4-82S strain in Malaysia or Singapore in 2008, giving rise to a strongly supported nsP4-R82S sublineage with a posterior probability value of 1.0.

The chronological and geographical distribution of the nsP4-R82S mutation across Southeast Asia is depicted in Figure 4.2. CHIKV sequences of the putative Indian source carried nsP4-82R. This variant was detected at the beginning of the Southeast Asian outbreak in southern Malaysia in 4 out of 21 reported sequences before April 2008, and was later replaced by nsP4-82S (17/21 sequences) from August 2008 onwards. CHIKV spread south into neighbouring Singapore, where the first isolate in May 2008 carried nsP4-82S. In Thailand, CHIKV strains with nsP4-82R and genetically related to CHIKV from Malaysia were first reported in October 2008. The nsP4-82S

(n=1/4) was detected later in December 2008 and became predominant (n=6/7) in 2009, and remained detectable (n=3/4) in 2013. In China, nsP4-82R (n=2/2) was detected in 2008 in travellers to Malaysia (102) but in 2010, the only sequenced isolate has nsP4-82S. All isolates sequenced in Indonesia (n=1) and Myanmar (n=4) in 2010 and Cambodia (n=7) in 2012 had nsP4-82S. Overall, the phylogenetic analysis indicated that following the introduction of the IL epidemic strain from India, the nsP4-R82S mutation arose uniquely in Southeast Asia in 2008. During spread from Malaysia to other countries in the region, the nsP4-82S strain became predominant over the early nsP4-82R strain, suggesting that this mutation may facilitate the spread of CHIKV.

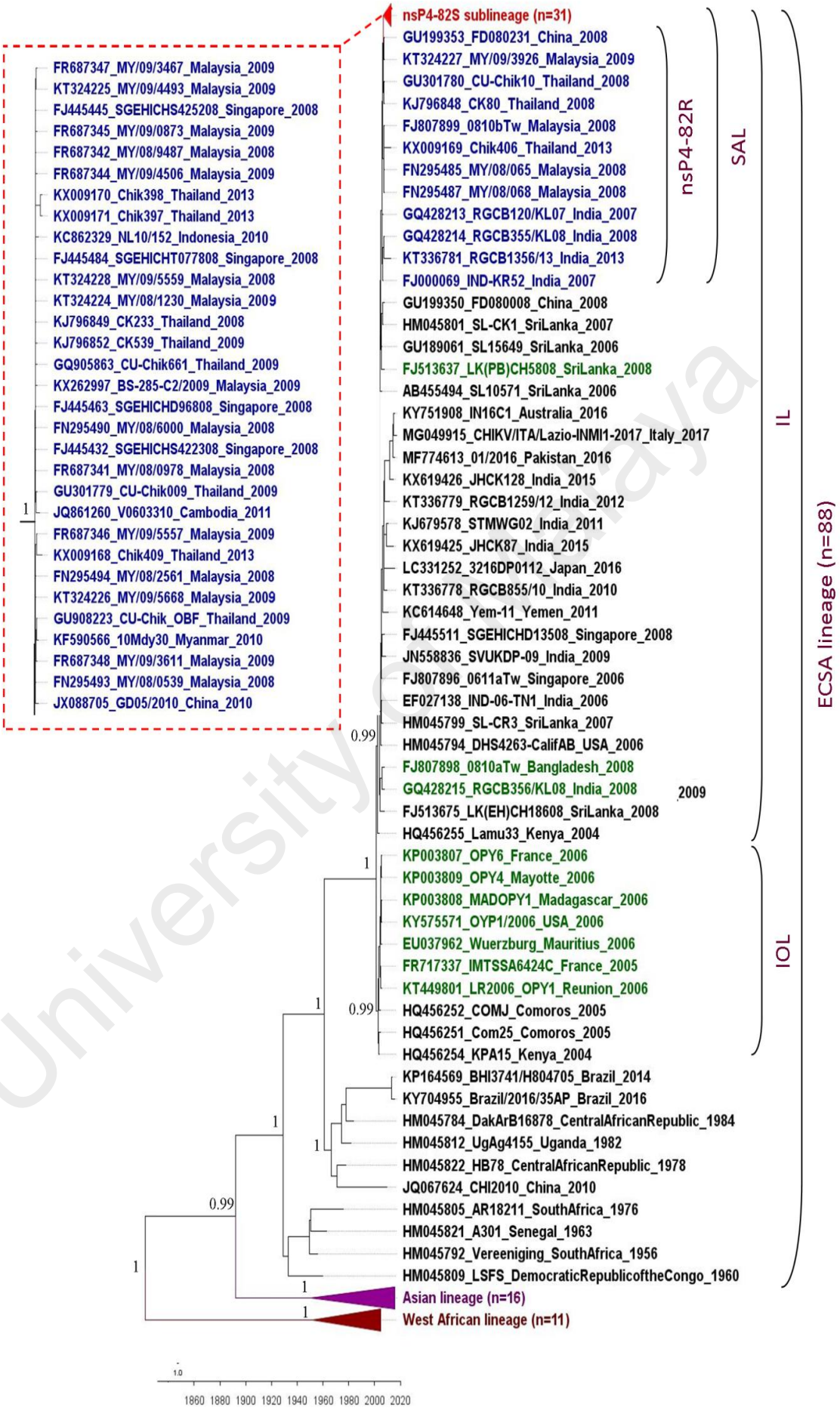


Figure 4.1: Phylogenetic tree of 115 CHIKV whole genome sequences. The three major genotypes are ECSA, Asian and West African. Two distinct sublineages, Indian Ocean sublineage (IOL) and Indian sublineage (IL) emerged within the ECSA genotype. Sequences with E1-226V (blue and green) and E1-226A (black) are shown. The Southeast Asian sublineage (SAL) emerged within the IL, where the nsP4-82S formed an independent sublineage, which is separately expanded for clarity. Strains are labelled as follows: accession number_strain name_country of origin_year of isolation. The estimated posterior probability values are shown at key nodes.

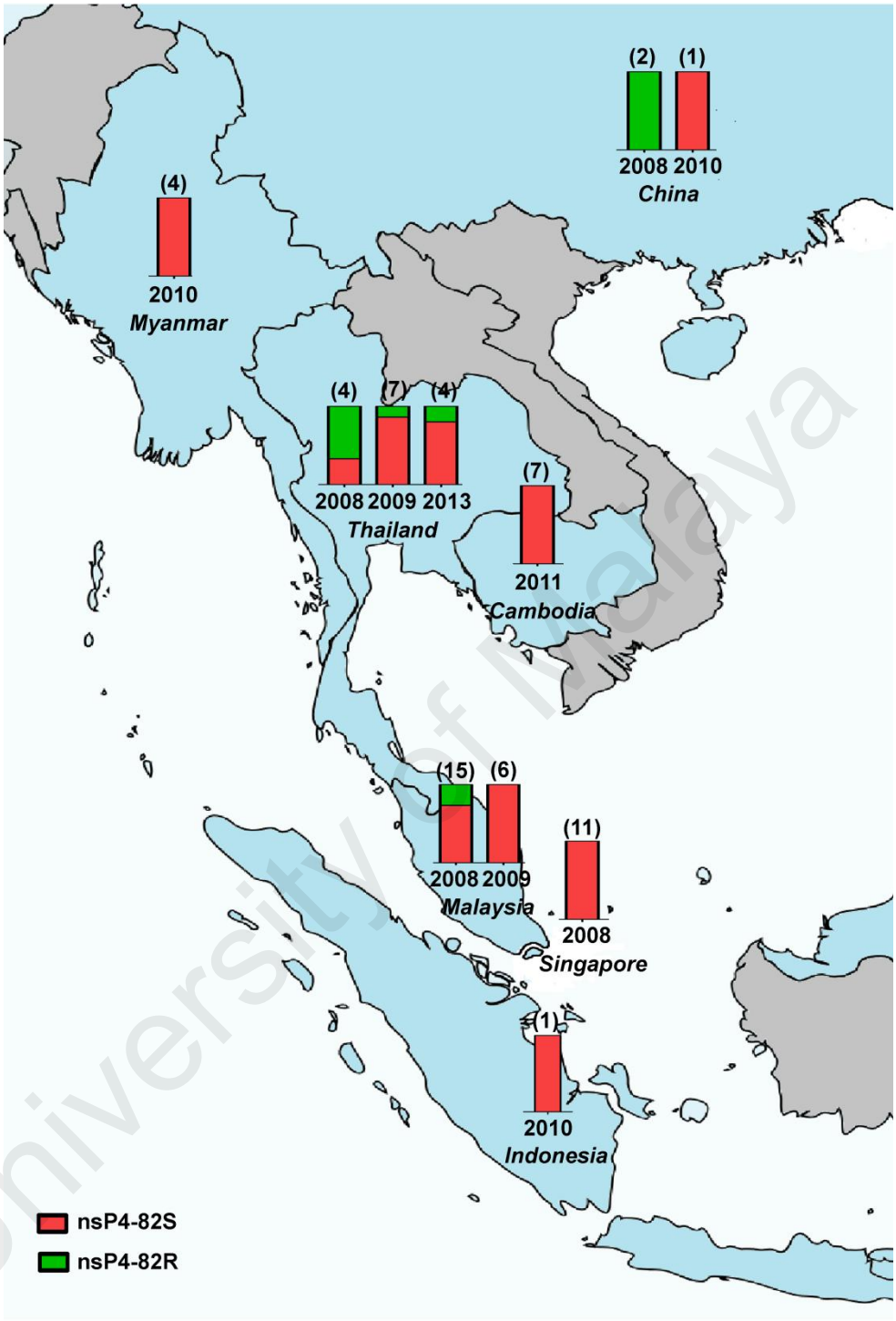


Figure 4.2: Relative distribution of CHIKV sequences with nsP4-82S and nsP4-82R in Southeast Asia from 2008-2013, following introduction of CHIKV with nsP4-82R from India. The numbers in brackets represent total number of isolates collected while the numbers below the graphs represent the year of isolation.

4.2 CONFIRMATION OF RESCUED INFECTIOUS VIRUSES

CHIKV infectious clones (nsP4-82S and nsP4-82R) based on an epidemic Singapore human isolate were used, including four infectious clones expressing green and red fluorescent protein (nsP4-82S-mCherry, nsP4-82R-mCherry, nsP4-82S-ZsGreen and nsP4-82R-ZsGreen). The constructed infectious clones were electroporated into BHK-21 cells and titrated by plaque assay. The electroporated viral titres (Figure 4.3) were comparable ($p>0.05$), ranging between 7.4 log pfu/ml to 7.9 log pfu/ml. Expression of fluorescent proteins observed in electroporated infectious clones (Figure 4.4) indicated successful viral replication in the cells. Since the titres of the fluorescent viruses were comparable ($p>0.05$), nsP4-82S-mCherry and nsP4-82R-ZsGreen viruses were selected for downstream work. All electroporated viruses were used for mosquito and tissue culture infection without further passaging.

Figure 4.3: Viral titres of electroporated CHIKV viruses in BHK-21 cells. All viruses have comparable viral titres with no significant difference (Mann-Whitney test, $p>0.05$).

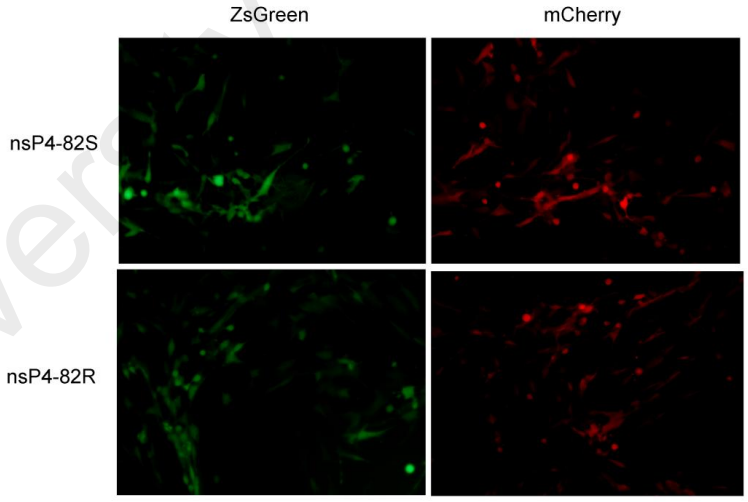


Figure 4.4: Visualization of ZsGreen and mCherry fluorescence signal at 24 hpi after electroporation of CHIKV fluorescent infectious clones (nsP4-82S-mCherry, nsP4-82R-mCherry, nsP4-82S-ZsGreen and nsP4-82R-ZsGreen) in BHK-21 cells.

4.3 REPLICATION KINETICS IN CELL LINES

The replication kinetics of nsP4-82S and nsP4-82R viruses were evaluated in *Ae. albopictus* C6/36 and U4.4 (Figure 4.5) and mammalian RD, HEK-293T and Vero cell lines (Figure 4.6). The nsP4-82S-mCherry and nsP4-82R-ZsGreen were only evaluated in mosquito cell lines (Figure 4.5). The infected cells were collected at 0, 8, 12, 24, 48, 72 and 96 hpi and titrated on BHK-21 cells.

The replication kinetics of all viruses showed similar ($p>0.05$) growth patterns in mosquito cells. In C6/36 cells (Figure 4.5a), all viruses displayed rapid growth within the first 24 hpi before peaking at 48 hpi (7.7 to 8.1 log pfu/ml), and viral replication was sustained at high titre until 96 hpi. In U4.4 cells (Figure 4.5b), the replication pattern of all viruses similarly peaked at 48 hpi (4.5 to 5.6 log pfu/ml), followed by a slight decrease in viral fitness. The growth curves of all viruses in C6/36 cells were generally higher compared to U4.4 cells, likely due to lack of an intact RNAi system in C6/36 cells to restrict viral replication (239). The presence of functional antiviral immune responses in U4.4 cells could also restrict viral growth compared to C6/36 cells (240).

In RD cells (Figure 4.6a), the nsP4-82S showed higher replication compared to the nsP4-82R within the first 24 hours of infection, although differences were not significant ($p>0.05$). Both viruses reached a peak titre of 7 to 7.2 log pfu/ml at 72 hpi before declining. In HEK-293T cells (Figure 4.6b), the nsP4-82S reached peak titre (7.6 log pfu/ml) earlier at 48 hpi, compared to nsP4-82R that peaked (7.6 log pfu/ml) at 72 hpi. In Vero cells (Figure 4.6c), both viruses reached a similar peak titre of about 7.1 to 7.6 log pfu/ml at a similar rate by 48 hpi, before declining. There were no significant differences ($p>0.05$) in the overall growth kinetics of both viruses in RD, HEK-293T and Vero cells.

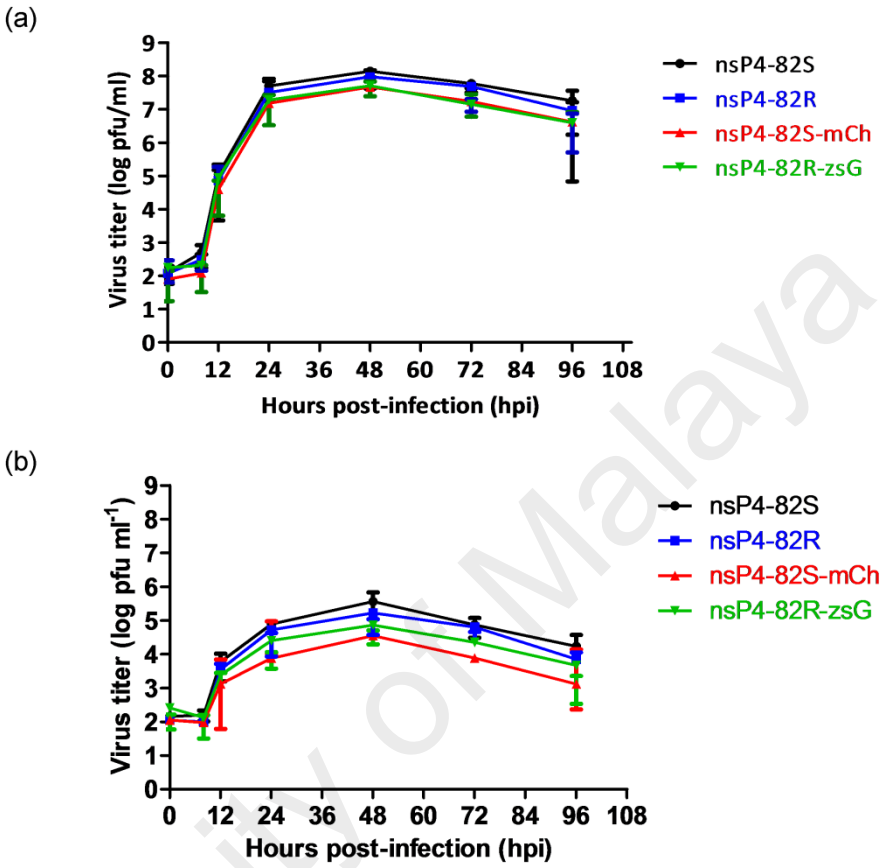


Figure 4.5: Replication kinetics of CHIKV viruses in mosquito cell lines. The virus growth curves in C6/36 cells (a) and U4.4 cells (b) are presented. Means ± SD of 2 independent experiments are plotted. No significant differences were observed between the replication kinetics of the viruses (two-way ANOVA with Bonferroni post hoc test, $p>0.05$).

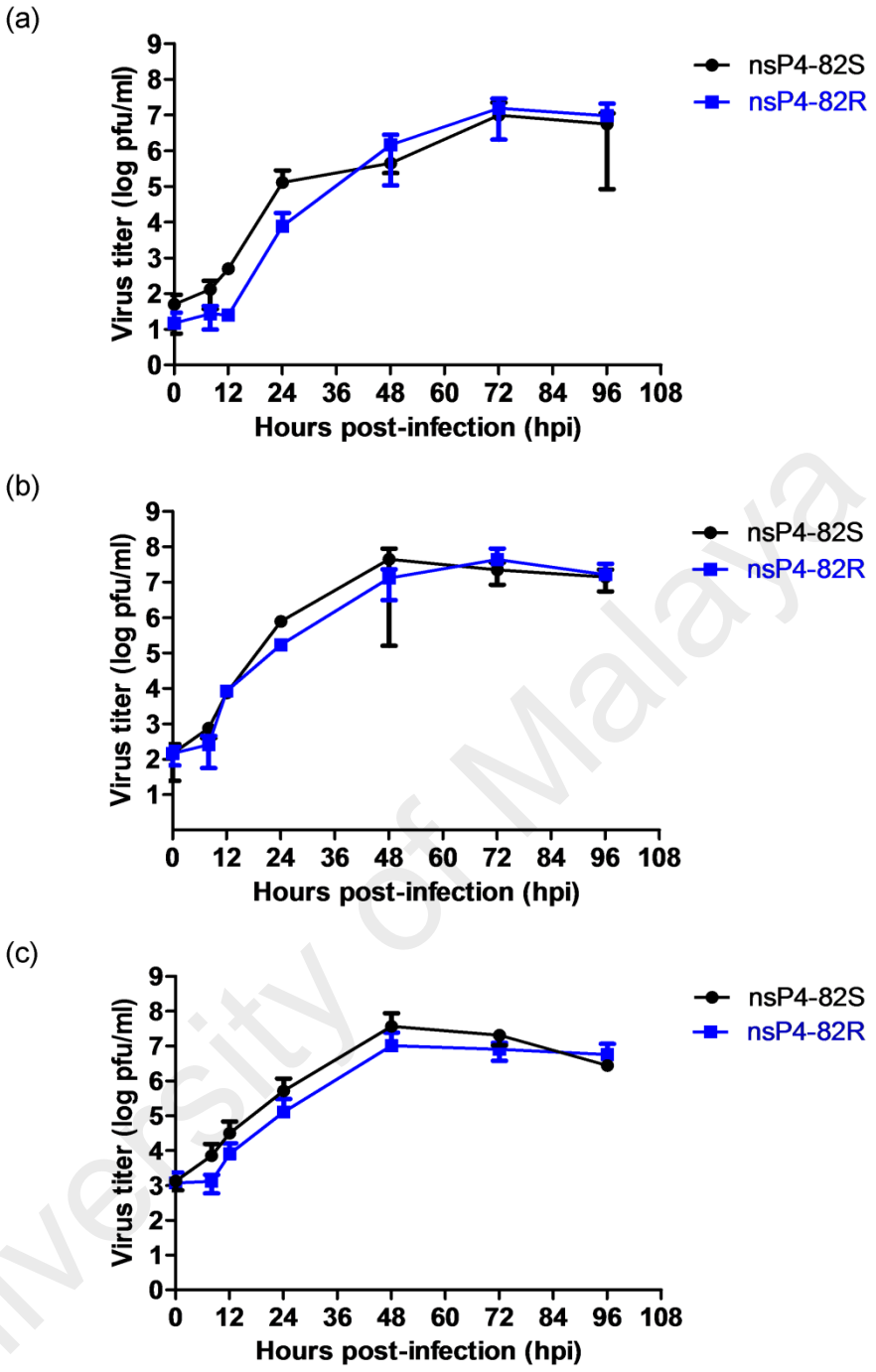


Figure 4.6: Replication kinetics of CHIKV viruses in mammalian cell lines. The virus growth curves in RD cells (a), HEK-293T cells (b) and Vero cells (c) are presented. Means ± SD of 2 independent experiments are plotted. No significant differences were observed between the replication kinetics of the viruses (two-way ANOVA with Bonferroni post hoc test, $p>0.05$).

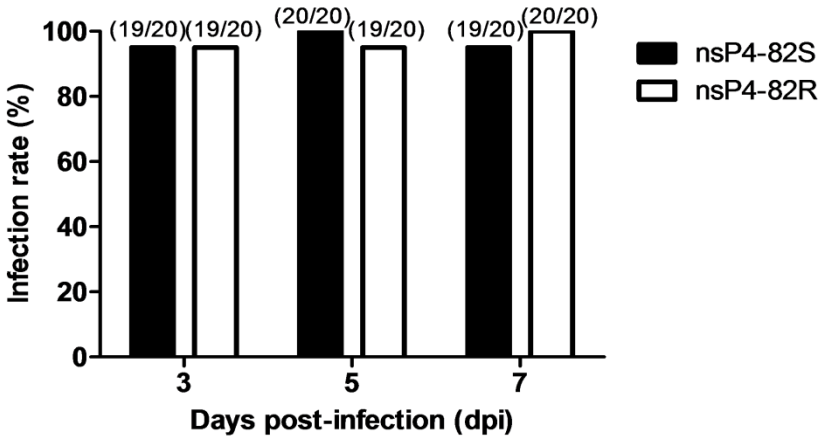
4.4 CHIKV INFECTIVITY IN *AE. ALBOPICTUS*

To investigate whether the apparent selection of nsP4-R82S mutation in CHIKV is due to increased fitness in *Ae. albopictus*, the mosquito species implicated during the Malaysia 2008 outbreak, the growth kinetics of nsP4-82S and nsP4-82R viruses in the mosquitoes were compared. Virus in the midgut represents positive infectivity of the virus. Salivary glands were harvested to determine potential dissemination from the midgut. Saliva was titrated to determine the rate of transmission and absolute viral titre. In addition, as the mutation occurred in the CHIKV polymerase (nsP4), the genetic diversity of CHIKV in *Ae. albopictus* at 7 dpi was also compared.

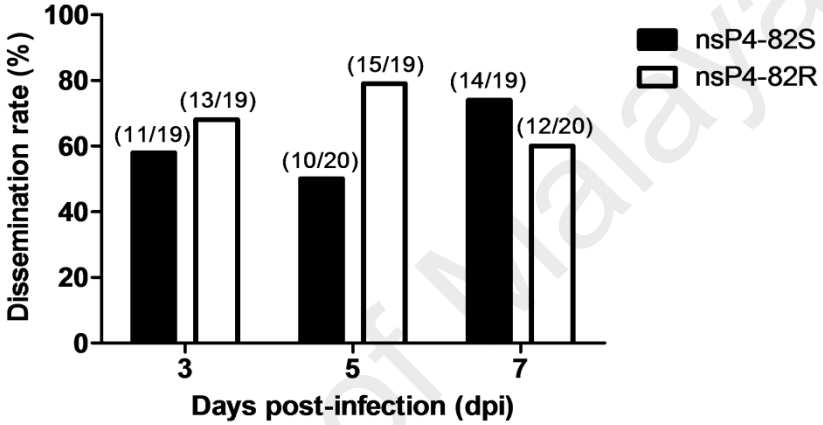
4.4.1 CHIKV GROWTH KINETICS IN *AE. ALBOPICTUS*

Both nsP4-82S and nsP4-82R viruses replicated equally well in midgut with 95% (n=19/20) to 100% (n=20/20) infected from as early as 3 dpi, and remained consistent at 5 and 7 dpi (Figure 4.7a). The dissemination rates of nsP4-82S and nsP4-82R into salivary glands were not significantly ($p>0.05$) different at 3, 5 and 7 dpi (Figure 4.7b). The transmission rates into saliva were considerably lower throughout the time-points (Figure 4.7c), ranging between 0-20% and showed insignificant ($p>0.05$) difference between nsP4-82S and nsP-82R viral titre (Figure 4.7d). Overall, the growth kinetic data showed that the nsP4-R82S mutation did not have significant effect on infection, dissemination and transmission of CHIKV in *Ae. albopictus*.

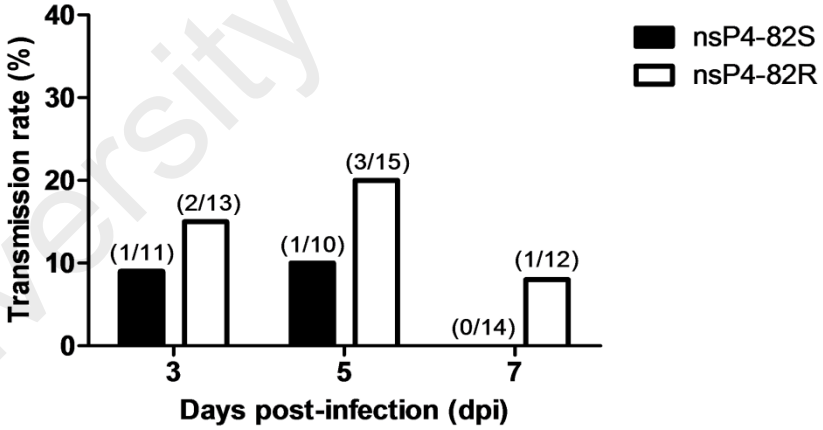
(a)



(b)



(c)



(d)

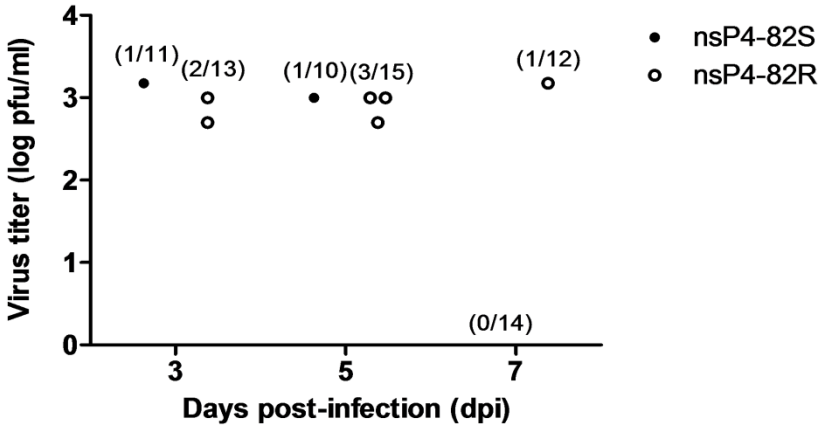


Figure 4.7: Growth kinetics of nsP4-82S and nsP4-82R viruses in *Ae. albopictus*. Infection rates in midgut (a), dissemination rates to salivary gland (b), transmission rates into saliva (c), and viral titre in mosquito saliva (d) are presented. The numbers of mosquitoes tested are shown in brackets. No significant differences were observed between nsP4-82S and nsP4-82R growth kinetics and viral titres (Fisher's exact test, $p>0.05$).

4.4.2 CHIKV GENETIC DIVERSITY IN *AE. ALBOPICTUS*

Next generation sequencing was performed directly on pooled midguts and salivary glands collected at 7 dpi to determine the effect of the nsP4-R82S mutation on CHIKV genetic diversity in *Ae. albopictus*. Two replicates were included for each pooled sample. The electroporated virus stocks of nsP4-82S and nsP4-82R were also sequenced to represent the input virus population into *Ae. albopictus*.

Mutation frequency (Figure 4.8), which identifies the proportion of mutant virus present in a population and is normally used to represent polymerase error (241), were compared between nsP4-82S- and nsP4-82R-infected midguts and salivary glands. Both nsP4-82S and nsP4-82R virus stock (input) have an average mutation frequency of 2.2 mutations per 10^4 nucleotides. In both nsP4-82S- and nsP4-82R-infected midguts, the mutation frequencies were comparable ($p>0.05$) at an average of 2.6 and 2.4 mutations per 10^4 nucleotides, respectively. In salivary glands, the mutation frequencies were also similar ($p>0.05$), ranging between 2.3 to 2.7 mutations per 10^4 nucleotides.

Low frequency amino acid variants in the midgut and salivary gland were analysed to identify potential mutations that may arise due to CHIKV adaptation in mosquitoes. The relative frequency of each variant was presented as the difference between the frequency rate of variant in input virus and the frequency rate of variant in midguts and salivary glands. Variants with significantly ($p\leq0.05$) increased frequency (compared to the input population) or which were >1% (and not present in input virus) were identified in both midguts and salivary glands; all changes were in the nsPs except for an E2-A336V mutation in nsP4-82R-infected midguts.

In nsP4-82S-infected mosquitoes (Table 4.1), 6 variants were detected in nsP1, nsP3 and nsP4 of CHIKV. Of note, the nsP4-82S-infected mosquito midguts showed an increase in the nsP4-82R population from 0.16% at input to 0.48% at 7 dpi, but the

reversion mutation was not detected in the salivary glands. The remaining variants detected have not been mentioned in the literature.

For nsP4-82R-infected mosquitoes (Table 4.2), 17 variants were detected in nsP1, nsP2, nsP3, nsP4 and E2 of CHIKV. There was no emergence of nsP4-82S in midguts or salivary glands. The one mutation with highest frequency at 7 dpi was the nsP1-L407P mutation in the midguts (4.77% and 0.137%) and salivary glands (4.32%). Variants at frequency >1% were not reported in any studies except the nsP2-S539L mutation (212).

Overall, the sequencing data showed both variants produced similar mutation frequency and genetic diversity in *Ae. albopictus*, suggesting that the nsP4-R82S mutation was not selected in *Ae. albopictus* midguts or salivary glands at 7 dpi.

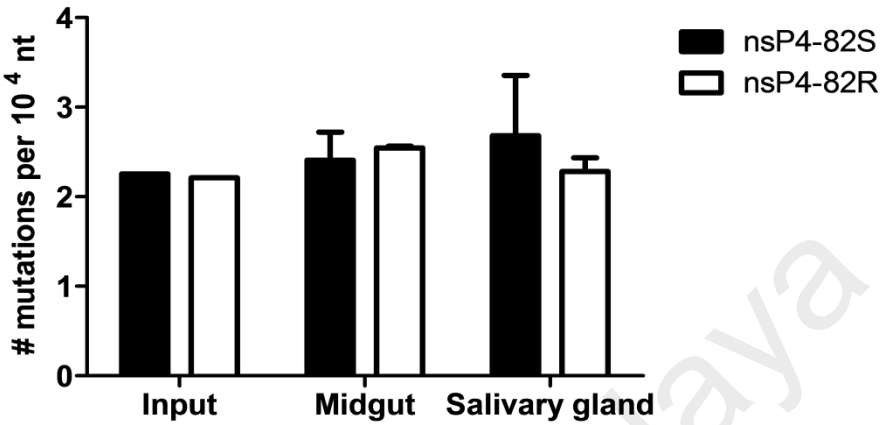


Figure 4.8: Mutation frequency of nsP4-82S and nsP4-82R in midguts and salivary glands of *Ae. albopictus* at 7 dpi. Two replicates were generated from pooled midguts and salivary glands, and the mutation frequencies are represented as mean number of mutations per 10^4 nucleotides sequenced ± SD. No significant differences were observed between the nsP4-82S and nsP4-82R mutation frequencies (t-test, $p>0.05$).

Table 4.1: Variants arising during nsP4-82S infection in *Ae. albopictus* at a significantly increased rate compared to input virus, or at >1% when not present in the input virus.

Region	Reference amino acid	Position	Variant input frequency (%)	Amino acid variants *Difference in frequency (%)			
				Midgut 1	Midgut 2	Salivary gland 1	Salivary gland 2
nsP1	Met	314	0.041	Ile 0	Ile 0.377	Ile 0	Ile 0.105
nsP1	Phe	450	0.069	Leu 0	Leu 0.109	Leu 0	Leu 0
nsP3	Ala	55	0.085	Thr 0	Thr 0.152	Thr 0	Thr 0
nsP3	Thr	376	0	Ala 1.381	Ala 0	Ala 0	Ala 0
nsP4	Ser	82	0.160	Arg 0.320	Arg 0	Arg 0	Arg 0
nsP4	Ala	162	0	Val 1.250	Val 0	Val 0	Val 0

*Difference in frequency (%) represents differences between frequency rate of input virus and mosquito samples.

Table 4.2: Variants arising during nsP4-82R infection in *Ae. albopictus* at a significantly increased rate compared to input virus, or at >1% when not present in the input virus.

Region	Reference amino acid	Position	Variant input frequency (%)	Amino acid variants *Difference in frequency (%)			
				Midgut 1	Midgut 2	Salivary gland 1	Salivary gland 2
nsP1	His	157	0.030	Ala 0.124	Ala 0	Ala 0	Ala 0
nsP1	Leu	260	0.045	Met 0	Met 0.136	Met 0	Met 0
nsP1	Met	314	0.143	Val 0.166	Val 0	Val 0	Val 0
nsP1	Leu	407	0.142	Pro 4.772	Pro 0.137	Pro 4.320	Pro 0
nsP2	Ile	3	0.017	Leu 0.536	Leu 0	Leu 0	Leu 0
nsP2	Glu	146	0.017	Val 0	Val 0.139	Val 0	Val 0
nsP2	Tyr	166	0.049	His 0	His 0.118	His 0	His 0.148
nsP2	Leu	277	0.061	Pro 0	Pro 0.062	Pro 0	Pro 0
nsP2	Lys	507	0	Glu 1.341	Glu 0	Glu 0	Glu 0

Table 4.2 continued.

Region	Reference amino acid	Position	Variant input frequency (%)	Amino acid variants *Difference in frequency (%)			
				Midgut 1	Midgut 2	Salivary gland 1	Salivary gland 2
nsP2	Ser	539	0	Leu 1.501	Leu 0	Leu 0	Leu 0
nsP2	Asp	594	0	Tyr 1.029	Tyr 0	Tyr 0	Tyr 0
nsP2	Ile	602	0	Leu 1.044	Leu 0	Leu 0	Leu 0
nsP3	Ala	36	0	Val 0	Val 1.592	Val 0	Val 0
nsP4	Asn	252	0	Ser 2.501	Ser 0	Ser 0	Ser 0
nsP4	Phe	589	0.061	Leu 0	Leu 0.221	Leu 0	Leu 0
nsP4	Ser	594	0	Pro 1.034	Pro 0	Pro 0	Pro 0
E2	Ala	336	0	Val 2.516	Val 0	Val 0	Val 0

*Difference in frequency (%) represents differences between frequency rate of input virus and mosquito samples.

4.5 COMPETITION ASSAYS IN *AE. ALBOPICTUS*

Since each variant on its own had comparable CHIKV fitness in *Ae. albopictus*, the competitive fitness when both variants were present at a 1:1 ratio in the same mosquito was investigated using a competition assay. The infection status of co-infected mosquitoes were determined using two methods; real-time PCR assay and fluorescence detection.

4.5.1 REAL-TIME PCR ASSAY VALIDATION

Oligonucleotide probes containing locked nucleic acid (LNA) residues were used to develop the real-time PCR assay due to reported strong affinity to complementary targets and high sensitivity in differentiating polymorphisms (242). The two mutation-specific probes were designed to independently detect a single nucleotide change between nsP4-82S and nsP4-82R, thus allowing a competitive analysis of both viruses in the same sample.

A single-probe assay was first performed and the LOD, LOQ, amplification efficiency, regression coefficient (slope) and correlation coefficient of nsP4-82S and nsP4-82R standard curves fell within the acceptable range (Appendix B). The cross-reactivity test showed the probes were specific enough to differentiate between nsP4-82S and nsP4-82R in a mixed population sample (3 log to 7 log cDNA copy number/reaction), whereby the observed cDNA copy number correlates to the expected cDNA copy number of single-probe standard curve (Appendix C).

The probe sensitivity assay was performed using both variants cDNA mixed at ratios of 1:1, 1:4, 1:8, 1:10, 1:100 and 1:1000 to determine the sensitivity of the assay in detecting the ratio difference. The real-time PCR assay was sensitive enough to pick up 1:8 differences between nsP4-82S and nsP4-82R but not at 1:10 and above. In addition,

the Ct values for both variants cDNA only corresponded to the single-probe standard curves when mixed at a ratio of 1:1, rendering the assay incapable of quantifying the variants beyond a 1:1 ratio despite high specificity.

Therefore, the real-time PCR assay was used to analyse the infection status of CHIKV in *Ae. albopictus* qualitatively. In the competition assay, detection of a single variant (e.g. nsP4-82S) in a sample was interpreted as the presence of nsP4-82S at a concentration of at least eight times that of nsP4-82R, and vice versa. Meanwhile, detection of both variants ("co-infection") indicated that both variants were present at a ratio of up to 1:8.

4.5.2 COMPETITION ASSAYS USING REAL-TIME PCR ASSAY

Competition assay was performed by infecting *Ae. albopictus* with blood meal containing a mixture of nsP4-82S and nsP4-82R at equal titres. The midgut, head and saliva was collected and analysed to determine the infection status of nsP4-82S and nsP4-82R. Figure 4.9(a) showed that at 3 dpi, all midguts (n=10/10, $p \leq 0.001$) were co-infected with nsP4-82S and nsP4-82R, and that co-infection remained consistently high at 90% (n=9/10, $p \leq 0.001$) at 7 dpi. However, at 10 dpi, the nsP4-82R was predominant in 20% (n=2/10, $p \leq 0.001$) of the midguts while the remaining midguts (n=8/10, $p \leq 0.001$) were co-infected.

The dissemination rate of nsP4-82S and nsP4-82R were not significantly different ($p > 0.05$) at 3 dpi and 7 dpi (Figure 4.9b), ranging between 10% and 30%. At 10 dpi, only 10% (n=1/10) of the heads were positive for co-infection, followed by another 10% (n=1/10) of the heads with predominant nsP4-82S infection. A significant 80% (n=8/10, $p \leq 0.01$) of the heads were dominated by nsP4-82R infection, most likely a direct consequence of the predominant nsP4-82R infection observed in midgut at 10

dpi. Viral RNA was not detected in the saliva, possibly due to low transmission rate. Overall, these data showed that the nsP4-82R was preferably disseminated at 10 dpi in *Ae. albopictus* compared to nsP4-82S in a competitive environment, suggesting that the nsP4-R82S mutation most likely was not selected in the mosquito vector.

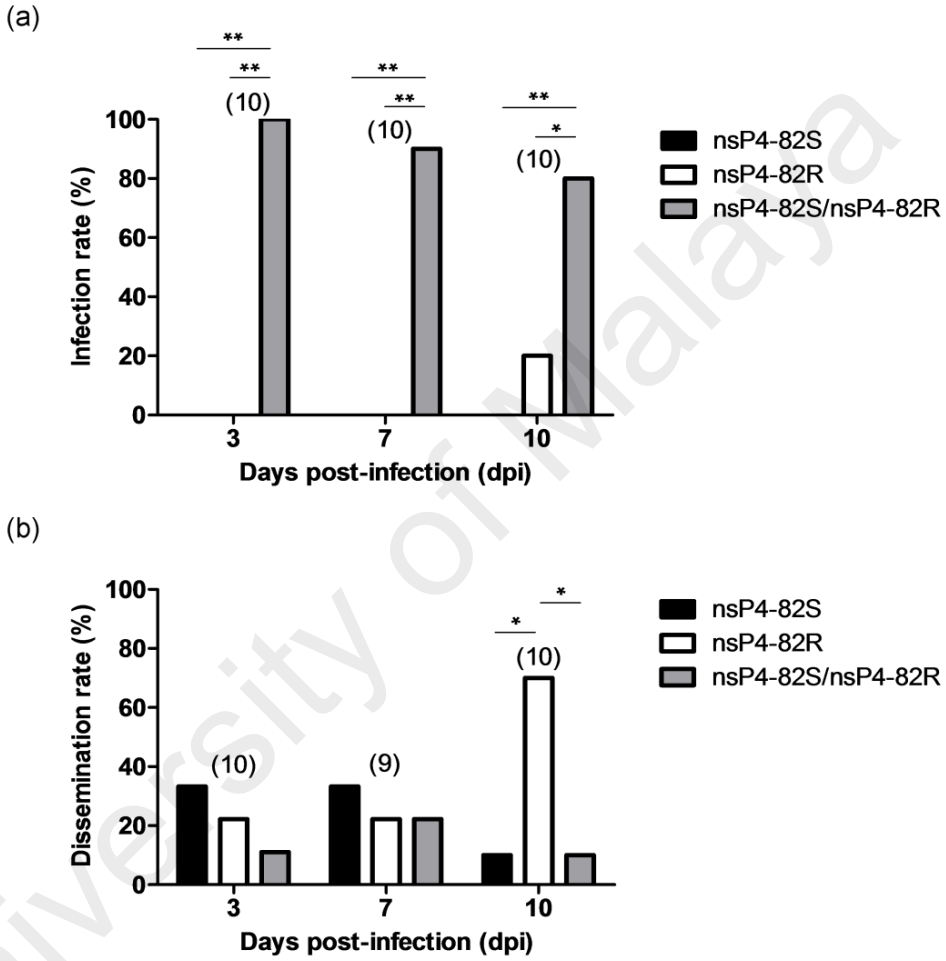


Figure 4.9: Competition assays comparing the competitive fitness of nsP4-82S and nsP4-82R in *Ae. albopictus* using real-time PCR assay. Infection rates (a) and dissemination rates (b) of nsP4-82S and nsP4-82R after co-infection are presented. Bars show single detection of either nsP4-82S or nsP4-82R, or both. Significantly different rates are shown (Fisher's exact test, **, $p \leq 0.001$ and *, $p \leq 0.01$). Numbers in brackets show the number of mosquitoes tested.

4.5.3 COMPETITION ASSAYS USING FLUORESCENT CHIKV

The nsP4-82S-mCherry and nsP4-82R-ZsGreen viruses were used in a further competition assay, to confirm detection of viable, replicating virus and to determine the proportion of nsP4-82S and nsP4-82R in midguts and salivary glands. Three to four midguts and salivary glands were dissected at 3, 7 and 10 dpi, and viewed under a fluorescence microscope.

Visually, the midgut cross-sections at 3 dpi showed even distribution of both nsP4-82S-mCherry and nsP4-82R-ZsGreen, indicating both variants had equal infection efficiency in the midgut (Figure 4.10a). The nsP4-82S-mCherry and nsP4-82R-ZsGreen infection peaked at 7 dpi (Figure 4.10b) and was sustained until 10 dpi (figure 4.10c). The absence of overlapping fluorescence signals indicated the variants typically do not co-infect a single cell and that their replication occurred independently from one another. Two out of three salivary glands showed positive infection of nsP4-82R-ZsGreen at 10 dpi (Figure 4.10d), indicating better dissemination compared to nsP4-82S-mCherry.

The expression of nsP4-82S-mCherry and nsP4-82R-ZsGreen fluorescence signals was measured to compare the relative fitness of both variants in the midgut (Figure 4.11). With an average fluorescence signals ratio ranging from 1.3 to 1.9, no significant differences ($p>0.05$) were observed between the nsP4-82S-mCherry and nsP4-82R-ZsGreen fitness. Taken together, these results showed that the nsP4-R82S mutation did not confer a fitness advantage in *Ae. albopictus*, and that nsP4-82R may have a slight advantage to disseminate to salivary gland, supporting the real-time PCR assay findings in *Ae. albopictus*.

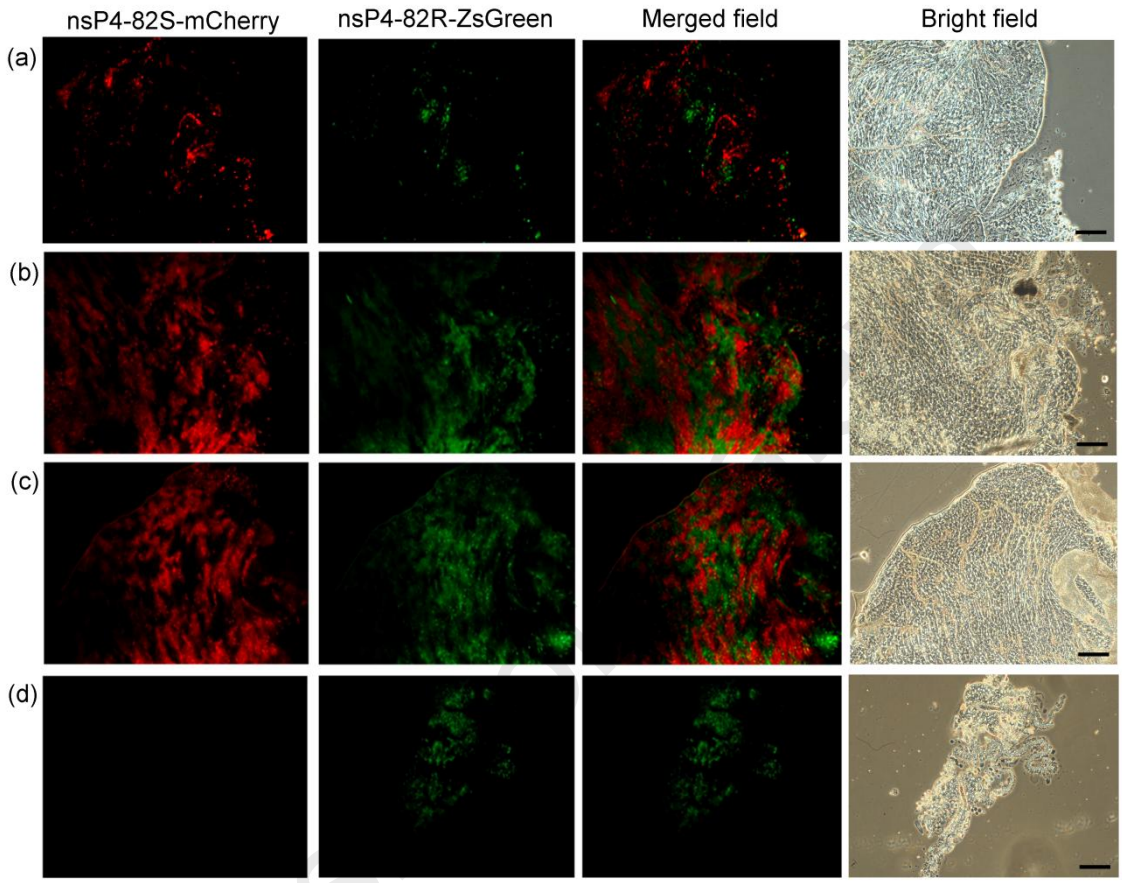


Figure 4.10: Competition assays comparing the competitive fitness of nsP4-82S-mCherry and nsP4-82R-ZsGreen in *Ae. albopictus* using fluorescent viruses. The representative images showed co-infected midguts dissected at 3 dpi (a), 7 dpi (b), 10 dpi (c), and an nsP4-82R-positive salivary gland collected at 10 dpi (d). Scale bar, 0.13 mm. Objective magnification: 20 ×

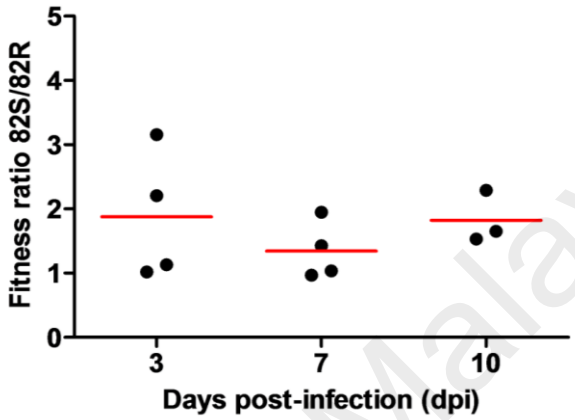


Figure 4.11: Relative fitness of nsP4-82S-mCherry and nsP4-82R-ZsGreen in *Ae. albopictus* midguts, following competition assay. The CHIKV fitness ratio is represented by nsP4-82S-mCherry/nsP4-82R-ZsGreen fluorescence signal, with each dot representing an individual mosquito midgut and the red horizontal lines representing the average fitness ratio. No significant differences were observed between the nsP4-82S-mCherry and nsP4-82R-ZsGreen fluorescence signals (two-tailed Wilcoxon matched-pairs signed rank test, $p>0.05$).

4.6 COMPETITION ASSAYS IN MAMMALIAN CELL LINES

Since previous experiments had confirmed that the nsP4-R82S mutation was not selected in the *Ae. albopictus* mosquito vector, the competitive fitness of nsP4-82S and nsP4-82R was investigated in alternate hosts of CHIKV, using the mammalian cell lines RD, HEK-293T and Vero cells. The cells were infected with three different ratios 1:1, 10:1 and 1:10 using nsP4-82S:nsP4-82R. The cells were infected with the virus mixtures and serially passaged three times. Each passage was sequenced to determine the proportion of nsP4-82S and nsP4-82R in a virus population.

In RD and HEK-293T cells infected with both variants at ratio 1:1 (Figure 4.12a and Figure 4.12d), nsP4-82S gradually outcompeted nsP4-82R over the three passages to predominate at P3. In Vero cells (Figure 4.12g), the nsP4-82S was replaced by nsP4-82R at P3 in the first replicate while the nsP4-82S appears to predominate over nsP4-82R in the second replicate.

At ratio 10:1 of nsP4-82S:nsP4-82R, nsP4-82S remained predominant over three passages in all cell lines (Figure 4.12b, Figure 4.12e and Figure 4.12h). At ratio 1:10, nsP4-82R remained predominant at P3 in RD cells (Figure 4.12c) and one of the replicates in HEK-293T cells (Figure 4.12f); however, in the second HEK-293T replicate, nsP4-82S outcompeted nsP4-82R by P3. In Vero cells (Figure 4.12i), the nsP4-82R predominated over nsP4-82S by P3 in both replicates.

While arginine (R) appeared to be mainly encoded by AGA and serine (S) was mainly encoded by AGT, low frequency polymorphisms were also seen in all three positions of the codon (Figure 4.12) which may potentially have resulted in nsP4-82S (AGC), nsP4-82R (CGC, CGA and CGT), nsP4-82I (ATA, ATT and ATC) and nsP4-82L (CTC and CTT).

Overall, the nsP4-82S appears to have a competitive fitness advantage against nsP4-82R in human (RD and HEK-293T) cell lines, from as early as the first passage when competing at initially equal ratios, but not in monkey (Vero) cells. In addition, nsP4-82S could outcompete nsP4-82R at a lower starting ratio of 1:10 in HEK-293T cells, but at a lower likelihood.

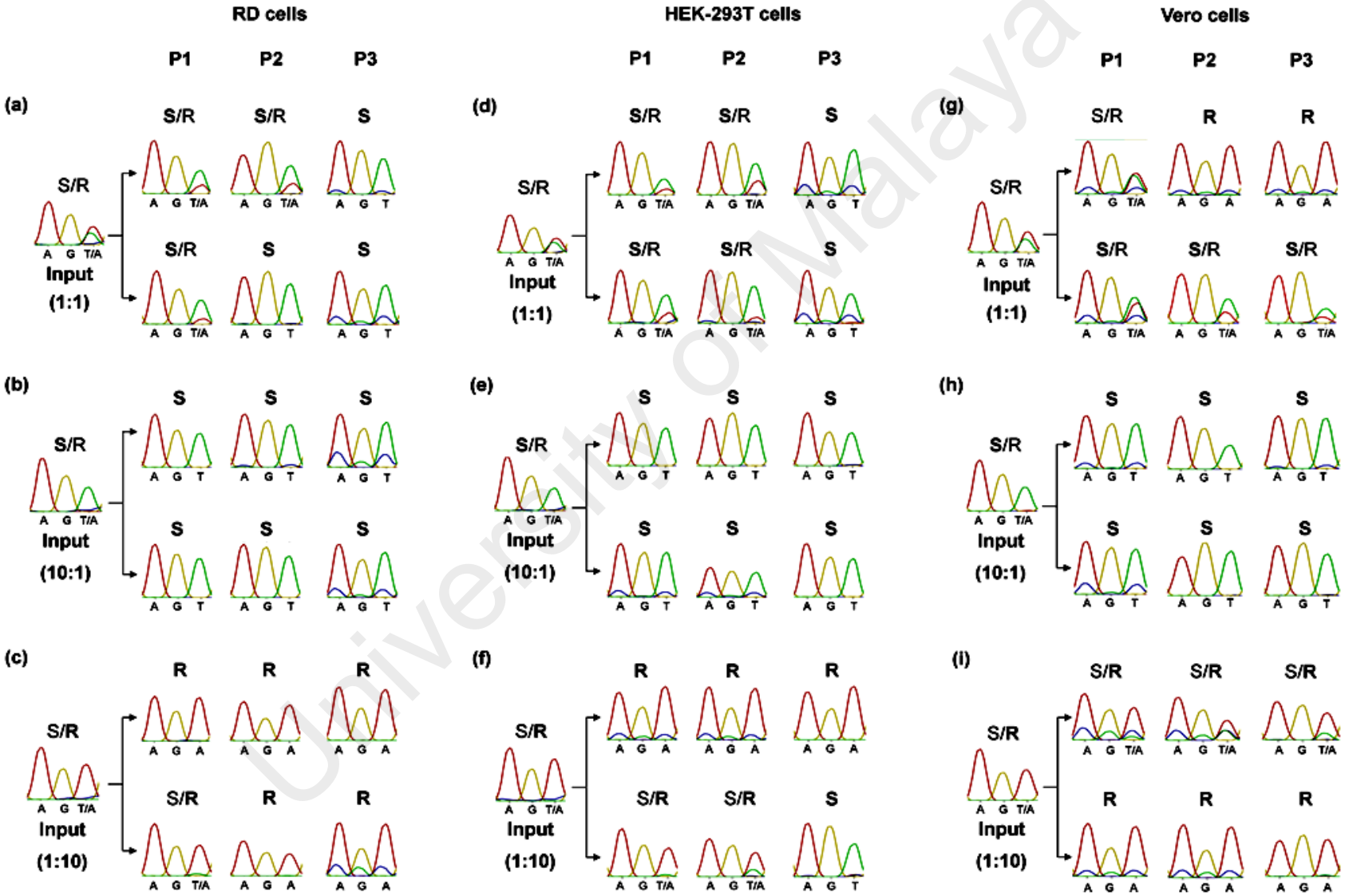


Figure 4.12: **Sequencing chromatograms of the codons encoding the amino acid at nsP4-82, following serial passage of nsP4-82S:nsP4-82R in RD, HEK-293T and Vero cells.** The input viruses were used to initiate passage 1 (P1) and two replicates were performed for each passage series in RD cells at ratios of 1:1 (a), 10:1 (b) and 1:10 (c), in HEK-293T cells at ratios of 1:1 (d), 10:1 (e) and 1:10 (f), and in Vero cells at ratios of 1:1 (g), 10:1 (h) and 1:10 (i). The amino acids and nucleotides are shown above and below the chromatograms, respectively. In the input viruses, arginine (R) is encoded by AGA and serine (S) is encoded by AGT. Amino acids highlighted in bold represent the predominant nsP4-82 variant. Low-frequency polymorphisms subsequently detected in all three positions of the codon may potentially result in nsP4-82S (AGC), nsP4-82R (CGC, CGA and CGT), nsP4-82I (ATA, ATT and ATC) and nsP4-82L (CTC and CTT).

CHAPTER 5: DISCUSSION

CHIKV is an RNA virus that is constantly evolving under the presence of selective pressure due to its alternation between mosquito vectors and vertebrate hosts (198). The RNA virus polymerase lacks proofreading ability to repair errors, leading to diverse viral quasispecies that are crucial for virus evolution, fitness and pathogenesis (243). Although the mutations are often lethal for viruses in the presence of selective pressure, some variants may emerge as specific viral populations with fitness advantage in nature. In addition, a viral quasispecies that is more adapted to the transmission cycle could also displace the wild-type strain. The most notable example of this was the emergence of the E1-A226V mutation in the Indian Ocean and India and the subsequent microevolution of CHIKV which led to global expansion into new geographical regions (132).

This study demonstrated that a mutation in the CHIKV polymerase (nsP4-R82S) could be associated with increased virus fitness in the human host without causing a deleterious effect in fitness in the *Ae. albopictus* vector. This adaptation may have facilitated the CHIKV outbreak in Southeast Asia in 2008.

5.1 EMERGENCE OF NSP4-82S IN THE SOUTHEAST ASIAN SUBLINEAGE

Prior to 2005, CHIKV in Southeast Asia was restricted to sporadic outbreaks caused by viruses belonging to the Asian genotype which were mainly transmitted by *Ae. aegypti* (97). Epidemic ECSA viruses first appeared in India in 2005 (244), followed by the emergence of E1-A226V mutation that soon became the source of spread of CHIKV into Malaysia in 2008 (18). Malaysia first reported CHIKV outbreaks in April 2008,

when the ECSA genotype (E1-226V) carried two unique amino acid substitution (nsP2-L539S and E2-K252Q), which were also present in earlier sequences from Kerala, India (89). Singapore subsequently reported an outbreak in May 2008, and epidemiological investigations and sequenced isolates showed the outbreak was most likely initiated by travellers arriving from neighbouring Johor state in Malaysia, where the Malaysian CHIKV outbreak was first reported (88).

The nsP4-R82S mutation is assumed to have emerged at some point during the initial months of the outbreaks in Malaysia and Singapore, but the exact point of emergence remains unconfirmed due to lack of detailed sequences from the early stages of the outbreak in Malaysia. In subsequent years, this nsP4-R82S sublineage spread throughout the rest of Malaysia and into countries of Southeast Asia, including Indonesia, Thailand, China, Cambodia and Myanmar. Although CHIKV whole genomes are not available from outbreaks in Laos (245) and Vietnam (109) in 2012, the sequenced CHIKV strains showed high similarity to the ECSA strain from Cambodia carrying the nsP4-82S variant.

The phylogenetic analysis showed that the nsP4-R82S mutation is unique to Southeast Asian CHIKV isolates, as CHIKV of ECSA and Asian lineages otherwise have nsP4-82R while CHIKV of West African lineage have nsP4-82G. There are precedents where an amino acid shift was detected in an outbreak region. The emergence of distinct sublineages within the IOL was associated with specific outbreak regions, with each sublineage defined by an amino acid shift (212). Studies have shown that amino acid shifts are associated with CHIKV adaptation to local mosquito vectors to further facilitate the spread of the virus (12, 23, 213). It is therefore interesting that a single amino acid shift (nsP4-R82S) was associated with a specific geographical spread of CHIKV outbreak in Southeast Asia.

The widespread CHIKV outbreak in India and Southeast Asia was likely due to the decline of herd immunity in human populations as a result of long intervals between epidemics (e.g. it had been 33 years since the last Indian outbreak) (130). The emergence of the nsP4-R82S mutation during the CHIKV outbreak across Southeast Asia gives rise to the question of whether the mutation could provide fitness advantage to the virus and further facilitate the spread of the outbreak. To determine which specific host selects the nsP4-R82S mutation, this study investigated the mutation in the *Ae. albopictus* vector and mammalian cell lines by measuring changes in fitness, genetic diversity, and adaptability to competitive pressure.

5.2 THE NSP4-R82S MUTATION DOES NOT PROVIDE FITNESS ADVANTAGE IN *AE. ALBOPICTUS*

To date, the majority of naturally occurring adaptive mutations in alphaviruses involve the envelope glycoproteins E1 and E2 (11, 208, 211, 212), while adaptive mutations in the nsPs are rare (207). In this study, the fitness of the nsP4-82S and nsP4-82R viruses were compared in *Ae. albopictus*, the primary vector identified during CHIKV outbreaks in Southeast Asia (15, 88, 99, 246). The mosquitoes were orally fed with infectious blood meal to mimic the natural route of feeding (247). The midguts, salivary glands and saliva were processed to compare efficiency of the virus in establishing infection and overcoming tissue barriers associated with the organs (193).

Results showed that the nsP4-R82S mutation does not exact a fitness cost in *Ae. albopictus in vitro* or *in vivo*. The comparably high infection and dissemination rates observed in midguts and salivary glands, respectively, showed that both nsP4-82S and nsP4-82R variants could efficiently establish and maintain infection from as early as 3 dpi up to 7 dpi. Although transmission rates of the virus into saliva were low for both

variants, the viral titres achieved were similar to other study (172). The low transmission rate with the forced salivation method used here could be improved with alternative methods of collecting saliva such as allowing the mosquitoes to feed on hanging blood drops (160) or honey-baited nucleic acid preservation cards (248).

The lack of fitness change observed suggests there is a minimal selection pressure against the nsP4-82S when the virus is present independently in the mosquito. However, growth competition assays for CHIKV (198) and human immunodeficiency virus type 1 (249, 250) have been shown to give a more sensitive and valid measure of fitness differences, whereby two or more viral variants compete for the same cell population *in vivo*. Therefore, the competitive fitness of both nsP4-82S and nsP4-82R variants was further compared in the midguts and salivary glands of *Ae. albopictus*.

Interestingly, the increase in number of midguts infected with nsP4-82R at a later stage of infection (10 dpi) correlates with the increase in dissemination rate to salivary glands at the same time point. This increase was not seen in the individual variant replication kinetics, which covered a shorter incubation period of 7 dpi. Alternatively, the significant difference in dissemination rates suggested that the nsP4-82R variant could disseminate more efficiently when directly competing against the nsP4-82S, but only in the late stage of infection. This fitness advantage in the wild-type may reflect the detection of an nsP4-82R residue in the only available sequence of CHIKV (GenBank accession number: GU908223) isolated from wild *Ae. albopictus*, which was reported from Thailand (251). These findings differ from nsP mutations (nsP2-L539S, nsP3-Y38H, nsP3-T444M) observed during CHIKV epidemics that do not increase fitness in *Ae. albopictus* even at late stages of infection (10 dpi) (212).

To demonstrate the infection pattern of the two variants in *Ae. albopictus*, nsP4-82S-mCherry and nsP4-82R-ZsGreen viruses were used to infect the mosquito using competition assay. Dissection of the midgut showed the variants typically do not infect the same midgut cell. This observation is similar to E2-L210Q mutation on CHIKV infection pattern in *Ae. albopictus*. While the same study demonstrated that the E2-L210Q mutation increased viral dissemination by increasing CHIKV infectivity in *Ae. albopictus* midguts (14), the nsP4-R82S mutation does not seem to affect CHIKV infectivity in the midgut. However, it is possible that the small sample size limited this ability of this experiment to accurately estimate the virus competitive fitness in the midguts of *Ae. albopictus*. Nevertheless, the detection of only nsP4-82R in the salivary glands of 2 out of 3 mosquitoes was similar to the real-time PCR result.

Overall, the wild-type nsP4-82R appears to disseminate better when competing against the nsP4-82S. However, this apparent *in vivo* advantage is not seen during single infections, which are far more likely in nature. Studies have shown that RNA viruses like CHIKV optimize replication efficiency through balance between replication speed and replication fidelity (252, 253), and since the nsP4-R82S mutation does not affect viral fitness in the mosquito, the effect of the mutation on replication fidelity was investigated.

5.3 POLYMERASE FIDELITY WAS NOT AFFECTED BY THE NSP4-R82S MUTATION

Mutation in the polymerase is often associated with a change in replication fidelity, which in turn changes the diversity of quasi-species produced (254-256). Therefore, the midguts and salivary glands infected with nsP4-82S and nsP4-82R variants were deep sequenced to compare the relative diversity in these viral populations. The possibility

that the absence of fitness cost, observed in mosquito cell culture and *Ae. albopictus* mosquito, was due to the reversion to wild-type was also addressed.

First, single-host viruses such as poliovirus (257) and coxsackievirus B3 (258) with altered polymerase fidelity present attenuated phenotype, either due to lack of genetic diversity or accumulation of deleterious mutations. While arbovirus genetic diversity is mainly restricted in the insect host due to strict population bottlenecks and selective pressure (193, 198), polymerase fidelity variants of CHIKV (19) and WNV (259) which generated increased or decreased mutation frequency compared to their wild-type counterparts have attenuated fitness and virulence. In this study, the comparable mutation frequency in the midguts and salivary glands indicated that the genetic diversity of CHIKV was not affected by the nsP4-R82S mutation, which in turn suggests that the mutation does not affect the replication fidelity of CHIKV polymerase.

Secondly, the nsP4-R82S mutation is relatively stable in the mosquito, as only a very low percentage of reversion (0.32%) to wild-type nsP4-82R was observed in the midgut. In support of this, a single available sequence of CHIKV (GU908223) isolated from wild *Ae. albopictus* in Thailand has an nsP4-82S residue (251). This contrasts with nsP4-C483A/G/W mutations which alter fidelity of CHIKV polymerase; variants with low polymerase fidelity demonstrate defective replication in *Ae. albopictus* and greatly favour the reversion (39% to 93%) to wild-type (20).

Thirdly, while minority variants have been reported to alter arbovirus emergence, transmission and pathogenesis (196, 260), mosquito infection with nsP4-82S and wild-type nsP4-82R variants resulted in a number of low frequency variants (<2.516%) that have not been previously reported and were unlikely to be biologically significant. Interestingly, the nsP4-82R-infected mosquitoes had two mutations (nsP1-H157A and

nsP1-L407P) previously identified as amino acid positions that are subjected to positive selection, contributing to CHIKV evolution (213, 261). However, study showed that nsP1-L407P increased CHIKV replication capacity in BHK-21 cells but has no effect on *Ae. albopictus* infection (14), suggesting that this mutation is a laboratory adaptation.

At this point, this study result showed that the nsP4-R82S mutation does not affect viral fitness or polymerase fidelity, suggesting that the presence of R or S at the nsP4-82 site has a neutral effect in *Ae. albopictus*. This led to a hypothesis that the nsP4-R82S mutation could be selected in an alternate host instead.

5.4 THE NSP4-R82S MUTATION ENHANCES VIRAL FITNESS IN HUMAN CELL LINES

The effect of the nsP4-R82S mutation was tested in human cell lines (RD and HEK-293T cells) to represent the main mammalian hosts. The competition assay was conducted in these cell lines according to previous reports that the target organs of CHIKV infection and replication include muscle (causing myalgia) and kidney (239, 246, 262). The comparison was also carried out in Vero cells, which represent the model for non-human primates, to investigate whether non-human primates could serve as the selective pressure for the nsP4-82S variant.

Despite producing similar replication kinetics in RD, HEK-293T and Vero cells when the two variants were assayed individually, the nsP4-82S was robustly selected against the wild-type when competed against each other, especially in RD and HEK-293T cells. In a similar study of the emergence of NS4B-V116A/M mutations during a dengue epidemic in Vietnam in 2013, DENV viruses with these mutations inhibited host interferon response in human cell lines during early infection, leading to higher replication (263). Another example includes the emergence of the NS1-A188V mutation

during the Zika outbreaks, which inhibits interferon-β induction in mammals, leading to increased NS1 antigenaemia and enhanced infection of *Ae. aegypti* (264, 265).

The rapid displacement of wild-type by the nsP4-82S in human cell lines suggested there is a strong selection pressure favouring the variant, which contrasts with the result observed in *Ae. albopictus*. A potential explanation for this is that the nsP4-R82S variant can adapt to a given host (in this case human) without undergoing major trade-off of fitness in its alternate host (*Ae. albopictus*). A similar result was reported for E2-K252Q and E2-L210Q mutations in CHIKV, except that the mutations increased viral fitness in *Ae. albopictus* without deleterious effect in mammalian hosts (14, 212).

The fitness advantage observed in RD and HEK-293T cell lines suggest that human muscle and kidney may play critical roles in selection of the nsP4-R82S mutation, and that these cell lines could be used to further elucidate the molecular mechanisms responsible for enhanced CHIKV fitness. Certainly, future work could include investigating the effect of the nsP4-R82S mutation *in vivo*, since cell lines are still of limited relevance to the *in vivo* situation (239).

In Vero cells, both variants replicated with equal efficiency, which indirectly suggest that the nsP4-R82S mutation was selected as a result of CHIKV adaptation to humans rather than non-human primates. This observation is supported by previous studies in Malaysia (126, 134), Thailand (110) and Mauritius (136) which demonstrated that non-human primates such as long-tailed macaques typically possess low seroprevalence rate even after the onset of an outbreak, and are unlikely to play an important role in CHIKV transmission.

This study's findings indicated that the selection of the nsP4-R82S mutation most likely occurred in the human host, and the persistence and accumulation of the mutant

nsP4-82S in the transmission cycle led to its establishment as the predominant circulating strain in the Southeast Asian CHIKV outbreaks. As signature changes in other geographic clusters often include both structural and non-structural proteins (18), co-evolution of amino acids in CHIKV nsPs may influence the emergence of adaptive mutations in other proteins through epistatic interaction (27). Since the Southeast Asian sublineage is characterised by a triad of substitutions (nsP4-R82S, nsP2-L539S and E2-K252Q), the possible interactions and co-dependence between apparently selected nsP mutations and other proteins would be an interesting area of future study.

5.5 POSSIBLE MOLECULAR FUNCTIONS OF THE NSP4-R82S MUTATION

The polymerase encoded by nsP4 is the most highly conserved protein in alphaviruses, but its structure is not yet known. The nsP4-82 site is located in the N-terminal domain (~150 amino acids in length), which is predicted to be unstructured (266, 267). The N-terminal domain is important for virus replication as it interacts with other nsPs in the replication complex (36, 266) and initiates minus-strand RNA synthesis through template recognition (37, 38). During replication, the flexible nature of this domain forms different interactions with viral or host proteins during early stages of minus-strand synthesis and later stages of plus-strand synthesis in infection (268, 269) to allow synthesis of different RNA species (22).

Substitution of arginine (polar, positively-charged) with the smaller serine (polar, uncharged) at nsP4-82 could result in changes to protein folding and efficiency in viral-host protein interaction during replication (270). Alternatively, serine located within an intracellular protein could be phosphorylated as part of a signal transduction process, which may be critical for protein functionality (271). Mutation in the amino acid (nsP4-

G83L) adjacent to nsP4-82 has been shown to cause defective minus-strand synthesis and delayed shutoff of host cell protein synthesis in SINV despite producing similar viral protein expression as the wild-type virus (22). The proximity of the two amino acids (nsP4-82 and -83) may indicate that nsP4-82 is associated with this function. Future studies could explore the potential significance of the mutation on minus-strand synthesis, viral protein function and host cell protein synthesis upon infection. These mechanisms could be elucidated using muscle or liver cells (known to be important sites of CHIKV replication), as initial replication kinetics (Figure 4.6) and competition assays (Figure 4.12) in RD and HEK-293T cell lines showed increased viral fitness of nsP4-82S compared to nsP4-82R.

CHAPTER 6: CONCLUSION

In conclusion, this study demonstrated that the nsP4-R82S in CHIKV emerged in Malaysia or Singapore in 2008, and become predominant in the Southeast Asian CHIKV lineage. The variant provides fitness advantage to CHIKV in human cells rather than the *Ae. albopictus* vector, without causing a fitness cost in the mosquito. Deep sequencing data showed the mutation is not selected in the mosquito, and does not affect genetic diversity (and hence, CHIKV polymerase fidelity). The mutation may have increased CHIKV fitness in the human host through other unknown mechanisms such as minus-strand synthesis, changes in viral protein function or host cell protein synthesis. This is an unusual example of a naturally occurring nsP mutation which impacts human host fitness, and which may have contributed to extensive transmission in the region. The effects of mutations in nsPs during outbreaks are understudied, as attention has primarily focused on structural proteins. It is important to continue surveillance of CHIKV sequences in patients and mosquitoes, to gain insight into host-adaptive changes which may develop as the virus evolves.

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