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ADAPTATION OF CHIKUNGUNYA VIRUS TO *AEDES ALBOPICTUS* MOSQUITOES: THE ROLE OF MUTATIONS IN THE E1 AND E2 GLYCOPROTEINS

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ADAPTATION OF CHIKUNGUNYA VIRUS TO *AEDES ALBOPICTUS* MOSQUITOES: THE ROLE OF MUTATIONS IN THE E1 AND E2 GLYCOPROTEINS

by
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To my family who encouraged and believed in me.

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ADAPTATION OF CHIKUNGUNYA VIRUS TO *AEDES ALBOPICTUS* MOSQUITOES: THE ROLE OF MUTATIONS IN THE E1 AND E2 GLYCOPROTEINS

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Chikungunya virus (CHIKV) is a positive sense single-stranded RNA virus in the family *Togaviridae* that between 2005 and 2007 caused its largest outbreak/epidemic in documented history, affecting parts of Africa, the Indian Ocean islands, India, and Europe. An unusual feature of this epidemic was the involvement of the previously unrecognized vector of CHIKV: *Ae.* (*Stegomyia*) *albopictus* mosquitoes. It was postulated that genetic changes in the virus might have contributed to the scale of these epidemics by facilitating CHIKV transmission by *Ae. albopictus* mosquitoes. In order to characterize genetic factors that might influence the ability of CHIKV to be transmitted by *Ae. albopictus,* I developed full-length infectious clone (i.c.) (pCHIKV-LR i.c.) and an i.c. that expressed enhanced green fluorescent protein (eGFP) from either a 3' or 5' additional sub-genomic promoters (pCHIKV-LR 3'GFP and pCHIKV-LR 5'GFP respectively) based on a CHIKV strain isolated during 2005-2006 epidemic on Reunion Island (LR2006 OPY1). The viruses produced from these i.c. were characterized in cell culture and in *Ae. aegypti* and *Ae. albopictus* mosquitoes. I concluded that, pCHIKV-LR i.c. and pCHIKV-LR 5'GFP infectious clones are suitable for investigation of the genetic factors influencing CHIKV fitness in the mosquito and vertebrate hosts.

Previous phylogenetic analysis had demonstrated that the 2005-2006 epidemic on Reunion Island was associated with a strain of CHIKV with a mutation in the E1 glycoprotein (E1-A226V). Using viral infectious clones of Reunion and West African strains of CHIKV, into which either the E1-226 A or V residues were engineered, I demonstrated that the E1-A226V mutation was directly responsible for a significant increase in CHIKV infectivity for *Ae. albopictus*, and led to more efficient viral dissemination into mosquito secondary organs and transmission to suckling mice. I also demonstrated that increased CHIKV infectivity of *Ae. albopictus* midgut cells associated with the E1-A226V mutation is directly responsible for more efficient virus replication in the mosquito, more rapid dissemination of the virus into salivary glands and more efficient transmission. Interestingly, this mutation caused a marginal decrease in the ability of CHIKV to infect the *Ae.* (*Stegomyia*) *aegypti* midgut, but had no effect on viral dissemination, and was associated with a slight increase in transmission by *Ae. aegypti*. These findings demonstrate that the E1-A226V mutation confers CHIKV adaptation to transmission by *Ae. albopictus,* and provide a plausible explanation of how this mutant virus caused an epidemic in a region lacking the typical vector.

I also demonstrated that the E1-A226V mutation is associated with an increase in cholesterol-dependency of CHIKV for growth and entry into C6/36 cells, and is responsible for increase in the pH dependency of CHIKV fusion reaction. However, analysis of viruses with specific mutations at position E1-226, and at other CHIKV genomic regions that modulate cholesterol dependency of CHIKV, demonstrated that there is no clear mechanistic correlation between dependency for cholesterol and increased infectivity to *Ae. albopictus* mosquitoes. Also no correlation was observed between pH dependency of CHIKV fusion and infectivity to *Ae. albopictus* mosquitoes. Based on these data, I conclude that the E1-A226V mutation probably acts at different steps of the CHIKV life cycle, affecting multiple functions of the virus.

Using i.c. of Reunion and Ugandan strains of CHIKV, I demonsrated that mutations at positions 60 and 211 of the E2 glycoprotein of CHIKV are responsible for modulating the effect of the E1-A226V mutation on CHIKV infectivity for *Ae. albopictus*. Analysis of the effect of the mutations at E2-211 on CHIKV replication in cell culture and on CHIKV binding to the brush border membrane proteins of *Ae.albopictus* midgut cells, indicated that different residues at E2-211 might differentially affect the ability of CHIKV to interact with specific proteins expressed on the surface of midgut epithelial cells. I hypothesized, that after internalization by endocytosis, these interactions might determine the particular location within endosomal compartments where the CHIKV membrane fusion and release of virus nucleocapsid occur.

The information from the present study provides insight into the processes of CHIKV adaptation to a new vector species, which would determine the potential threat for spreading and establishment of CHIKV in tropical and temperate regions populated with *Ae. albopictus* mosquitoes.

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aa	amino acid
Ab	antibody
Ae.	*Aedes*
An.	*Anopheles*
ATP	adenoside triphosphate
BBM	brush border membrane
BHK	baby hamster kidney
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CHIKV	Chikungunya virus
CIP	Calf Intestine Alkaline Phosphatase
cm	centimeter
C6/36	*Aedes albopictus* larval cells
CNS	central nervous system
cpe	cytopathic effect
Cs.	*Culiseta*
Cx.	*Culex*
DAPI	4',6-Diamidino-2-phenylindole
DI	domain I
DII	domain II
DIII	domain III
DENV	dengue virus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpe	days post-electroporation
dpi	days post-infection
E. coli	*Escherichia coli*
ECSA	Eastern Central South African clade
EEEV	Eastern equine encephalitis virus
eGFP	enhanced green fluorescent protein
EM	electron microscopy
ER	endoplasmic reticulum
EtOH	ethanol
FBS	fetal bovine serum
h	hour
hpi	hours post-infection
HRP	horseradish peroxidase
HS	heparan sulfate
i.c.	infectious clone
IC	Infectious center
IFN	interferon
i.t.	intrathoracic
kDa	kiloDalton

L	liter
LB	Luria-Bertani
MAYV	Mayaro virus
MeOH	methanol
min	minute
ml	milliliter
mM	millimole
MOI	multiplicity of infection
mRNA	messenger RNA
NCR	non-coding region
ng	nanogram
nsP	non-structural protein
nt	nucleotide
Oc.	*Ochlerotatus*
OID	Oral infectious dose
ONNV	O'nyong-nyong virus
ORF	Open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PNK	T4 polynucleotide kinase
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
rpm	revolutions per minute
RRV	Ross River virus
RT	room temperature
RT-PCR	reverse transcriptase PCR
sec	second
SFV	Semliki forest virus
SG	Sub-genomic prototer
SINV	Sindbis virus
TBEV	tick-borne encephalitis virus
TCID	tissue culture infectious dose
TEM	transmission electron microscopy
TAP	Tobaco Acid pyrophosphatase
ts	temperature sensitive
U	unit
UTMB	University of Texas Medical Branch
µl	microliter
UV	ultraviolet
VEEV	Venezuelan equine encephalitis virus
VOPBA	virus overload protein binding assay
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WNV	West Nile virus

YFV	yellow fever virus

CHAPTER 1: INTRODUCTION TO CHIKUNGUNYA VIRUS

1.1 ALPHAVIRUSES BACKGROUND

The *Alphavirus* genus in the family *Togaviridae*, consists of a geographically widespread group of mainly mosquito-borne viruses, many of which are represented by numerous geographic variants or strains (Strauss and Strauss, 1994). Aside from two alphaviruses with no known vector (southern elephant seal virus and salmon pancreas disease virus (Weaver et al., 2000) all alphaviruses are typically maintained in the natural transmission cycle between specific mosquito vectors and susceptible vertebrate hosts, including reptiles, amphibians, birds and mammals. Based on geographic distribution, alphaviruses are divided into Old and New world viruses and antigenically are classified into seven complexes (Calisher et al., 1988) that mostly accurately reflect clades of viruses that share medically important characteristics. The most medically important New world viruses including Venezuelan (VEEV), eastern (EEEV) and western equine encephalitis virus (WEEV) are considered etiologic agents of encephalitis in humans and equids, whereas Old World alphaviruses are typically characterized by a severe arthralgia and rash syndrome.

Of the Old World alphaviruses, Chikungunya virus (CHIKV) is the most important human pathogen, which has caused numerous outbreaks of arthritic disease in people since it was first isolated in 1953 (Jupp and McIntosh, 1988). The most recent outbreak/epidemic of CHIKV which started in Kenya in 2004 (Kariuki Njenga et al., 2008), then spread to the islands of the Indian Ocean, India, Italy, Indonesia and Gabon, was the largest in the documented history and has rapidly elevated the virus to the status of a major global health problem (Simon et al., 2008).

1.1.1 Genome organization and virion structure of alphaviruses

Alphaviruses are positive sense RNA viruses approximately 12 kb in length, with a 5'methyl-guanosine cap and polyadenylated tail at the 3' end (Figure 1.1). The genome consists of two open reading frames (ORF), which are flanked by 5' and 3' non-coding

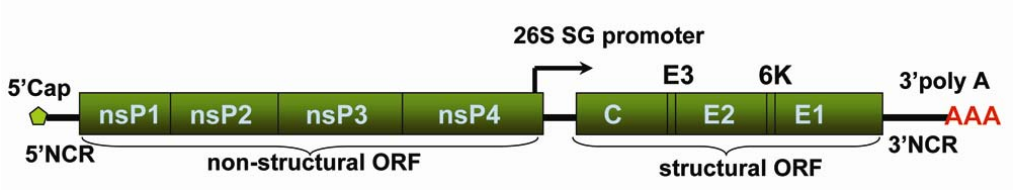

Figure 1.1 Genomic structure of Alphaviruses.

regions (NCR). Both NCRs contain signals important for replication of the viral RNA.

The first ORF (so-called non-structural ORF) is translated directly form full-length genomic RNA located in the 5' two-thirds of the genome and encodes four non-structural proteins (nsP1-4) that are involved in viral replication and processing. The second ORF located at 3' end of genome encodes three major structural protein genes: the capsid and envelope glycoproteins E2 and E1 (Figure 1.1) and two small proteins E3 and 6K. This ORF is translated from a 26S subgenomic RNA, which is transcribed from 26S subgenomic promoter located at the 3' end of the non-structural ORF.

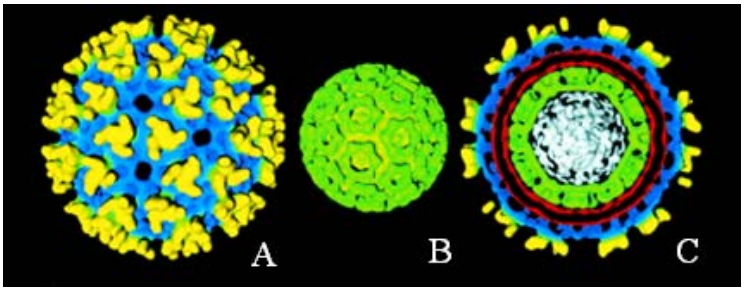

Figure 1.2 Cryo-EM reconstruction of alphavirus particle.

A) External view of the virion particle.
B) Nucleocapsid.
C) Cross Section of Virus Particle; white is the viral RNA, green is the nucleocapsid, red is the viral membrane, blue/yellow is the envelope.
Adapted from (Paredes et al., 2001) .

The mature virion of alphaviruses is spherical, about 70 nm in diameter and consists of an icosahedral nucleocapsid (T=4) surrounded by lipid bilayer and outer shell formed by E2-E1 glycoproteins (Figure 1.2). The nucleocapsid consists of genomic positive sense RNA, complexed with 240 copies of capsid protein. The lipid bilayer is derived from the host cell plasma membrane and is 4.8 nm in thickness. Viral glycoproteins E2 and E1 form heterodimers on the surface of the virus particle and are embedded in the lipid bilayer by conversional membrane-spanning anchors in the C-terminus. Two hundred and forty dimers, necessary to form a virion, are organized in 80 trimers of E1-E2 heterodimers that form spikes on the surface of the virion. E2 lies on top

of E1 and is believed to interact with cellular receptors (Strauss and Strauss, 1994). E1 underlying E2 mediates fusion of viral and cellular membranes during virus entry and is responsible for formation of icosahedral architecture of the virus envelope with T=4 triangulation (Kielian, 2006).

1.1.2 Non-structural proteins (synthesis)

Upon cell entry and uncoating, genomic RNA of alphaviruses serves as mRNA for translation of the viral non-structural proteins of alphaviruses and synthesis of the complementary minus RNA strand ($_{(-)}$RNA). $_{(-)}$RNA in turn provides the template for synthesis of both new genomic and subgenomic RNA. Depending on the virus and presence of the opal codon between nsP3 and nsP4, the genomic RNA is translated into one or two non-structural polyproteins (Strauss and Strauss, 1994). The shorter version P123 consisting of sequences of nsP1, nsP2 and nsP3 is produced when translation is terminated at an opal stop-codon at position 1857 (for many strains of CHIKV) of the nonstructural ORF at the C terminus of the nsP3 protein. The longer version P1234, containing in addition the sequence of nsP4, is produced upon read-through of this opal codon. Not all alphaviruses have the opal stop-codon and its role is not fully understood. The fact that sense and opal codon are present in closely related strains of Sindbis virus (SINV) suggests that the advantage of a sense codon is transient (Strauss and Strauss, 1994). Interestingly it has been shown that presence of an opal codon versus arginine in the O'nyong-nyong virus (ONNV) genome caused more efficient viral infection and led to earlier dissemination of the virus in its vector *Anopheles (An.) gambiae* mosquitoes (Myles et al., 2006), indicating that polymorphism at this position may play an important role in adaptation of alphaviruses to its mosquito vectors.

1.1.3 Non-structural proteins (functions)

nsP1 – experiments with temperature sensitive (*ts*) mutants indicated that nsP1 is specifically required for synthesis of $_{(-)}$RNA strand (Hahn et al., 1989a; Sawicki et al., 1981; Wang et al., 1991b). Also it is believed that nsP1 acts as a methyltransferase and guanyltransferase in the process of 5'methyl-guanosine cap formation (Durbin and Stollar, 1985; Mi and Stollar, 1990; Mi and Stollar, 1991). Lastly, it has been shown that

nsP1 can regulate the activity of the nsP2 protease. The presence of nsP1 significantly reduced cleavage between nsP2 and nsP3 in SINV by nsP2 (de Groot et al., 1990).

nsP2 – the nsP2 protein of alphaviruses is the largest among its non-structural proteins and possesses multiple enzymatic activities. It consists of 2 structural and functional domains (Russo et al., 2006). The N-terminal domain has been shown to possess ATPase, GTPase (Rikkonen et al., 1994) and RNA 5'-triphosphatase activity (Vasiljeva et al., 2000) and serves as a RNA helicase (Gomez de Cedron et al., 1999) to unwind the RNA-RNA duplex during RNA replication and transcription.

The C-terminal part of the protein has been associated with proteolytic processing of the alphavirus nonstructural polyprotein. It was suggested that nsP2 is a cysteine protease with C-481 and one of five conserved histidine residues in the region, defined by deletion mapping, form the catalytic dyad. The crystal structure of nsP2 later confirmed this finding (Russo et al., 2006). Also it was shown that the C terminal domain is involved in regulating the 26S subgenomic RNA synthesis (Suopanki et al., 1998), downregulating minus-strand RNA synthesis late in infection (Sawicki et al., 2006; Sawicki and Sawicki, 1993), targeting nsP2 for nuclear transport (Peranen et al., 1990), and RNA 5'-triphosphatase activity (Vasiljeva et al., 2000; Vasiljeva et al., 2003).

nsP2 has been shown to play an important role in suppression of the type I interferon response in the infected cells. The infection of NIH 3T3 cells with SINV bearing the mutation nsP2-P726G (which was originally selected for persistent SINV replication in BHK-21 cells) causes higher levels of IFN secretion and the activation of 170 cellular genes that are induced by IFN and/or virus replication (Frolova et al., 2002). A large proportion of nsP2 of SFV was found to localize in the nucleus, but inactivation of the nuclear localization sequence with the nsP2-R649D mutation leads to the virus that induces a significantly more robust IFN response in infected cells as compared with parental virus (Breakwell et al., 2007).

nsP3 – the role of this protein in alphavirus replication is not well understood. The nsP3 protein comprises three domains; the first is conserved among alphaviruses, coronaviruses, *Hepatitis E virus* and *Rubella virus* (Koonin and Dolja, 1993), the second is conserved among alphaviruses and the third C-terminal domain is hypervariable (Strauss and Strauss, 1994). nsP3 is a phosphoprotein and the phosphorylation sites have been mapped to serine and threonine residues, which are located mainly in the

hypervariable domain (Li et al., 1990; Peranen, 1991; Vihinen and Saarinen, 2000). The phosphorylation of this domain could play a role in negative-strand RNA synthesis (De et al., 2003). It is known that nsP3 plays a role in subgenomic 26S and negative-strand RNA synthesis (Hahn et al., 1989b; LaStarza et al., 1994b; Lemm and Rice, 1993; Shirako and Strauss, 1994; Wang et al., 1994). It has been proposed that it acts in concert with nsP1 to mediate association of the replication complex with cytoplasmic membrane structures (Peranen and Kaariainen, 1991; Peranen et al., 1988). It is also known to affect the cleavage specificity of the nsP2 proteinase (de Groot et al., 1990; Strauss and Strauss, 1994).

The role of the hypervariable domain is even less-well understood, however, several recent studies demonstrated that this region of nsP3 might modulate replication of SINV *in vitro* (Lastarza et al., 1994a), virulence and pathogenesis of Semliki forest virus (SFV) in mice model (Vihinen et al., 2001). It was suggested that, for VEEV, the hypervariable domain reflects adaptation to growth in different hosts or vectors species (Oberste et al., 1996).

nsP4 – is thought to be the RNA polymerase of the alphaviruses. It contains the GDD motif characteristic of viral RNA polymerases (Kamer and Argos, 1984), and its concentration is tightly regulated in most alphaviruses by read-through of the opal codon at the end of nsP3 and efficient degradation by the N-end rule pathway (Strauss and Strauss, 1994). However, a small fraction of nsP4 remains stable in infected cells, and it was suggested that nsP4 associated with the replicative complex is protected from rapid degradation.

1.1.4 Non-structural polyprotein processing and regulation of genome replication

Early studies of viral genome replication have shown that it is controlled by cleavage of the viral nonstructural protease. During, or shortly after translation, the full-length nonstructural polyprotein P1234 cleaves itself in *cis* to produce P123 and nsP4. This complex is inefficient for synthesis of $_{(+)}$RNA, but can make complementary $_{(-)}$RNA. Slow accumulation of P123 leads to cleavage of the polyprotein in *trans* between nsP1 and nsP2, which leads to formation of the complex capable of synthesis of both $_{(+)}$RNA and $_{(-)}$RNA, and is very efficient in cleavage between nsP2 and nsP3. This final step in

proteolytic processing gives rise to the complex that can synthesize only $_{(+)}$RNA and subgenomic RNA using the $_{(-)}$RNA strand as a template (Strauss and Strauss, 1994).

This tight regulation of polyprotein processing was also shown to be important in regulating cell antiviral responses by alphaviruses. Mutant SINV that are incapable of either P23 or P123 cleavage, induce a high level of type I interferon production by infected cells. These viruses were also incapable of spreading among cells that have no defects in α/β interferon (IFN-α/β) production and signaling; however they were capable of efficient growth in α/β interferon-deficient cell lines. Moreover, P123-cleavage-deficient virus was readily eliminated, even from the already infected cells with no defects in α/β interferon (Gorchakov et al., 2008).

1.1.5 Structural proteins (synthesis)

Structural proteins of alphaviruses are translated from 26S subgenomic RNA as a single polyprotein, which is co- and post-translationally processed leading to formation of 3 main structural proteins (capsid, E2 and E1) and 2 minor proteins E3 and 6K which are not required in the mature virion.

The capsid protein self-releases from the nascent polyprotein chain due to serine protease activity located at the C-terminal part of the protein (Strauss and Strauss, 1990). After release of the capsid protein, the N-terminal signal sequence leads to insertion of nascent polyprotein into the endoplasmatic reticulum to produce the E2 glycoprotein precursor PE2. The carbohydrate chain is added early to the signal sequence of PE2, while the polypeptide is nascent, suggesting that the N terminus of PE2 is released from the ER membrane into the lumen very soon after translation. The signal sequence, therefore, plays no role in further translocation of the protein (Garoff et al., 1990; Sefton, 1977). The translocation stops at the hydrophobic stretch at the C terminus of the PE2 protein, which serves as an anchor for E2. Downstream sequence contains another translocation signal, which targets nascent polypeptide back into the ER where it is cleaved by host cell signalase releasing PE2 from the 6K peptide. The sequence of 6K consists of an N-terminal translocation stop signal, small cytoplasmic domain and hydrophobic stretch, which serves as translational signal for E1 protein. 6K is released from E1 by host cell signalase (Schlesinger and Schlesinger, 2001b).

PE2, 6K and E1 move together as a complex through secretory vesicles from their synthesis site to their final destination at the cell plasma membrane (Schlesinger and Schlesinger, 2001b). During this movement, carbohydrate chains on PE2 and E1, which were added to the proteins in the ER during synthesis, are modified by the enzymes located at the various compartments of the secretory system in the manner resembling modification of the cellular secreted proteins. The precise nature of the modifications varies depending on the host cells in which virus is replicating.

The heterodimers of PE2 and E1 form soon after synthesis in the ER and are relatively stable to low-pH exposure. It is believed that PE2 acts as a chaperone for the E1 glycoprotein. It prevents E1 from adapting a thermodynamically stable conformation (Andersson et al., 1997; Doms et al., 1993). Activation of the heterodimers occurs after proteolytic cleavage of PE2 by furin protease to form E3 and E2 (Moehring et al., 1993; Molinari and Helenius, 1999; Sariola et al., 1995). E3 is a small protein that is lost in most alphaviruses into the culture fluid whereas E2 in complex with E1 stays anchored to the membrane.

1.1.6 Structural proteins (structure and functions)

Capsid – is a multifunctional protein, acting at different stages in the viral life cycle. Capsid protein interacts with genomic RNA and forms a nucleocapsid core structure berneath the viral membrane. It has been shown to act as an autoprotease, to recognize the genomic RNA, and to assemble into an ordered protein shell (Warrier et al., 2008). The crystal structure of the C terminal protease domain has been solved, revealing chymotrypsin-like organization (Choi et al., 1991; Skoging et al., 1996; Tong et al., 1993), suggesting that this protein was derived from a cellular serine protease during evolution of the virus (Strauss and Strauss, 2002). The crystal structure also revealed a hydrophobic pocket in the capsid protein, which appears to interact with the cytoplasmic tail of the E2 protein, and which is critical for virion formation and virus budding. The same hydrophobic pocket also appears to play a role in nuclocapsid assembly (Skoging et al., 1996). The regions in the capsid protein that bind specifically to RNAs have been extensively studied, revealing that a stretch of 76-107 aa in SINV is responsible for interaction with the encapsidation signal in the genomic RNA (Geigenmuller-Gnirke et al., 1993) and the region of 68 amino acids (residues 1 to 10 and 75 to 132) possess

almost full binding activity (Geigenmuller-Gnirke et al., 1993). Interestingly it was found that the region between residues 99 and 113 can interact with the large ribosome subunit (Wengler et al., 1992), which is now believed to play a critical role in nucleocapsid disassembly during the infection process (Strauss and Strauss, 1994).

The capsid protein of New World alphaviruses recently was found to regulate cell antiviral mechanisms (Aguilar et al., 2007; Garmashova et al., 2007a; Garmashova et al., 2007b), in the manner similar to those which were found for the nsP2 protein in Old World alphaviruses (Ryman and Klimstra, 2008).

6K – is a small (58-61 amino acids), hydrophobic protein associated with membranes and incorporated in small amounts into the virion. The function of the 6K protein in the virus life cycle is not fully understood: virions lacking 6K are indistinguishable from wild-type particles, indicating that 6K is not required for infectivity. It was found that mutations in 6K affect virus trafficking, virus assembly, and budding (Liljestrom et al., 1991) (Loewy et al., 1995). It was postulated that 6K influences the selection of the lipids that interact with transmembrane domains of the E2-E1 heterodimers, which might control deformability of the lipid bilayer (Cadd et al., 1997). Recently it has been shown that 6K protein can form an ion channel in the lipid bilayer, which could influence the plasma membrane electric potential (Melton et al., 2002). Considering that alphavirus maturation requires a high intracellular concentration of Na^+ ions (Ulug et al., 1989), and that the alterations in ion transport induced by virus infection may facilitate the efficient release of virus particles (Garry et al., 1979; Ulug et al., 1984) it is tempting to suggest that the 6K ion channel might be intricately involved in this process.

E2 – is a ~50 kDa type I transmembrane protein. It consists of an N-terminal (1–364, SINV numbering) hydrophilic region, followed by a 26 residue membrane-spanning region (365–390) and a 33 residue (391–423) cytoplasmic endodomain. SINV E2 is glycosylated with two asparagine (N)-linked carbohydrate chains at N-196 and N-318 (Mayne et al., 1985). In addition, certain cysteine residues in the cytoplasmic domain and membrane-spanning region of E2 have been implicated as sites for palmitoylation (Schmidt et al., 1979). It is believed that the main function of the E2 glycoprotein during the course of the alphavirus life cycle is interaction with specific cell surface receptor(s) (will be discussed below). Although alphaviruses have long served as a model system for

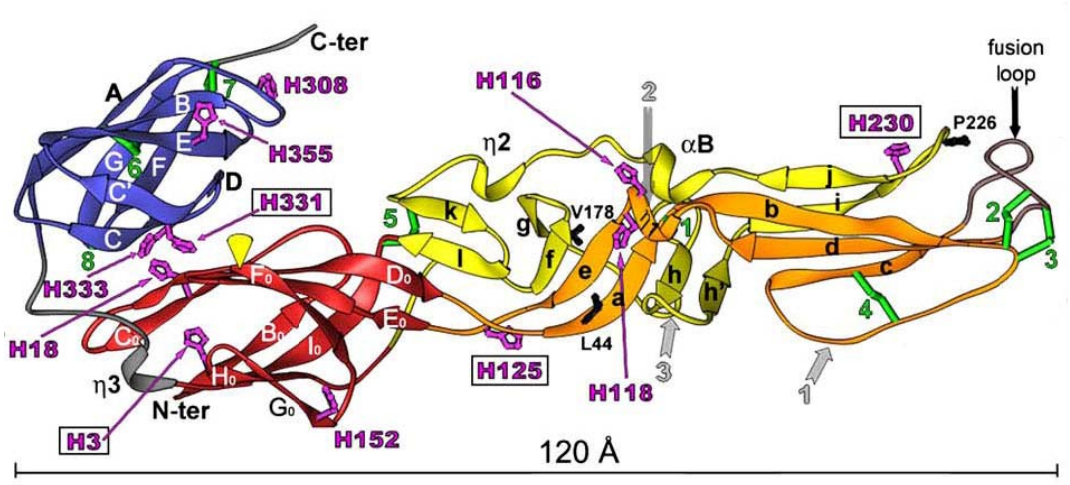

Figure 1.3 Structure of SFV E1.

Ribbon diagram colored according to domains, by using the standard class II color coding (red, yellow, and blue for DI, DII, and DIII, respectively). Adapted from (Roussel et al., 2006)

the study of virus structure and assembly, an atomic resolution structure for E2 has yet to be produced (Navaratnarajah and Kuhn, 2007).

E1 –is responsible for triggering fusion of the viral and target cell membranes during entry (discussed in section 1.1.7). The crystal structure of the E1 protein of SFV has recently been solved (Figure 1.3) revealing a striking similarity between the E1 protein of alphaviruses and the E protein of flaviviruses, supporting the hypothesis of the common origin of these proteins in two very distant virus genera (Lescar et al., 2001; Strauss and Strauss, 2001). The similarities in structure and mechanisms of action of the E and E1 proteins set them well apart from previously well-characterized membrane fusion proteins such as influenza virus haemagglutinin that introduced the concept of a separate class of fusion proteins, class II (Kielian and Rey, 2006).

The E1 protein of SFV follows a complex path, resulting in three globular domains (DI, DII and DIII) conprised primarily of β-sheets (Figure 1.3). The C terminus of E1 and the fusion peptide are found at the two ends of a rod-like molecule of ~120 Å in length. The DI is an eight-stranded β-sheet sandwich with simple up-and-down topology (Kielian and Rey, 2006). Two of the connections between adjacent strands in this sandwich are long and elaborated, and comprise the 'finger-like' DII with the fusion loop at the tip of the molecule and adjacent ij loop that are responsible for interacting with target membranes. The mutations in fusion peptide significantly shift the pH threshold for fusion (Duffus et al., 1995), and mutation of E1-226 located at the tip of the

ij loop affects the lipid dependence of alphavirus entry into and exit from cells (Chatterjee et al., 2000; Lu et al., 1999; Vashishtha et al., 1998). DIII, which lies at the opposite end of domain I, has an immunoglobulin-superfamily fold and is connected to the C terminus of DI by an ~12 amino-acid polypeptide (Kielian and Rey, 2006). The subsequent membrane region of E1 interacts with the membrane region of E2, which plays a role in virus assembly and fusion (Sjoberg and Garoff, 2003). The connection between DI and DII called "the hinge" forms a flexible region, which allows rotations of DI and DII along the axis passing between the connecting and central β sheets. This flexibility is believed to be important for the biological function of the molecule. Mutations in this region were shown to modulate sphingolipid and cholesterol dependence of the SFV (Chatterjee et al., 2002).

1.1.7 Virus entry into the cell

Alphaviruses infect cells by receptor-mediated endocytosis followed by low pH-triggered membrane fusion reaction (Helenius et al., 1980). Alphaviruses are known to replicate in a wide range of hosts both *in vitro* and *in vivo* (Schlesinger, 2001). It has been speculated that the receptor is either a single surface molecule that is common in a variety of host cells or a series of several different molecules that are used for attachment to the host cell (Schlesinger, 2001). A number of potential cellular receptors/viral-binding molecules for alphaviruses have been examined and partially characterized. DC-SIGN and L-SIGN lectins have been shown to be attachment receptors for SINV on human monocytic THP-1 cells, possibly by binding of virions to carbohydrate moieties (Klimstra et al., 2003). Interaction with heparan sulfate (HS), a negatively charged glycosaminoglycan (GAG) found on the surfaces of most cells, has been implicated for a number of alphaviruses including: SINV (Byrnes and Griffin, 1998; Klimstra et al., 1998; Smit et al., 2002), VEEV (Bernard et al., 2000), and Ross River virus (RRV) (Heil et al., 2001) as an attachment mechanism preceding virus entry. The results of several studies have indicated that non-HS-binding strains of SINV (Byrnes and Griffin, 2000; Klimstra et al., 1998), VEEV (Bernard et al., 2000; Wang et al., 2003), foot-and-mouth disease virus (Hulst et al., 2000; Neff et al., 1998), tick-borne encephalitis virus (Mandl et al., 2001), and classical swine fever virus (Hulst et al., 2000) are more virulent in animal models than their HS-binding counterparts, suggesting that for these viruses, the HS

binding phenotype is a tissue culture adaptation that lowers virus fitness *in vivo*. Binding of alphaviruses to heparan sulfate is largely dependent on positively charged amino acids in E2 (Bernard et al., 2000; Byrnes and Griffin, 2000; Klimstra et al., 1998). It was suggested that binding to heparan sulfate is probably insufficient for viral entry into cells and the entry receptor is most likely to be a distinct molecular entity (Heil et al., 2001).

A cellular receptor for SINV in BHK-21 cells has been reported to be the 67-kDa high-affinity laminin receptor (Wang et al., 1992); another laminin receptor was identified for VEEV in mosquito C6/36 cells (Ludwig et al., 1996). Laminin receptor genes are highly conserved among vertebrates (Wang et al., 1992) and share significant sequence similarity between mammals and insects (Tsetsarkin K. unpublished data) which may be in part responsible for the broad host range exhibited by the virus, which must replicate in both vertebrate and insect hosts in nature. Additionally, two unknown proteins, a 74- and a 110-kDa proteins, have also been reported as possible receptors for SINV on mouse neuroblastoma cells (Ubol and Griffin, 1991); as well as a 63-kDa protein on chicken cells (Wang et al., 1991a). La Linn et al. (2005) provide evidence that RRV (and possible other arthritogenic alphaviruses) uses the collagen-binding α1β1 integrin as its cellular receptor (La Linn et al., 2005). Using virus overlay immunoassay Mourya et al. (1998) found two proteins (putative receptors) of 60 and 38 kDa, for CHIKV in the brush border membrane fraction of the normal population of *Ae. aegypti* mosquitoes (Mourya et al., 1998). Unfortunately, no follow up work has been done to characterize these proteins biochemically and physiologically to confirm that these proteins indeed serve as a cell surface receptors for CHIKV. No mosquito midgut proteins have been proven to serve as cellular receptors for alphaviruses.

The uptake pathway has been particularly well studied for SFV, SINV and VEEV. Early studies identified that internalization of alphaviruses into endosomes occurs through clathrin-coated pits (Helenius et al., 1980; Marsh et al., 1984; White and Helenius, 1980) and is dependent on the cellular proteins dynamin, Eps15 and Rab5 (DeTulleo and Kirchhausen, 1998; Earp et al., 2003; Sieczkarski and Whittaker, 2002; Sieczkarski and Whittaker, 2003). The acidic pH of endosomes triggers the fusion reaction between viral and endosomal membranes and release of viral nucleocapsid in cytoplasm. It is believed that fusion of Old World alphaviruses occurs in early endosomes, whereas entry of VEEV (New World alphaviruses) into mammalian and

mosquito cells is dependent on presence of functional Rab7 protein and requires late endosome formation (Colpitts et al., 2007; Kolokoltsov et al., 2006).

It has been shown that fusion of SFV and SINV is cholesterol-dependent and is promoted by presence of low concentrations of sphingolipids in the target membrane (Lu et al., 1999; Nieva et al., 1994; Phalen and Kielian, 1991; Smit et al., 1999). However, the effects of cholesterol and sphingolipids on viral entry do not appear to be due to their combined physical effects on the membrane such as changes in membrane fluidity or formation of lipid rafts (Waarts et al., 2002). Interestingly, studies of VEEV and CHIKV demonstrated that cell entry of these viruses is insensitive to cholesterol sequestration from cell membranes (Kolokoltsov et al., 2006; Tsetsarkin et al., 2007). Furthermore, it has been shown that mutations in the E1 protein can modulate the requirements of cholesterol (and sphingolipids) for fusion of SFV, SINV (Chatterjee et al., 2002; Lu et al., 1999; Vashishtha et al., 1998), which suggests that cholesterol dependence is not absolute and can be acquired or lost by alphaviruses as a result of adaptation to specific conditions present in the natural transmission cycle.

The low pH of endosomes triggers a series of conformational changes in viral glycoproteins, which result in the membrane fusion reaction. The reaction is initiated by dissociation of E2-E1 dimers resulting in the exposure of the E1 fusion loop, which then inserts into the target membrane of host cell. The trimeric form of E1 is required for fusion (Kielian et al., 1996), and the kinetics and pH dependence of trimer formation closely correlate with those of membrane fusion (Bron et al., 1993; Glomb-Reinmund and Kielian, 1998b; Smit et al., 2001). Studies with soluble form of the E1 protein lacking the transmembrane region demonstrate that membrane insertion is highly cooperative and leads to the formation of a lattice composed of rings of 5 and 6 trimers on the surface of the liposomes (Gibbons et al., 2003).

The atomic structure of the low-pH induced E1 homotrimer has been solved, revealing that the homotrimer is composed of a central ‘‘core trimer’’ made up of domains I and II of the E1 protein, with the three fusion loops at the tip of the trimer (Gibbons et al., 2004b). The two adjacent E1 molecules form the groove along the tangential side of the trimer, which is occupied by stem region that connects the domain III of E1 to the virus transmembrane anchor. It is thought that after E1 homotrimer formation, the domain III and the stem region fold back towards the fusion loop, which

brings viral and cellular membranes in to close proximity (hairpin conformation). The cooperative interaction of the trimers through their fusion loops distorts the target and virus membranes leading to mixing of the outer leaflets (hemifusion), which subsequently results in the complete fusion with mixing of the inner membrane leaflets. This model of fusion very closely resembles the fusion mechanism used by class I fusion proteins, which strongly suggest that both classes act by a single, universal mechanism to cause membrane fusion (Kielian, 2006; Kielian and Rey, 2006).

Released nucleocapsids undergo uncoating in the cytoplasm, which is triggered by binding to ribosomes. Once released from its coat, the viral RNA is accessible to ribosomes for initiation of translation (Schlesinger and Schlesinger, 2001a).

1.1.8 Replication in mammalian and mosquito cells

Alphaviruses multiply rapidly in a diverse variety of cell cultures, including most mammalian cell lines as well as mosquito and tick cells (Strauss and Strauss, 1994). Alphaviruses generally persistently infect insect cells, but in mammalian cells they cause acute cytolytic infection, which is accompanied by inhibition of the macromolecular synthesis on the level of translation and controlled by a variety of mechanisms. The proposed mechanisms for translational shut-off include: inhibition of host cell factors required for protein synthesis, competition for translation of viral mRNA, changes in ionic intracellular environment, and direct inhibition by the viral capsid protein.

Replication of alphaviruses in mammalian cells usually induces apoptosis. Block of cellular apoptosis by expression of anti-apoptotic gene *bcl-2* in BHK-21, mouse neuroblastoma (N18), and rat prostatic adenocarcinoma (AT-3) cells results in long-term persistent productive viral infection (Levine et al., 1993). Interestingly, the single amino acid change E2-Q55H in SINV is sufficient to overcome the anti-apoptotic effect of the *bcl-2* gene in AT-3 by an unknown mechanism (Ubol et al., 1994).

In mosquito cells, alphaviruses usually cause persistent infection with efficient viral replication during the first 48 h, followed by a decline in virus production, which can be maintained indefinitely. The mechanisms responsible for persistent infection are unknown; however, several cellular factors have been characterized that restrict virus production. These factors appear to be cell and virus specific in that they act only in particular cell types and against only specific viral species (Condreay and Brown, 1986;

Condreay and Brown, 1988). For SINV the antiviral factor was shown to be 3.2kDa polypeptide. Treatment of the cells with this factor leads to establishment of an antiviral state (Luo and Brown, 1993). It has been suggested that lysosome associated 55-kDa protein is involved in resistance to viral infection (Luo and Brown, 1994). The existence of intracellular factors that restrict virus production was also supported by an experiment showing that infection of U4.4 cells at extremely low MOIs does not affect progression of the infection to the high acute phase (Karpf et al., 1997). Also, recently is has been shown, that RNA interference is a likely mechanism, that controlls alphavirus replication in mosquitoes (Keene et al., 2004; Sanchez-Vargas et al., 2004).

1.1.9 Full-length cDNA clones of alphaviruses

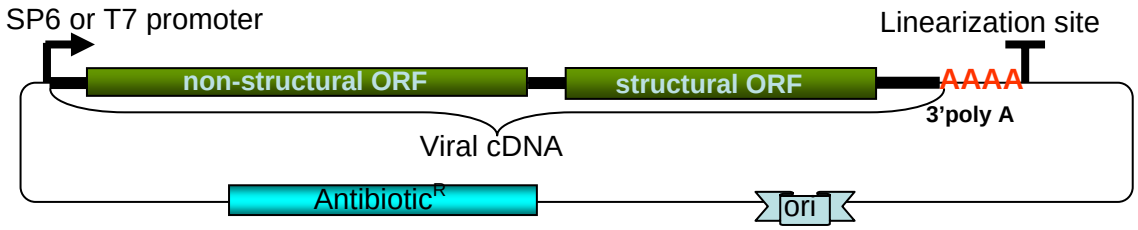

Figure 1.4 Infectious cDNA clone of alphaviruses.

The significant advancements in our knowledge about the mechanisms of alphavirus entry into cells, replication within and exit, along with understanding of the mechanisms of pathogenesis, transmission by mosquito vectors and viral evolution would not have been impossible without development of full-length cDNA clones corresponding to the entire genome of alphaviruses. The typical genetic organization of such clones is depicted in Figure 1.4. Viral cDNA is placed under control of a promoter for RNA polymerase, usually T7 or SP6. At the 3' end of genome after a short polyA tract the unique restriction endonuclease site allows precise termination of the *in vitro* transcription reaction initiated from the RNA polymerase promoter. The rest of the clone contains the antibiotic resistance gene and the origin of replication that allows propagation of the clone in the *E. coli*. Using standard molecular biology techniques, viral cDNA can be easily genetically manipulated and serve as a template for *in vitro* transcription. The alphaviral RNA is infectious and can be transfected into susceptible

cells where it leads to replication and virion production. The full-length infectious clones have been developed for almost all medically important alphaviruses including SINV (Klimstra et al., 1998; Rice et al., 1987), SFV (Liljestrom et al., 1991) VEEV (Davis et al., 1989), EEEV (Aguilar et al., 2008), RRV (Kuhn et al., 1991), ONNV (Brault et al., 2004a), CHIKV (Vanlandingham et al., 2005b) and Mayaro virus (Tsetsarkin K., Forrester N. unpublished data).

1.2 MOSQUITO VIRUS INTERACTION

CHIKV, like the most alphaviruses is an arbovirus, which by definition is maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by haematophagus arthropods. It multiplies and produces viremia in the vertebrates, multiplies in the tissues of arthropods, and is transmitted to new vertebrates by the bites of arthropods after an extrinsic incubation period (EIP).

1.2.1 Basic mosquito biology

Mosquitoes are classified in the class *Insecta*, order *Diptera*, family *Culicidae*. Adult female mosquitoes have piercing-siphoning mouthparts adapted for drawing blood out of vertebrate animals. Mosquitoes have three main body regions, the head, thorax, and abdomen, and adults possess three pairs of legs and a pair of wings attached to the thorax. These insects possess an open circulatory system within the body cavity called the hemocoel and respire via a tracheal system (Kondratieff and Black, 2005). Mosquito reproduction is sexual, and during development they undergo complete metamorphosis, which includes morphologically distinct life stages: egg, larvae, pupae, and adult. The length of each life stage depends on environmental conditions such as ambient temperature and availability of food sources. For *Ae. albopictus* and *Ae. aegypti* it takes approximately 10 days during warmer months. Adult male and female mosquitoes feed on nectar or other carbohydrate sources, and only females feed on the blood of vertebrates to facilitate high-energy-requiring egg production (Calvo et al., 2004).

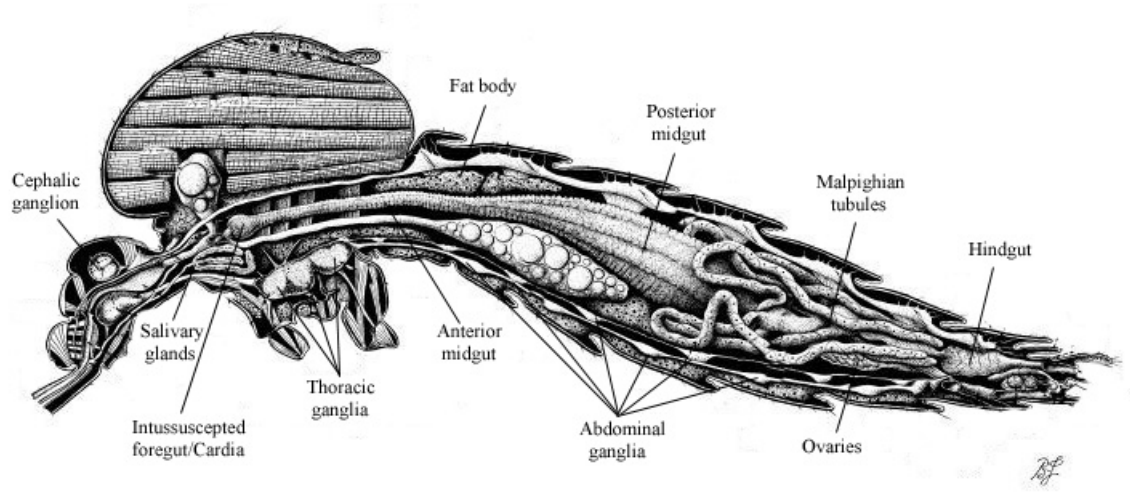

Figure 1.5 Anatomy of the adult female mosquito.

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1.2.2 Anatomy

The anatomy of a female adult mosquito is depicted in Figure 1.5. The successful biological viral transmission by mosquito vectors requires infection of and replication in the midgut epithelial cells, dissemination from the midgut to hemocoel and eventually infection of the mosquito salivary glands, from where alphaviruses can be transmitted to the next vertebrate host during subsequent mosquito feeding. Therefore, the most important organs with respect to viral transmission are the mosquito midgut and the salivary glands.

The mosquito digestive system consists of foregut, midgut and hindgut. The foregut contains cibarial and pharengial pumps, which via contraction enable blood to be sucked into the oesophagus and passed directly to the midgut. The mosquito midgut is a tubular organ comprised mainly of columnar epithelial cells with an underlying basement membrane, or basal lamina. This organ is divided into bulbous cardia, narrow anterior and flask-shaped posterior sections, and it is the posterior midgut which collects and digests the bloodmeal (Pennington and Wells, 2005). Viral infection of the midgut epithelium is most likely a receptor-mediated event, which follows virus attachment, penetration, and uncoating.

Following uptake of a blood meal, midgut epithelial cells initiate secretion of a number of enzymes to facilitate digestion, such as the protease trypsins, aminopeptidases and estrases. It was shown that presence of these enzymes may affect the midgut infection for some arboviruses (Hardy et al., 1983; Ludwig et al., 1989; Molina-Cruz et al., 2005). Also midgut epithelial cells are responsible for secretion of the peritrophic matrix in response to feeding. The peritrophic matrix is made up of a number of compounds including chitin that surrounds the bloodmeal several hours after feeding and serves to protect midgut epithelia from chemical and mechanical damage. It has been hypothesized that this matrix inhibits virus infection by blocking adsorption of virions by the midgut epithelium [reviewed by (Chamberlain and Sudia, 1961; Hardy et al., 1983; Higgs, 2004)]. However, dsRNA-mediated inhibition of chitinase activity or chitin synthetase activity (resulting in thicker or thinner-to-no peritrophic matrix formation, respectively) had no effect on DENV-2 infection or dissemination rates in *Ae. aegypti*, indicating that the peritrophic matrix does not interfere with arbovirus infection processes, at least for this virus-mosquito pair (Kato et al., 2008).

Mosquito salivary glands are morphologically paired tubular organs located in the thorax flanking the oesophagus, with glands connected to the intima. Each gland includes two lobes; lateral and median lobes, which consists of central ducts surrounded by single layers of epithelial cells that are bound by a basal lamina (James and Rossignol, 1991). A common lateral duct connects the pair of salivary glands and opens at the base of the hypopharinx. The main functions of salivary glands are synthesis and secretion of saliva and readsorption of salts (James and Rossignol, 1991).

With respect to viral infection, all sections of salivary glands appear to be susceptible to virus infection, although the sections infected vary by virus-mosquito pair. For example, DENV infection of *Ae. albopictus* salivary glands appears to be confined to the lateral lobes (Gubler and Rosen, 1976), which are involved in blood-feeding, wheras YFV, RVFV and WNV are also able to infect the median lobes of *Ae. aegypti*, *Cx. pipiens*, and *Cx. p. quinquefasciatus* salivary glands, which are involved in sugar-feeding (Girard et al., 2004; Miller and Mitchell, 1991b; Romoser et al., 1992).

1.2.3 Viral infection processes in a permissive mosquito vector

In general, the processes of virus-vector interaction are controlled by viral, vector, and environmental factors. The vector factors include the genetic control of susceptibility to virus infection. For CHIKV it was established that different geographic strains of *Ae. aegypti* and *Ae. albopictus* have different levels of susceptibility to the virus (Banerjee et al., 1988; Tesh et al., 1976; Turell et al., 1992). The genetic analysis of the Rosy eye strain of *Ae. aegypti*, which was found to be refractory to CHIKV infection revealed that gene(s) for refractoriness to CHIKV is dominant and appears to be closely linked with a rosy eye mutation (Mourya et al., 1994). Unfortunately no work followed this study to further characterize genes involved in refractoriness to CHIKV.

It was shown that susceptibility of South American strains of *Ae. alpbopictus* to VEEV is significantly higher as compared to North American strains (Beaman and Turell, 1991). The same authors showed that the Gentilly strain of *Ae. alpbopictus* collected in New Orleans, LA, was even more susceptible to VEEV as compared to previously tested North American and South American strains. More importantly it was shown that *Ae. alpbopictus* strains, which demonstrated higher susceptibility to VEEV infection, were also more prone to infection with CHIKV. This may indicate shared receptor sites for these two alphaviruses in *Ae. albopictus* (Turell and Beaman, 1992). Significant differences in the susceptibility of *Ae. alpbopictus* to VEEV subtype IC were also found between rural and urban populations of the species in Sao Paulo state Brazil (Fernandez et al., 2003).

Environmental factors include temperature, availability of food sources, and co-existence of a virus circulation in nature with a susceptible vector. Of these, the effect of ambient temperature on the extrinsic incubation period has been well documented, with higher temperatures resulting in more rapid dissemination of virus in a mosquito and, therefore a shorter EIP, whereas lower temperatures result in a longer EIP. These effects have been documented for EEEV in *Ochlerotatus. triseriatus* (Chamberlain and Sudia, 1955), RRV in *Oc. vigilax* (Kay and Jennings, 2002) YFV in *Ae. aegypti* (Davis, 1931), Rift Valley fever virus (RVFV) in *Cx. pipiens* (Turell et al., 1985), DENV-2 in *Ae. aegypti* (Watts et al., 1987), WNV in *Cx. tarsalis* (Reisen et al., 2006). The effect of temperature has obvious consequences for viral transmission in nature, the seasonality of outbreaks of some viruses, and virus distribution in nature. Finally, the viral factors are

discussed in subsequent sections of this chapter (section 1.2.5) with particular emphasis on viral genetics.

1.2.4 Microscopic analyses of virus-mosquito interactions

Among alphaviruses, VEEV and EEEV have been the most characterized with respect to infection processes in their principal vectors. Larsen at al. (1971) demonstrated that VEEV penetrates the digestive tract and reaches the brain within 48 hours after the infective feeding (Larsen and Ashley, 1971). They concluded that VEE virus appears to be pantrophic in tissues of *Ae aegypti* and it showed a preference for the salivary glands, where it was markedly concentrated by the 8th day after infection. Scott and Burrage (1984) determined that EEEV was localized in the hindgut, nervous tissue and salivary glands following replication in the posterior midgut of *Culiseta (Cs) melanura* mosquitoes, and they hypothesized that salivary gland infection occurred via the hemolymph (Scott and Burrage, 1984). Similar conclusions were obtained by Weaver et al. (1990) indicating that dissemination of the EEEV to salivary glands is a relatively rapid process (Weaver et al., 1990). Weaver et al. (1991) also characterized early infection processes of EEEV in *Cs. melanura* following oral infection with radiolabeled virus and found that the posterior midgut was most heavily infected, while the anterior midgut was also infected at a low rate 1-2 hpi. and virus was deposited in the folds of the cardia (Weaver et al., 1991). Dissemination was hypothesized to be facilitated by virus trapped in the cardia, which could then spread to the intussuscepted foregut. Interestingly a small number (4/25) of mosquitoes examined showed signs of leaky abdominal midguts, with virus detected in the abdominal hemocoel. Weaver (1986) studying the processes of VEEV infection of its enzootic vector *Cx. taeniopus*, found that virus accumulates in the abdominal fat body within 1 hr following the infectious blood meal engorgement by some unknown mechanism and without any evidence of mechanical disruption of the posterior midgut prior to replication within the vector (Weaver, 1986). Rapid dissemination of virus in these studies was hypothesized to have been facilitated by a physical break in the posterior midgut, described as a“leaky midgut”.

Investigation of EEEV infection of *Cs. melanura* by light and electron microscopy revealed that replication of EEEV is not a benign process and causes significant pathological changes in midgut cells associated with disruption of the midgut

basal lamina (Weaver et al., 1988). These authors concluded that the observed phenomena may result in bypassing of barriers to virus dissemination within the mosquito and allow rapid transmission to occur. Similar pathological changes were observed in the midgut of *Cx. tarsalis* infected with Western equine encephalomyelitis virus (WEEV), suggesting that alphaviruses in general may adversely affect their mosquito vectors in nature (Weaver et al., 1992).

1.2.5 Role of viral genetics in infection processes

It has been shown that different strains of alphaviruses may vary considerably with respect to their ability to infect particular species of mosquito. The first evidence that viral genetics could affect mosquito infectivity came from study of VEEV and *Ae. taeniorhynchus* (Kramer and Scherer, 1976). It was shown that *Ae. taeniorhynchus* mosquitoes transmitted two epizootic strains of VEEV with a higher rate than two enzootic strains (subtype IE). A study of WEEV and *Ae. trivittatus* (Green et al., 1980) showed that *Ae. trivittatus* mosquitoes were more susceptible for a WEEV strain isolated from this species than a WEEV strain isolated from *Cx. tarsalis*, even though the site, date of collection, and passage history of these isolates were identical. Examination of the ability of epizootic (subtype IAB) and enzootic (subtype IE) VEEV strains to infect and disseminate in an enzootic vector *Cx.* (*Melanoconion*) *taeniopus*, revealed that the two epizootic strains were able to infect mosquitoes at a low rate (17 to 20%) following oral infection and did not disseminate, compared with 100% infection following intrathoracic inoculation. In contrast the enzootic viruses efficiently infected mosquitoes even at low doses (> 90%). The authors hypothesized that the enzootic viruses absorb more efficiently to *Cx*. *taeniopus* posterior midgut epithelial cells than do the epizootic viruses, accounting for the low infection rate. It was also hypothesized that escape from the midgut is dependent on the virus titer that reached in that organ, i.e, threshold effect, which explains the inability of the epizootic virus strains to disseminate (Weaver et al., 1984). The differences in virus infectivity for enzootic and epizootic strains of VEEV were later confirmed for Central American strains of *Cx. taeniopus* and *Ae. taeniorhynchus* (Turell et al., 1999; Turell et al., 2003).

Also differences in infectivity for and transmissibility by colonized strains of *Ae. aegypti* and *Ae. albopictus* were observed for different strains of CHIKV (Mourya et al., 1987; Tesh et al., 1976).

The rapid advances in molecular biology in the eighties facilitated the following studies focused on the detailed role of the viral genes in infection processes. Woodward et al. (1991) identified that a single aa change, E2-I207F, in monoclonal antibody-resistant variant of VEEV is sufficient to decrease the infection rate from 80% to 60% and the dissemination rate from 38% to 0% in *Ae. aegypti* as compared with wild-type parent virus (Woodward et al., 1991). However the artificial conditions of selection of the E2-I207F mutation with respect to natural transmission of the VEEV made it difficult to interpret the biological role of these findings.

The new insights into the role of viral genes in adaptation of alphaviruses to particular mosquito species came form studies of VEEV and its epizootic vector *Ae. taeniorhynchus*. As was mentioned earlier, VEEV subtype IAB and IC easily infect this vector whereas enzootic subtype IE is significantly less infectious. The analysis of the mosquito infectivity of the chimeric viruses generated between IAB and IE VEEV subtypes, by exchanging the structural and nonstructural portions of the genome, revealed that viral determinants of *Ae. taeniorhynchus* mosquito infectivity lie within structural proteins. To map the determinants of mosquito infectivity more precisely, additional chimeric viruses were generated which contained PE2 gene of subtype IAB in the backbone of IE and PE2 gene of subtype ID in the backbone of the IAB and IC. Mosquito infectivity profile of these viruses clearly demonstrated that PE2 gene of VEEV harbors the major determinants of infectivity for *Ae. taeniorhynchus* (Brault et al., 2002). Unfortunately, the high degree of sequences divergence between enzootic and epizootic subtypes precluded further analysis of the precise locations of determinants of vector infection. This work also provided the first evidence that viral adaptation to a new mosquito vector could mediate its emergence (Brault et al., 2002). This hypothesis was further supported by studies of the 1993 and 1996 outbreaks of VEEV virus in the Mexican states of Chiapas and Oaxaca. The unusual feature of these outbreaks was involvement of the typically enzootic strains of VEEV subtype IE. These strains produce relatively low viremia in the equids, which is inconsistent with VEE emergence mechanisms proposed for epizootic strains in the subtypes IAB and IC (Weaver and

Barrett, 2004). The analysis of infection rates of enzootic and epizootic strains of VEEV subtype IE in *Ae. taeniorhynchus* (epizootic vector) mosquitoes, revealed that mosquito infectivity was significantly higher for epizootic strains of VEEV as compared to enzootic strains. The E2 protein sequence of enzootic and epizootic strains differs only by three positions and introduction of these mutations from epizootic into the background of enzootic strain of VEEV was sufficient to increase viral infectivity for *Ae. taeniorhynchus* to levels of epizootic strains, supporting the previous finding of the major role of the E2 protein in determining alphavirus infectivity for its mosquito vector. Subsequent detailed analysis of the roles of each mutation implicated the E2-S218N substitution in the E2 envelope glycoprotein as the major determinant of the increased vector infectivity phenotype. This finding demonstrated for the first time that single amino acid mutation could be sufficient for efficient adaptation of the alphaviruses to a new vector species which may result in the emergence of the disease in the new areas (Brault et al., 2004b).

Later analysis of the mechanisms of VEEV adaptation to *Ae. taeniorhynchus* using recombinant VEEV and virus replicons indicated that epizootic strain IC binds and infects midguts significantly more efficiently than the enzootic strain IE, and determinants of mosquito infectivity are encoded in the structural genes. More importantly this study showed that initial viral infection of the midguts is accommodated by a very small number of susceptible cells (~ 100 per midgut). Levels of replication of both VEEV strains in intrathoracically injected mosquitoes were similar, indicating that the midgut is the primary organ determining virus adaptation strategy (Smith et al., 2008).

The role of the alphavirus genes in infection and dissemination in mosquito vectors was also extensively studied in SINV. It was shown that virus produced from double subgenomic TE/3’2J or TE/3’2J/Δ2SGP (lacking the second sub-genomic promoter) infectious clones of SINV is poorly infectious to *Ae. aegypti* mosquitoes whereas Malaysian strain MRE16 infects more then 95% of mosquitoes and can efficiently disseminate to the salivary glands (Seabaugh et al., 1998). The TE/3’2J clone was derived from the clone TE12 which was constructed based on neurovirulent strain of SINV with substitution of the E2 and E1 genes form HRSP (heat-resistant small plaque) strain (Kuhn et al., 1992). Introduction of the structural genes of MRE16 into the

backbone of TE/3'2J/Δ2SGP clone resulted in the virus indistinguishable from MRE16 in its ability to infect and disseminate in *Ae. aegypti*, suggesting that structural genes of SINV harbor the determinants of mosquito infectivity and dissemination (Seabaugh et al., 1998). Later (Myles et al., 2003) found that deletion of a portion of the SINV E2 (ΔE200-Y229) which arose during propagation of MRE 16 strain in C6/36 cells, was responsible for a decrease in infection rates in *Ae. aegypti* from 100% for the wild-type parent to 23-35% in mutant variant. Dissemination also decreased from 93-100% to 2-20% at equivalent titers. Interestingly, this deletion occurred in the region of the E2 protein that has been postulated to constitute the receptor binding domain of alphaviruses (discussed in more details in the Chapter 6). This led to the suggestion that a SINV variant with deletion in E2 protein has impaired ability to interact with a cellular receptor on the mosquito midgut epithelium, thus explaining decreased infectivity for *Ae. aegypti* (Myles et al., 2003). In support of this hypothesis are findings of (Arcus et al., 1983; Houk et al., 1990; Smith et al., 2008) which showed that more efficient viral binding to the brush border membrane of midgut epithelial cells is usually associated with increased viral infectivity to the particular mosquito vector.

Subsequent studies of SINV focused solely on the role of the E2 glycoprotein in infection processes. TR339 virus derived from a cDNA clone, representing the consensus sequence of strain AR339 of SINV (Klimstra et al., 1998; McKnight et al., 1996), was shown to be highly infectious to *Ae. aegypti* as opposed to virus derived from TE/5'2J. The TE/5'2J strain was constructed based on neurovirulent and HRSP sub-strains of AR339 that differs from TR339 at two positions in E2 gene (E2-55 and E2-70). Genetic analysis demonstrated that introduction of the E2-H55Q and the E2-A70E mutations in the TE/5'2J virus increased viral infectivity to *Ae. aegypti* both independently and in combination. Both mutations E2-Q55H and E2-E70K were also found to be involved in increased SINV binding to heparan sulfate (Levine and Griffin, 1993; Smit et al., 2002) which could prevent effective receptor recognition by SINV on the midgut cells of *Ae. aegypti* (Pierro et al., 2007).

Comparison of the E2 protein sequence of MRE16 and TE/5'2J strains of SINV identified 60 positions which differ between these two viruses, making analysis of the precise locations of determinants of vector infectivity highly problematic. However, Pierro at al. (2008) was able to demonstrate that substitution of aa 95-96 and 116-119

from strain MRE16 into the TE/5'2J virus increased viral infectivity for *Ae. aegypti* both independently and in combination. These two regions are predicted to be loop domains that are separated by the highly conserved sequence among alphaviruses and flaviviruses. These authors hypothesized that the surface-exposed loop structures of the E2 glycoprotein work synergistically to enhance the overall affinity of the virus for cellular ligands (Pierro et al., 2008).

The paradigm that vector specificity of alphaviruses is only determined by the set of specific mutations in the E2 protein was questioned in the studies of mosquito infectivity determinants for CHIKV and ONNV in *An. gambiae*. These two viruses are closely related phylogenetically. Indeed, for a long period of time ONNV was even considered to be subtype of CHIKV (discussed in more detail in the following section 1.3.3). A unique property of ONNV is that the virus is not transmitted by culicine mosquitoes, but rather by anophelines, typically *An. funestus* and *An*. *gambiae*, whereas CHIKV is only able to infect *Aedes* spp. mosquitoes (Powers et al., 2000; Vanlandingham et al., 2005a).

To elucidate the genetic determinants of ONNV infectivity for *An*. *gambiae* mosquitoes we constructed the chimeric virus containing the entire structural protein genes region of ONNV and CHIKV non-structural protein genes and tested in *An*. *gambiae*. The infection rate of the resultant chimera was determined to be 38%, which is significantly lower than for parental ONNV. However, the reverse chimera containing ONNV non-structural and CHIKV structural gene regions, was able to replicate in cell culture to the high titers and efficiently infect *Ae. aegypti* mosquitoes, but failed to infect *An*. *gambiae*. These results showed that both structural and non-structural gene regions are necessary for efficient ONNV infection of *An*. *gambiae,* but structural genes play a more important role in this process. The subsequent analysis showed that genes encoding capsid and viral glycoproteins both contain important determinants of ONNV infectivity for *An*. *gambiae*. The role of the non-structural genome region in the ONNV infection and dissemination in *An. gambiae* was supported by Myles et al. (2006), who showed that the presence of an opal codon versus arginine between nsP3 and nsP4 is associated with more efficient viral infection and leads to earlier dissemination of the virus in *An. gambiae* (Myles et al., 2006).

1.3 CHIKUNGUNYA VIRUS

1.3.1 History and geographic distribution

The word "chikungunya" comes from ChiMakonde, the language spoken by the Makonde people, an ethnic group in southeast and northern Mozambique meaning "that which contorts or bends up" and refers to the stooping posture of infected patients due to severe joint pain. The first isolation and characterization of CHIKV was conducted in 1953 during an epidemic of febrile polyarthritis in Tanzania (formerly Tanganyika) (Robinson, 1955; Ross, 1956b). It was concluded that outbreaks were caused by a previously unknown mosquito-associated virus whose etiological role was confirmed by the presence of specific antibodies in the serum of recovered patients (Ross, 1956b). The transmission of the virus by *Ae. aegypti* mosquitoes was later documented using a bat-wing membrane technique (Ross, 1956a). Subsequent serological tests showed that CHIKV is a member of Group A of the arthropod-borne viruses and is more closely related to Mayaro virus (MAYV) and Semliki Forest viruses than to any of the other known members of the group (Spence and Thomas, 1959).

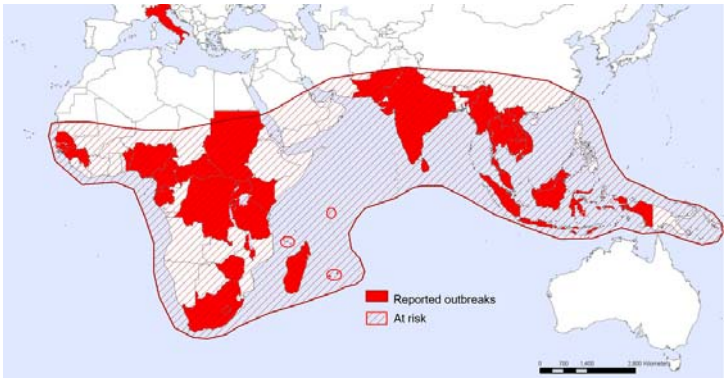


Figure 1.6 Global distribution of CHIKV.

Red areas - the countries where people have become infected with chikungunya virus. Dashed areas – the countries which are at risk. Modified form WHO (www.who.int), and CDC (www.cdc.com)

It was suggested that for a long time that outbreaks of CHIKV have been frequently diagnosed inaccurately as dengue; however, the presence of some clinical differences in the course of infections between these two diseases made it possible to retrospectively differentiate outbreaks caused by CHIKV and DENV. It appears that the first CHIKV outbreak can be traced to 1779 in Batavia-Jakarta, followed by outbreaks in Zanzibar 1823 and in India in 1824-1825 (Carey, 1971). In modern times, the CHIKV outbreaks have repeatedly occurred throughout numerous African countries including;

Sudan, Uganda, Democratic Republic of Congo (DRC), the Central African Republic (CAR), Malawi, Zimbabwe, Kenya, South African Republlic, Senegal, Benin, the Republic of Guinea, Cote d'Ivoire and Nigeria. In Southeast Asia, frequent outbreaks were reported in India, Malaysia, Indonesia, Cambodia, Vietnam, Myanmar, Pakistan and Thailand (Figure 1.6) (Jupp and McIntosh, 1988; Powers and Logue, 2007). The most recent large-scale outbreak in Africa occurred in 2002 in the DCR, where after seven years of absence, the virus reappeared and infected 50,000 people during three separate epidemics (Muyembe-Tamfum et al., 2003; Pastorino et al., 2004). In Asia the last reemergence of CHIKV was documented in 2001-2003 in Indonesia following increased *Ae. aegypti* and *Ae. albopictus* densities during the rainy season (Laras et al., 2005). The 2004-2007 CHIKV epidemics will be discussed later.

1.3.2 Transmission Cycles

CHIKV is endemic across tropical regions of Africa and Asia, however the mechanisms of CHIKV transmission and maintenance in nature appears to be very complex and vary significantly depending on the particular region where virus activity is detected.

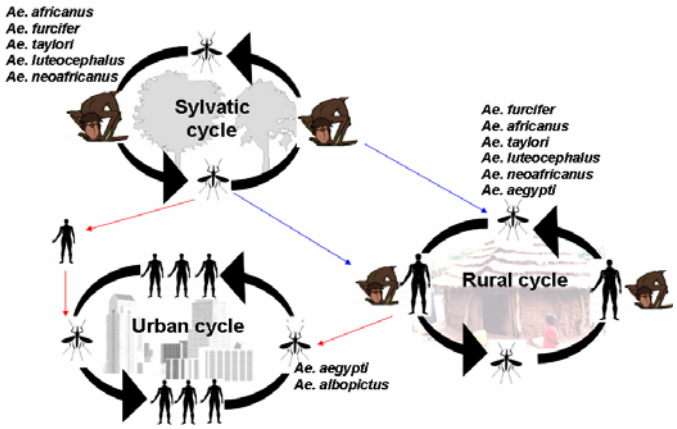


Figure 1.7 CHIKV transmission cycle in Africa.

African mosquito vectors

In Africa CHIKV is believed to be maintained in a sylvatic cycle involving wild non-human primates and forest-dwelling *Aedes* spp. mosquitoes (Figure 1.7). Several field studies conducted in Senegal, Nigeria, Uganda, Tanzania, Cote d'Ivoire, Central African Republic and South Africa concluded that the main sylvatic vectors of CHIKV are probably *Ae. furcifer-taylori*, *Ae. africanus*, *Ae. luteocephalus* and *Ae. neoafricanus* (Jupp and McIntosh, 1988). Based on isolation frequencies it appears *Ae. furcifer-taylori* is more important in southern and western Africa (Diallo et al., 1999; McIntosh et al., 1977) while *Ae. africanus* is more important in central regions (Jupp and McIntosh, 1988;

McCrae et al., 1971; Weinbren et al., 1958). In these primarily rural regions, the outbreaks have tended to be of smaller scale with few exceptions, including Tanzanian 1953, Nigerian 1969 and Congonese 2000 epidemics, and appear to be heavily dependent upon the sylvatic mosquito densities that increase with periods of heavy rainfall (Lumsden, 1955).

Laboratory studies have confirmed vector competence of *Aedine* African sylvatic mosquitoes. For *Ae. furcifer* from South Africa the oral infectious dose 50% (OID_{50}) - the titer of the virus in the blood meal sufficient to infect 50% of mosquitoes was found to be less than 6.2 log/ml resulting in a transmission rate of 25-32%. This is sufficient to sustain CHIKV transmission from velvet monkeys and baboons, which develop viremia up to 7-8 log/ml (Jupp et al., 1981; Paterson and McIntosh, 1964). *Ae. africanus* was also shown to be able to transmit virus between rhesus monkeys (Sempala and Kirya, 1973).

Vertebrate host

Although occasional isolation of CHIKV from various vertebrates has been documented, including the golden sparrow (*Auripasser luteus*), ground squirrel (*Xerus erythropus*), and bat (*Scotophilus* spp), only wild primates have been consistently implicated by a number of field studies to be primary CHIKV hosts in Africa. The presence of anti-CHIKV antibodies was shown in chimpanzees in DRC (Zaire) (Osterrieth et al., 1960), vervet monkeys (*Cercopithecus aethiops*) and Chacma baboons (*Papio ursinus*) in southern Africa (McIntosh, 1970; McIntosh et al., 1977; McIntosh et al., 1964). In Senegal CHIKV has been directly isolated from vervet monkeys, baboons and bushbabies (*Galago senegalensis*) (Bres et al., 1969; Cornet et al., 1979). In Nigeria seroprevalence of *Erythocebus patus*, *C. aethiops tantalus* and *C.mona* was found to be 10, 7 and 20% respectively (Boorman and Draper, 1968). In Uganda immunological and entomological studies provide evidence of a sylvatic cycle between *Ae. africanus* and red-tailed monkeys (*Cercopithecus ascanius*) (McCrae et al., 1971). Studies on the wild primates imported into the U.S. from Africa have shown a high prevalence of immunity to CHIKV in baboons, chimpanzees, gorillas, vervets monkeys (Harrison et al., 1967b).

Laboratory infections of rhesus macaques (*Macaca radiata*), vervet monkeys (*Cercopithecus aethiops*) and baboons (*Papio ursinus*) with CHIKV, showed high viremia followed by antibody development. In rhesus monkeys infected with an African strain of CHIKV, viremia lasted up to 6 days with titers of 3.3 to 7.5 log/ml determined

by newborn mouse brain titration (Paul and Singh, 1968) and 3.0 to 5.0 log/ml for African and 4.5 to 5.5 log/ml for Asian strains determined by Vero cell titration (Binn et al., 1967). In vervet monkeys and baboons viremia lasts up to 4 days with titers of 4.5 to 7.0 and 4.6 to 8.2 log/ml respectively (McIntosh et al., 1963). Cattle, sheep, goat, horses and various species of birds are refractory to infection but develop antibodies (Karabatos, 1985). Studies of wild African rodents showed that some species in the genera *Mastomys*, *Arvicanthis*, and *Aethomys* develop low viremia, while in *Mystromys* CHIKV produces high viremia followed by antibody development (McIntosh, 1961). The distribution of the rodents, however, does not coincide with distribution of CHIKV and so they cannot play an important role in sylvatic cycles. Additionally one out of nine Indian and two African bats inoculated with CHIKV demonstrated a low viremia (Bedekar and Pavri, 1969).

No CHIKV antibodies were detected in wild-captured bats, bird, rodents, and cattle in Uganda (McCrae et al., 1971; Simpson et al., 1968); however, in the recent study by (Diallo et al., 1999) conducted in rural areas of Senegal it was shown that frequencies of CHIKV isolation from strictly ornithophilic mosquitoes do not statistically differ from mosquitoes captured with human baits (2 of 77 and 154 of 12074). This suggests more complex CHIKV ecology, which may also involve secondary wild CHIKV cycles between birds and/or rodents and appropriate mosquito species (Cornet M. and others, unpublished data; Diallo et al., 1999).

Maintenance in Africa

The long-term studies of CHIKV activity in the Zika forest in Uganda revealed that CHIKV was not being constantly maintained in a red-tailed monkey-*Ae. africanus* cycle, but rather was being reintroduced with 5-7-year intervals. Disappearance of CHIKV is believed to occur because of the rising monkey herd immunity, which would not allow the virus to return until the sufficient numbers of non-immune monkeys replaced the old immune population. This resulted in observed periodicity in CHIKV activity and explains the failure to isolate virus during interepizootic years from *Ae. africanus.* This study led to the conclusion that in central Africa, CHIKV is maintained in only very large forest areas by a series of epizootic foci moving continually due to an ever-changing pattern of wild monkey immunity (McCrae et al., 1971). A similar transmission pattern was proposed for Yellow Fever virus in Africa (Monath, 1988). It

was suggested that after heavy rains CHIKV spills over from enzootic forest cycles into the savannah-woodland where it initiates an epizootic cycle. Similar periodicity in CHIKV activity but with shorter intervals (3-4 years) was documented for *Ae. furcifer-taylori* and *Ae. luteocephalus* in Kedougou, Senegal (Diallo et al., 1999). Interestingly, the same study established that CHIKV activity alternatively peaked in these two vectors, suggesting the possibility that enzootic viruses regularly shift among different monkey species.

The introduction of CHIKV into the rural areas due to spillovers leads to small outbreak maintained by sylvatic mosquitoes and in some cases by *Ae. aegypti,* which are usually abundant in the African villages. *Ae. aegypti* is strictly an anthropophilic daytime feeder that for a long time has been recognized as an important CHIKV vector. Laboratory studies documented that *Ae. aegypti* can be easily infected with the various strains of CHIKV (Banerjee et al., 1988; Gilotra and Shah, 1967; Mangiafico, 1971; McIntosh and Jupp, 1970; Mourya et al., 1987; Paul and Singh, 1968; Ross, 1956a; Turell and Malinoski, 1992): OID_{50} values varies form 6.7 to 9.2 log/ ml depending on the virus and mosquito strains. The rates of virus transmission vary form 13-100% depending on the study. CHIKV was also isolated numerous times from this vector (Diallo et al., 1999; Ross, 1956b).

It is believed that human migration between towns and villages leads to introduction of the virus into the urban areas where the highly anthropophilic *Ae. aegypti* may become infected and propagate an epidemic in the mosquito-human-mosquito urban cycle. The virus can be sustained in this cycle for a considerable period of time, moving from locality to locality depending on availability of sufficient non-immune human populations (Chevillon et al., 2008). In the recent years due to eradication programs, the population of *Ae. aegypti* in some parts of Africa is steadily declining, allowing a new species – *Ae. albopictus* to recolonize these areas.

Maintenance in Asia

In contrast to Africa in Asia, only urban CHIKV transmission cycle has been described. *Ae*. *aegypti* is the main vector of CHIKV, and *Ae*. *albopictus* is believed to play a secondary role in several outbreaks (Powers and Logue, 2007) (Figure 1.8). CHIKV epidemics in humans seem to be disconnected from zoonotic transmission (Peiris et al., 1993; Wolfe et al., 2001), however, the recent study of seroprevalence to CHIKV infection among wild monkeys in the Philippines showed presence of anti-CHIKV IgG in 59.3% of animals tested, suggesting existence of a possible sylvatic transmission cycles (Inoue et al., 2003). Currently it is believed that persistence of CHIKV in Asia results from viral migration back and forth among different locations sustained by the human-*Ae*. *aegypti* cycle (Chevillon et al., 2008)

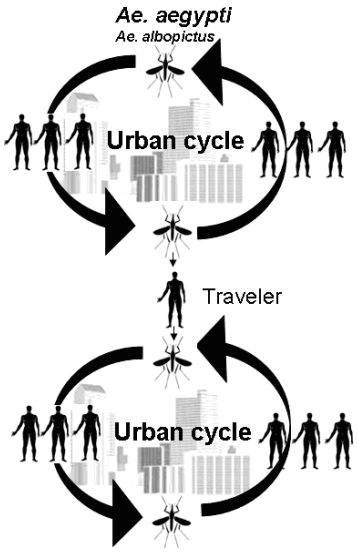


Figure 1.8 CHIKV transmission cycle in Asia.

1.3.3 Phylogenetic relations of CHIKV strains

The investigations of CHIKV transmission cycles in Africa and Asia suggest that CHIKV originated in Africa and spread later into Asia. This hypothesis is supported by the failure to confirm the existence of an enzootic cycle in Asia. This conclusion was further supported by phylogenetic studies (Powers et al., 2000). The phylogenetic tree constructed based on the partial nucleotide sequences of E1 gene showed that all CHIKV isolates form a monophyletic group within the Semliki Forest virus antigenic complex (Figure 1.9), and are most closely related to ONNV. The CHIKV phylogeny consists of 3 major clades [West African, Asian and East/Central/South African (ECSA)], which correspond to the geographic origin of the strains with exemption of strains from 2004-2007 CHIKV epidemics, which were isolated throughout parts of Africa, and countries of the Indian Ocean basin and Europe (discussed in the chapter 1.3.4).

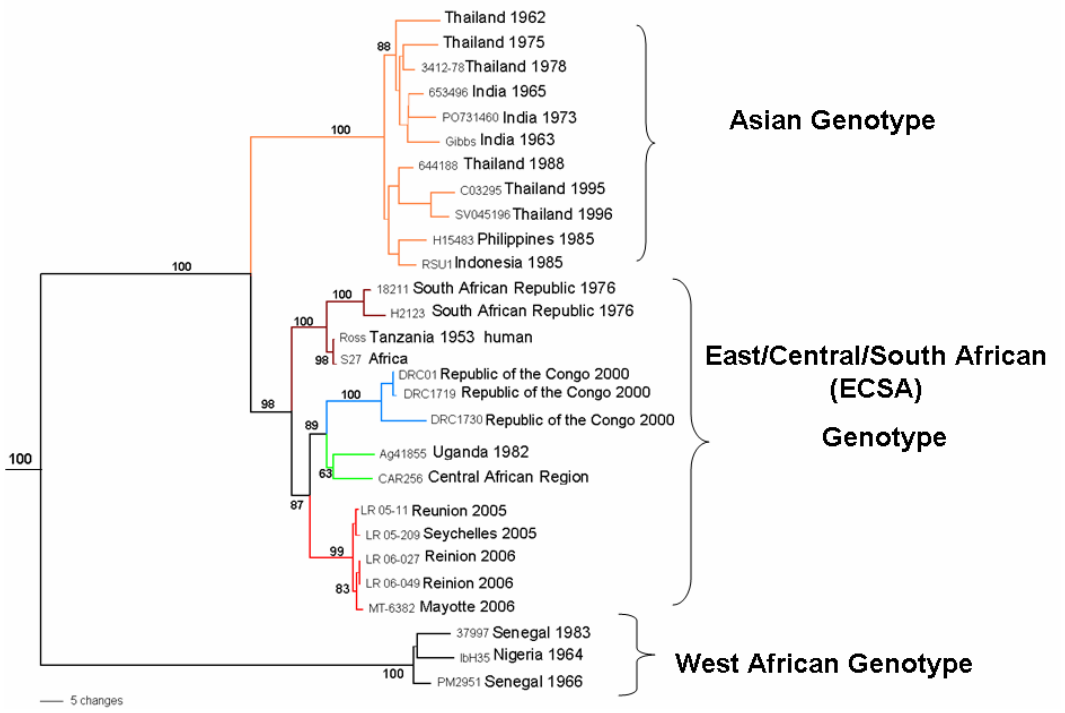


Figure 1.9 Phylogenetic relationships of the CHIKV strains.

Phylogenetic analysis was performed using Neighbor-Joining method with Kimura 2-parameter formula implemented in the PAUP 4.0 software (Swofford, 1998) on the 1050 bp partial E1 gene sequence. (Tsetsarkin K. unpublished data 2006)

The early study by Powers et al. (2000) using partial E1 gene sequence estimated that Asian clade evolved from hypothetical African ancestor 50-430 (±1 standard deviation) years ago and the common ancestor of all of the CHIKV has emerged between 150 and 1,350 years ago. Later investigation of full length genome sequences provided slightly different results: The Asian clade diverged form ESCA 87.7 (61.0 – 123.9) years ago and common ancestor exited 167.3 (71.4 – 299.3) years ago (Arankalle et al., 2007; Cherian et al., 2009).

1.3.4 The CHIKV outbreak of 2004-2007

In 2004-2007 CHIKV caused its largest outbreak/epidemic in documented history, affecting parts of Africa, the Indian Ocean islands, India, and Europe. The comparison of the CHIKV genome sequences obtained at different locations where CHIKV activity was documented allowed for the reconstruction of the predicted dispersal

pattern of CHIKV outbreaks during 2004-2007 (de Lamballerie et al., 2008; Schuffenecker et al., 2006) (Figure 1.10).

The phylogenetic analysis concluded that these outbreaks can be divided into two groups each caused by slightly different lineages of CHIKV, both evolved from a common ancestor in the ECSA clade (de Lamballerie et al., 2008).

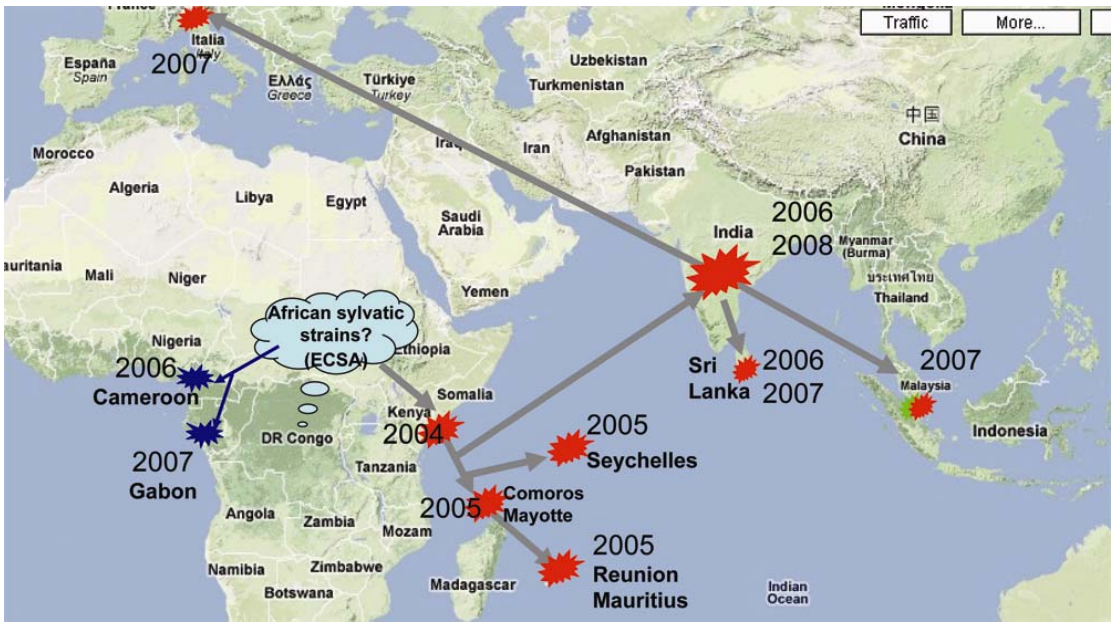


Figure 1.10 Predicted dispersal pattern of Chikungunya virus from Africa to the Indian Ocean and Europe during 2004-2007.
Modified from (de Lamballerie et al., 2008)

The initial outbreak in the first group started in coastal Kenya and Lamu Island (Kenya), in May 2004 and peaked in July 2004. A seroprevalence study revealed that the outbreak was widespread, affecting 75% of the Lamu population (13,500 people) (Sergon et al., 2008). The vector responsible for transmission of the virus was thought to be *Ae. aegypti* mosquitoes. Associated outbreaks occurred in Mombasa, Kenya, between November and December 2004 and in Comoros and Mayotte Islands from January to May 2005. Common feature of these outbreaks was involvement of *Ae. aegypti* as the principle vector (Sang et al., 2008). Also very high seroprevalence was observed among local populations in post-epidemics sera: on Grande Comore Island, CHIKV affected 63% of island population which could be translated into 215,000 infections (Sergon et al., 2007).

	2006					2007				2008				2009(P)*			
State	No. of districts affected	Total fever cases/ Suspected Chikungunya fever cases	No. of samples sent to NIV/NICD	No. of confirmed cases	No. of deaths	Total fever cases suspected Chikungunya fever cases	No. of samples sent to NIV/NICD	No. of confirmed cases	No. of deaths	Total fever cases suspected Chikungunya fever cases	No. of samples sent to NIV/NICD	No. of confirmed cases	No. of deaths	Total fever cases suspected Chikungunya fever cases	No. of samples sent to NIV/NICD	No. of confirmed cases	No. of deaths
1	2	3	4	5	6	7	8	9	10	11	12	13	14	11	12	13	14
Andhra Prd.	23	77535	1224	248	0	39	39	11	0	5	2	1	0	549	45	29	0
Karnataka	27	762026	5000	298	0	1705	1705	133	0	46510	2957	1008	0	1913	525	192	0
Maharashtra	34	270116	5901	804	0	1762	1762	135	0	853	339	238	0	102	21	10	0
Tamil Nadu	35	64802	648	116	0	45	45	10	0	46	0	0	0	16	16	16	0
Madhya Prd.	21	60132	892	106	0	0	0	0	0	0	0	0	0	0	0	0	0
Gujarat	25	75419	1155	225	0	3223	3223	122	0	303	163	53	0	0	0	0	0
Kerala	14	70731	235	43	0	24052	24052	909	0	24685	1356	492	0	36	25	18	0
A&N Islands	2	1549	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GNCT of Delhi	12	560	560	67	0	203	203	22	0	14	14	14	0	0	0	0	0
Rajasthan	1	102	44	24	0	2	2	2	0	3	3	3	0	0	0	0	0
Pondicherry	1	542	52	9	0	0	0	0	0	0	0	0	0	0	0	0	0
Goa	2	287	75	2	0	93	93	18	0	52	14	21	0	64	35	29	0
Orissa	13	6461	171	34	0	4065	4065	90	0	4676	238	11	0	0	0	0	0
West Bengal	1	21	0	21	0	19138	19138	347	0	17898	2789	593	0	35	35	11	0
Lakahsdweep	1	35	0	0	0	5184	5184	10	0	0	0	0	0	0	0	0	0
Uttar Prd.		4	4	4	0	4	4	4	0	11	11	7	0	0	0	0	0
Haryana		0	0	0	0	20	20	13	0	35	0	20	0	0	0	0	0
Total	212	1390322	15961	2001	0	59535	59535	1826	0	95091	7886	2461	0	2715	702	305	0

Table 1.1 Chikungunya situation in 2006-2009 India.

Adapted form http://www.nvbdcp.gov.in/Chikun-cases.html

The subsequent epidemics of CHIKV on Reunion Island and Seychelles islands started in May 2005, subsided in the dry season in August 2005 and returned in the winter of 2006, reaching their peak in February –March of 2006 (Renault et al., 2007). The early studies indicated that the number of infections on Reunion Island totaled 266,000 (34% of the total island population) (Renault et al., 2007), which was almost identical to the latter estimates of seroprevalence (38%) (Gerardin et al., 2008b). The interesting feature of the CHIKV epidemic on Reunion Island was the involvement of *Ae. albopictus* mosquitoes as the principal vector (discussed in more details in the section 1.4.5).

In December of 2005 CHIKV reemerged in India. In 2006 confirmed cases of CHIKV were detected in 14 states, affecting more than 1.3 million. In 2007 and 2008 CHIKV activity decreased compared with 2006, however, it was suspected that virus still caused 59,000 and 95,000 infections respectively (http://www.nvbdcp.gov.in/Chikun-cases.html; Table 1.1). The vectors responsible for viral transmission during these epidemics have not been definitely characterized. Both *Ae. aegypti* and *Ae. albopictus* are present in India and their epidemiologic significances for CHIKV transmission probably vary depending on the geographic location. It appears that *Ae. aegypti* was the predominant vector during 2006 in the most of the states (Yergolkar et al., 2006),

however, during 2007 at least in state of Kerala (South western India) *Ae. albopictus* was believed to be most important vector (Kumar et al., 2008). Another intriguing feature of the 2006-2008 CHIKV epidemic in India, beside the magnitude, is the fact that this epidemic was caused by virus from the ECSA complex. All previous outbreaks were caused by Asian genotype of CHIKV (Arankalle et al., 2007; Cherian et al., 2009). It was proposed that this shift in viral genotype was the major factor in the re-emergence of Chikungunya in an unprecedented outbreak in India after a gap of 32 years (Dash et al., 2007).

Continued expansion of CHIKV was reported in Sri Lanka (http://www.epid.gov.lk/pdf/chikungunya/OBOFCHIGYA.pdf) and Malaysia. In Sri Lanka, where CHIKV is believed to be brought by refugees from Indian cities affected by this disease (Seneviratne and Perera, 2006), CHIKV was detected in 11 districts causing 37,000 infections. *Ae. aegypti* and *Ae. albopictus* were the primary vectors of the virus. (http://www.aussieindolanka.com/news/sri_lanka/features/?newsid=56231&NewsDate=). In Malaysia it appears that 2006-2007 CHIKV outbreaks were caused by viruses belonging to the endemic Asian strain (AbuBakar et al., 2007) as well as by imported strains directly related to the CHIKV isolated on Indian Ocean Islands and in India (Noridah et al., 2007). CHIKV was detected in 6 states and caused 3,200 confirmed cases (http://www.prlog.org/10160200-chikungunya-has-expanded-to-6-states-in-malaysia.html).

In the summer of 2007, an outbreak of CHIKV was detected for the first time in the history of virus in Italian provinces of Ravenna, Cesena-Forli and Rimini (Angelini et al., 2007a; Angelini et al., 2007b; Rezza et al., 2007). Health authorities identified 214 laboratory-confirmed cases with date of onset from 15 July to 28 September 2007. Most cases (161) occurred in the two neighbouring villages of Castiglione di Cervia and Castiglione di Ravenna, but limited local transmission also took place in the cities of Ravenna, Cesena, Cervia, and Rimini. *Ae. albopictus* was the only vector responsible for CHIKV transmission in the region. Viral RNA was detected in *Ae. albopictus* captured in Castigliones Ravenna and Cervia (Bonilauri et al., 2008). Phylogenetic analysis of the CHIKV strain isolated in Italy showed that this outbreak was caused by CHIKV variant the most closely related to the strain of 2006-2007 epidemic in India (de Lamballerie et al., 2008).

Another series of outbreaks, caused by slightly different strain of CHIKV, started in 2006 in Cameroon and spread to Gabon in 2007. In 2006 CHIKV was isolated from French soldiers in Douala, Cameroon (Peyrefitte et al., 2007). Phylogenetic analysis revealed that Cameroon 2006 strain grouped with DRC strains from 1999-2000 outbreaks but not with Indian Ocean clade. The data indicated that continuous circulation of the genetically stable CHIKV strains has occurred in Central Africa during 6 years period. In 2006-2007 CHIKV was also isolated during dengue-like syndrome outbreak in several regions of Gabon (Peyrefitte et al., 2008). The outbreak generated 20,000 cases of febrile disease of which 80% probably were attributed to CHIKV and the remaining 20% were caused by DENV, whose activity in the region occurred simultaneously with CHIKV (Leroy et al., 2009). The sequence analysis revealed that a strain of CHIKV from Gabon 2007 was most closely related to the strain from Cameroon 2006, indicating that the epidemic originated from the emergence of a local strain, but not from the Indian Ocean variant. Interestingly *Ae. albopictus* was identified as the principal vector for the transmission of both CHIKV and DENV, although *Ae. aegypti* was present in the region simultaneously with *Ae. albopicus* (Leroy et al., 2009; Pages et al., 2009).

1.3.5 Clinical presentations of CHIKV

Typical signs for CHIKV infection are not specific and include fever, arthralgia and a rash that may or may not be accompanied by other signs and symptoms of the disease (Adebajo, 1996; Deller and Russell, 1967; McGill, 1995). The acute illness lasts 3-5 days, with recovery in 5 to 7 days. The incubation period following the bite of an infected mosquito is short (2-6 days) and ends with a sudden onset of fever reaching as high as 104 ºF that may last up to 10 days (Deller and Russell, 1967).

The fever almost always precedes the rash and joint pain and only very rarely has been reported as biphasic with recurrence noted on the fourth or fifth day of illness (Jadhav et al., 1965; Robinson, 1955). The rash, appearing primarily on the trunk, face, and limbs of the body is visible on day 2 to 5 post-infection, and may last up to 10 days. Examination of skin lesion revealed a perivascular lymphocytic infiltrate in the upper half of the dermis and red blood cells extravagations around skin capillary (Morrison, 1979). Minor and transient mucosal bleeding is possible, but severe hemmorage is very rare and in some cases has been misdiagnosed as dengue infection (Chastel, 1964).

One of the most common chikungunya disease symptoms - arthralgia - is mostly bilateral, symmetrical, and culminates within a few days usually affecting peripheral joints: ankles, toes, fingers, elbows, wrists and knees. The joints exhibit extreme tenderness and swelling with patients frequently reporting incapacitating pain that lasts for weeks or months (Powers and Logue, 2007). Retrospective studies of CHIKV infection in South Africa showed that 12.1% of patients experience persistent symptoms of joint stiffness, pain and effusion 3 years after the disease (Brighton et al., 1983; Calisher, 1999; McGill, 1995), which are accompanied by a very high antibody titres against CHIK virus. Pain and stiffness is more prevalent and severe in older patients and in those who had rheumatism previously.

Other common symptoms of CHIKV infection occurring at variable frequencies include headache, retro-orbital pain, photophobia, lumbar back pain, chills, weakness, malaise, nausea, vomiting and myalgia (Brighton et al., 1983; Calisher, 1999; McGill, 1995).

1.3.6 Rare clinical presentations associated with CHIKV infection

Paresthesia of the skin over the affected joints was commonly observed in early CHIKV outbreaks, suggesting neurological involvement, but no evidence of neurological sequellae was documented. The first neurological manifestations of CHIKV disease were reported during CHIKV outbreak in Madras, India, in 1964. Six patients developed signs of the meningeal irritation with nuchal rigidity and Kerning`s sign, sluggish papillary reaction, delirium and reduced consciousness, with no significant cerebrospinal fluid changes (Thiruvengadam et al., 1965). The more detailed descriptions of neurological manifestations of CHIKV infection in adults, fetuses and neonates came form clinical studies of 2005–2007 outbreaks in India and Reunion Island (Rampal et al., 2007; Robin et al., 2008) which are summarized in Table 1.2. In 73% of children neurological symptoms appeared less than in 24h after onset of fever (Robin et al., 2008) and less than in 2-3 days in the most adults (Rampal et al., 2007). Of the 30 children analyzed in (Robin et al., 2008), 2 died and 4 children had neurological sequelae at discharge. Of the 20 adults that were anylized in (Rampal et al., 2007), six died (Table 1.2).

The encephalitis and meningoencephalitis were the fourth cause of death among 65 patients with severe CHIKV in a hospital-based study in Reunion Island (Economopoulou et al., 2009). The ratio between CHIKV infections with apparent CNS diseases to the total number of cases for CHIKV was determined to be approximately 1:200, which is higher than that for several classical neurotropic viruses such as WEEV and Japanese encephalitis virus (Arpino et al., 2009).

Neurological sign	Adults (India)	Children (Reunion)	
		>1 year	<1 year
Altered level of consciousness	20 (100)	15 (68)	2 (25)
Cranial nerve deficit	20 (100)	-	-
Seizures	15 (75)	13 (59)	5 (62)
Decreased deep tendon reflexes with faintness	7 (35)	-	1 (12)
Psychosis	6 (30)	3 (14)	-
Hemi/paraparesis, paraplegia	4 (20)	-	-
Involuntary movements	4 (20)	1 (4)	-
Pyramidal syndrome Nuchal rigidity	-	2 (9)	-
Nuchal rigidity	-	7 (32)	-
Hypotonia	-	-	2 (25)
Tense fontanelle	-	-	2 (25)
Status epilepticus	-	-	1 (12)

Table 1.2 Frequency distribution of neurological signs and symptoms of CHIKV disease in 20 adults, 22 children (>1 year old) and 8 infants (<1 year old) recruited.

Aadapted from (Arpino et al., 2009)

CHIKV infections also have been infrequently associated with haemorrhagic manifestations including haematemesis and melaena. During the 1963–1964 outbreak in Calcutta, haemorrhagic manifestations of various grades of severity were documented in eleven patients (Sarkar et al., 1965). The CHIKV infection in these patients was confirmed either serologically or by direct virus isolation. However, potential complications of CHIKV co-infection with another virus infection such as dengue-2 virus may explain the recent deaths associated with CHIKV infection in Reunion Island and India (Powers and Logue, 2007).

1.3.7 Mother to Child transmission of CHIKV

Chikungunya represents a substantial risk for neonates born to viremic patients. It appeared that vertical transmission of the CHIKV from mother to child occurs in nearly 50% of women (19 cases of vertical transmission out of 39 women with intrapartum

viremia), and does not correlate with caesarean section frequencies. All infected neonates were asymptomatic at birth, and median onset of neonatal disease was observed on day 4 (range 3-7 d), characterized by pain, prostration, and fever present in 100% (n=19) and thrombocytopenia in 89% of cases. Severe disease was observed in more than 50% (n=10) of cases and majority of which (90%) consisted of encephalopathy. MRI findings showed brain swelling in 47.3% of severe cases, cerebral hemorrhages with scattered signs of supratentorial white matter in the early stage in 10.5%, and 21.5% evolved towards persistent disabilities (Gerardin et al., 2008a). In these studies hypotonia was the most common manifestation, followed by coma, seizures and epileptic status (Ramful et al., 2007; Robin et al., 2008). MRI aslo revealed scattered white matter lesions with areas of intraparenchymal haemorrhage.

1.3.8 Animal models

Infection of guinea pigs, hamsters and rabbits with CHIKV does not lead to viremia but causes development of antibodies. Early laboratory studies showed that 1- to 4-day-old mice die 2 to 3 days after inracerebral or intraperitoneal inoculation of CHIKV, while 3- to 4-week-old mice usually survive and develop antibodies (Karabatos, 1985).

Two mouse models for studying the pathogenesis of CHIKV have recently been described (Couderc et al., 2008; Ziegler et al., 2008). The first study demonstrated that CHIKV infection in <12 day-old neonatal outbred OF1 mice and in inbred C57BL/6 and 129s/v mice is age-dependent, and associated with severe morbidity and mortality. Adult mice with a partially (IFN-α/βR$^{+/-}$) or totally (IFN-α/βR$^{-/-}$) abrogated type-I IFN pathway develop a mild or severe infection, respectively. It was shown that in IFN-α/βR$^{+/-}$ mice, following a burst of viral replication in the liver, CHIKV primarily targets muscle, joint, and skin fibroblasts; a cell and tissue tropism similar to that observed in biopsy samples of CHIKV-infected humans. These findings are also consistent with the *in vitro* observation that primary mouse muscle and cultured human lung and mouse skin fibroblasts are susceptible to CHIKV infection (Couderc T. unpublished data). In contrast, CHIKV did not replicate in lymphoid and monocytoid cell lines, primary lymphocytes and monocytes, or monocyte-derived dendritic cells (Sourisseau et al., 2007). In the severe disease, CHIKV disseminates to other tissues including the CNS, therefore neonatal phase and inefficient type-I IFN signaling were concluded to be a risk

factors for severe CHIKV-associated disease. It was concluded that IFN-α/βR$^{+/-}$ mice could serve as a suitable model for the study of CHIKV infection in humans and for development of future vaccine and therapeutic strategies (Couderc et al., 2008).

In the parallel study by (Ziegler et al., 2008) it was shown that subcutaneous infection of newborn mice (strains ICR and CD-1) with $10^{4.6}$ pfu of CHIKV leads to high viremia and high virus titer in leg muscle and brain (10^6–10^8 pfu), which lasts 10-12 days. Mice also developed lethargy, difficulty walking, dragging of hind limbs, and reduced weight gain within 7–10 days after infection. The most pronounced histopathologic changes were in skeletal muscle, which were focal necrosis and inflammation, followed by fibrosis and dystrophic calcification. The 14-day-old mice developed much less intense viremia, which lasted for a shorter period, and had much less virus in the brain as compared to newborn mice. The authors concluded that young ICR and CD-1 mice could be a useful and realistic model for further study of the pathogenesis and treatment of CHIKV infection.

1.3.9 Vaccine and antiviral therapeutics against CHIKV

Several experimental inactivated cell culture based vaccine candidates were developed in the sixties and seventies (Harrison et al., 1967a; Harrison et al., 1971; Nakao and Hotta, 1973; White et al., 1972) although none of them have been used to control CHIKV outbreaks. The most promising was the vaccine developed and described by Harrison et al. (1971). This vaccine candidate was based on a CHIKV strain isolated from serum of an infected patient from 1962 outbreak in Thailand (designated as 15561) and propagated in bank-frozen green monkey kidney tissue culture. The candidate vaccine elicited protective immunity in mice and high level of neutralizing antibodies by day 42 in volunteers in a phase I trial. Also, no side effects or untoward reactions were noted in any volunteers (Harrison et al., 1971).

The second generation CHIKV vaccine derived from the same CHIKV 15561 strain was produced by 11 serial passages in green monkey kidney cells followed by 18 plaque-to-plaque passages in MRC-5 (human embryonic lung) cells. The resultant virus strain exhibited small plaques and a temperature sensitivity phenotype, it had decreased virulence for suckling mice and it showed a reduced viremia in monkeys. The vaccinated

mice and rhesus monkeys elicited neutralizing antibody and were protected against challenge with virulent CHIKV.

Use of a live-virus vaccine raised a concern, that mosquitoes feeding on vaccine recipients may result in virus transmission to a second vertebrate host and/or to reversion of the vaccine to a more virulent form. The safety of live–attenuated CHIKV vaccine was tested in urban vectors of CHIKV *Ae. aegypti* and *Ae. albopictus*. The vaccine and parental virus were capable of oral infection of *Ae. aegypti* and *Ae. albopictus* and were transmitted by the mosquitoes that received virus by intrathoracic inoculation. Both viruses replicated similarly in intrathoracically injected mosquitoes, but there was no evidence of reversion to virulence (Banerjee et al., 1988; Turell and Malinoski, 1992). Also considering the low viremias observed in vaccinees, it was postulated that it would be very unlikely for either mosquito vector to become infected from feeding upon a vaccinated human (Turell and Malinoski, 1992).

In the subsequent phase 1 and 2 clinical trials vaccine safety and immunogenicity were tested on 73 healthy adult volunteers (Edelman et al., 2000). Fifty-seven (98%) of 58 CHIKV vaccinees developed CHIKV neutralizing antibody by day 28, and 85% of vaccinees remained seropositive at one year after immunization, confirming high immunogenicity of the vaccine. The vaccine was relatively safe: only 8 % of volunteers developed transient joint pain and 13% experienced flu-like symptoms (Edelman et al., 2000). Unfortunately, limited resources and lack of commercial potential (prior to the 2005–2007 outbreaks) restricted the scope of additional testing.

A new generation of the recombinant chimeric live-attenuated vaccine candidates has been recently developed (Wang et al., 2008). These vaccines were based on SINV and naturally avirulent strain of EEEV and attenuated vaccine strain TC-83 of VEEV where structural genes were substituted with CHIKV, strain LR2006 OPY1. All vaccine candidates replicated efficiently in cell cultures, and were highly attenuated in mice as determined by absence of signs of neurologic disease, febrile responses, or growth delays as indicated by continued weight gain. All of the chimeras also produced robust neutralizing antibody responses, although the TC-83 and EEEV backbones appeared to be more immunogenic. Vaccinated mice were fully protected against disease and did not develop a detected viremia after CHIKV challenge. Another variant of the recombinant live-attenuated CHIKV vaccine utilizing the IRES-dependent structural gene expression

(Volkova et al., 2008) currently is in development. Usage of an internal ribosome entry site (IRES) blocks viral replication in mosquito cells and therefore solves the safety problems which were discussed earlier.

Currently there are no specific therapeutics against chikungunya disease, and only supportive treatment is recommended, including analgesics, antipyretics and anti-inflammatory agents (Powers and Logue, 2007). An early clinical study showed significant improvement of the Ritchie articular index and morning stiffness among patients with chronic joint symptoms treated which chloroquine phosphate (Brighton, 1984); however, the efficacy of the drug was not confirmed in later studies (de Lamballerie et al., 2009). It has been shown that chemical inhibitors of viral replication, including ribavirin, 6-azauridine, glycyrrhizin and interferon-α down-regulate CHIKV replication in cell culture (Briolant et al., 2004). Futher clinical studies are required to determine their use against CHIKV.

1.3.10 Control and prevention

Mosquito control remains the most effective means of prevention and protection against chikungunya disease (Jupp and McIntosh, 1988). Control include using insect repellents with substances like DEET, picaridin (also known as picaridin and KBR3023), PMD (p-menthane-3,8-diol, a substance derived from the lemon eucalyptus tree), or IR3535. Wearing bite-proof long sleeves and trousers (pants) also offers protection. Vector abundance can be minimized through source reduction strategies to eliminate mosquito breeding sites and via the aerosol application of adulticides.

1.4 *AE. ALBOPICTUS* MOSQUITO - EMERGING VECTOR FOR CHIKV

1.4.1 *Ae. albopictus* general introduction

Ae. albopictus, commonly known as the Asian tiger mosquito, was first described scientifically in 1895, by a British-Australian entomologist, Frederick A. Askew Skuse

(Knight and Stone, 1977). Adult *Ae. albopictus* mosquitoes are easily recognized by the bold black shiny scales and distinct silver white scales on the palpus and tarsi (Hawley, 1988). The mosquito is about 4.75mm (2 to 10 mm) in length with a striking white and black pattern. The body size variation depends on the density of the larval population and food supply within the breeding water. The males are roughly 20% smaller than the females. *Ae. albopictus* is a very aggressive outdoor daytime biter with activity peaks generally occurring during the early morning and late afternoon. It feeds on a number of hosts including man (indoors and outdoors), domestic and wild animals and birds. The mosquito is a container-inhabiting species which lays its eggs in any water-containing receptacle in urban, suburban, rural and forested areas such as tires, flower pots, cemetery urns/vases, buckets, tin cans, rain gutters, ornamental ponds, drums, even the finger holes of an abandoned bowling ball have been reported. Larvae are also found in natural water-holding sites such as treeholes, bamboo stems, and leaf axils. Depending on temperature and availability of food, *Ae. albopictus* larval development takes between 5 to 10 days; the pupal stage takes 2 days (Briegel and Timmermann, 2001). Though limited food is the primary cause of death, parasites (ciliates and neogregarines), and predators (*Toxorhynchites* spp. larvae) may exert substantial influence on population size.

1.4.2 *Ae. albopictus* geographic distribution

Native to the forests of Southeast Asia, where it is likely zoophilic (i.e., feeding on wildlife) *Ae. albopictus* has recently spread globally due to advent of modern shipment, with the current geographic range including Europe, Africa, the Middle East, North and South America and the Caribbean (Benedict et al., 2007; Gratz, 2004) therefore deserving the reputation of the most invasive mosquito in the world. Continuing adaptation of this species to the anthropogenic environment ("domestication"), which provides alternative blood sources (domestic animals and man) and water collections for larval habitats was the operative of species evolution during the last century.

The early expansion of *Ae. albopictus* into the Indo-Malayan Peninsula, and the Indian Ocean islands including Madagascar is believed to have occurred centuries ago simultaneously with human migration. This spread was further fueled by the increase in intercontinental trade that occurred in the second half of the 20th century. In 1979, an infestation of *Ae. albopictus* was documented for the first time in Europe, in Albania (Adhami and Murati, 1987; Adhami and Reiter, 1998). The mosquito was found breeding in tires in seven localities. The most likely source of the mosquitoes was China, one of Albania's few trading partners at the time. In 1990, *Ae. albopictus* was discovered in the city of Genoa, Italy (Sabatini et al., 1990) and rapidly spread throughout the country (Romi, 2001; Toma et al., 2003). Continued geographic expansion of the species throughout late 20th and early 21st century, led to establishment of the species in France (Schaffner et al., 2001) and Spain (Roiz et al., 2007; Roiz et al., 2008), and foci of breeding has been found in Croatia (Klobucar et al., 2006), Netherlands (Scholte et al., 2008), Serbia, Belgium, Slovenia, Bosnia and Herzegovina, Germany, Greece and Switzerland (Scholte and Schaffner, 2007) (Figure 1.11).

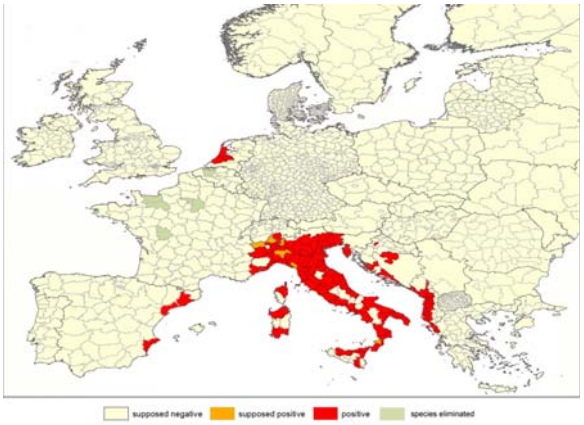


Figure 1.11 *Ae. albopictus* in Europe per province, as of January 2007.

Adapted form (Scholte and Schaffner, 2007)

In 1985, established populations of *Aedes albopictus* were found for the first time in the continental U.S.A. in Harris County, Texas (Sprenger and Wuithiranyagool, 1986). The species was introduced into Hawaii sometime before 1902. Since then *Ae. albopictus* has

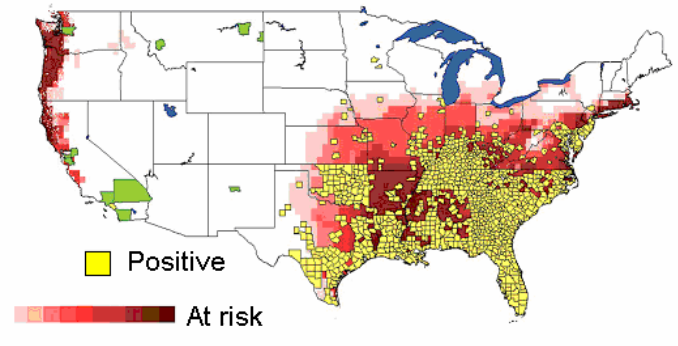


Figure 1.12 *Ae. albopictus* in USA as of 2001.
Adapted form (Benedict et al., 2007)

spread into 866 counties in 26 states (CDC) (Figure 1.12). Although isolated records of *Ae. albopictus* have been reported as far north as Chicago in the Midwest, the mosquito has been slow to expand its range northward along the Atlantic coast. The 0°C daily mean January isotherm has been used as a conservative estimate for the northern limit of this mosquito's overwintering range in North America. The ability of the species to resist cold temperatures is likely linked to its ability to synthesize a high amount of lipids for eggs yolk (Briegel and Timmermann, 2001). *Ae. albopictus* was also discovered in South and central America. Currently it is found in Brazil (Forattini, 1986), Argentina (Rossi et al., 1999), Mexico (Ibanez-Bernal and Martinez-Campos, 1994; Rodriguez Tovar and Ortega Martinez, 1994), Dominican Republic (Rodhain, 1996), Colombia, Bolivia, Cuba (Broche and Borja, 1999), Honduras (Woodall, 1995.), Guatemala (Ogata and Lopez Samayoa, 1996), El Salvador, Paraguay, Panama, Uruguay (Salvatella and Rosa, 2003) and Nicaragua (Cuellar-Jimenez et al., 2007; Lugo Edel et al., 2005).

In Africa, *Ae. albopictus* was first detected in 1989 in South Africa (where it has been controlled) and later in Nigeria, Cameroon, Equatorial Guinea and Gabon (Paupy et al., 2009).

1.4.3 Competition with pre-existing resident species

Upon introduction into a new area, the establishment of *Ae. albopictus* is dependent on the ability of the species to adapt not only to climatic and environmental factors, but also on the ability to successfully interact with endemic species of mosquitoes that occupy the same ecological niche. It was shown that in North and South America, Africa and Mayotte Island establishment of *Ae. albopictus* does not lead to displacement of the local populations of *Ae. aegypti* and both species co-exist sharing the same larval habitats (Braks et al., 2004; Juliano et al., 2004; Simard et al., 2005). However, an *Ae. albopictus*-attributed decline of local *Ae. aegypti* populations was observed in several countries (Barrera, 1996; Braks et al., 2004; Juliano et al., 2004). Interestingly, laboratory experiments in microcosms with plant detritus as a substrate showed that North American *Ae. albopictus* is superior in competition with *Ae. aegypti* (Barrera, 1996; Daugherty et al., 2000), probably due to its better population growth. However, competition between larvae of these species has tended to yield approximate competitive equality, or even an advantage for *Ae. aegypti* (Barrera, 1996; Daugherty et al., 2000). These discrepancies

were explained by differences in experimental conditions and mosquito strains used in these studies. Coexistence of *Ae. albopictus* with the native North American mosquito *Oc. triseriatus* across different ecological niches with no decline in the *Oc. triseriatus* population has been reported, even when *Ae. albopictus* exhibited greater fitness in laboratory experiments (Lounibos et al., 2001). A similar phenomenon was reported for *Ae. albopictus* and *Cx. pipiens* (Carrieri et al., 2003; Costanzo et al., 2005).

1.4.4 Laboratory studies of vector competence of *Ae. albopictus* to CHIKV

The presence of *Ae. albopictus* in the areas where CHIKV activity led to the suggestion that, although the species is not host-specific, it still could serve as a secondary vector (after *Ae. aegypti*) for CHIKV in several Asian outbreaks in the 20th century. The following laboratory studies that demonstrated high oral susceptibility and ability to transmit CHIKV by this mosquito species indirectly support this suggestion.

Singh and Parvi (1967) showed that an Indian strain of *Ae. albopictus* is susceptible to infection with Indian strain (Calcutta 1964) of CHIKV, when fed through a membrane in an artificial blood meal. The OID_{50} value of CHIKV for *Ae. albopictus* was 4.5-4.6 $Log_{10}LD_{50}$/ml (mouse lethal dose 50%), which is about 1 $Log_{10}LD_{50}$/ml lower (more infectious) than the OID_{50} values of CHIKV for *Ae. aegypti* (Singh and Pavri, 1967). Higher susceptibility of *Ae. albopictus* as compared to *Ae. aegypti* to CHIKV infection was later confirmed in several other studies. Paul and Singh (1968) demonstrated experimental infection of *Ae. albopictus* with an Indian strain of CHIKV by feeding on a viremic monkey *Macacca radiata*. The infection rates of *Ae. albopictus* were found to be ~ 50% when fed on monkeys with viremia of 4.5 $Log_{10}TCID_{50}$/ml (titrated on BHK-21 cells), however less than 20% of *Ae. aegypti* became infected when fed on monkeys with 5.0-5.5 $Log_{10}TCID_{50}$/ml virus in the blood (Paul and Singh, 1968). Similarly to Indian strains, the African strain of CHIKV was also found to be more infectious to *Ae. albopictus* than to *Ae. aegypti* (Mangiafico, 1971). In the same study (Mangiafico, 1971) demonstrated the ability of the experimentally infected *Ae. albopictus* mosquitoes to transmit CHIKV to suckling mice. The transmission rate was 78-80% at day 7 and 14 post infection, which gradually decreased at later times. At all time points the transmission rate of *Ae. albopictus* was higher as compared to *Ae. aegypti.* The experimental transmission of CHIKV to suckling mice by *Ae. albopictus* was also

documented in safety studies of a live attenuated vaccine candidate of CHIKV (Turell and Malinoski, 1992) and in the study of variation in CHIKV susceptibility among geographic strains of *Ae. albopictus* (Turell et al., 1992).

It was also found that geographic strains of *Ae. albopictus* differ in their susceptibility to infection with CHIKV when fed on an artificial blood-sugar-virus suspension (Tesh et al., 1976) or on viremic *M. radiata* monkeys (Turell et al., 1992). Tesh et al. (1976) showed that infection rates vary between 19 to 71% for African, and 28 to 97% for Indian strains of CHIKV depending on geographic origin of the mosquito strains. Generally, the mosquito strains that demonstrated higher susceptibility to African strains of CHIKV were also more susceptible to the Indian strain. Also, significant differences in ability to replicate virus after ingestion of infectious blood meal were detected between various mosquito strains. Although genetic inbreeding experiments to develop increasingly resistant or susceptible strain of *Ae. albopictus* did not succeed, the hybrids between strains with high and low susceptibility exhibited intermediate susceptibility phenotype, indicating involvement of vector genetic factors, which control the infectious process (Tesh et al., 1976). In the similar study by Turrel et al. (1992) using viremic monkeys as a virus source, a significant variation in susceptibility to and dissemination of CHIKV were observed among 10 geographic strains of *Ae. albopictus*. It was also shown that strains of *Ae. albopictus,* regardless of their geographic origin, were more susceptible to CHIKV infection and more prone to virus dissemination than strains of *Ae. aegypti* (Turell et al., 1992). These data altogether indicated that at least in laboratory conditions *Ae. albopictus* appears to be a more competent vector for CHIKV than *Ae. aegypti.*

1.4.5 Role of *Ae. albopictus* mosquitoes in 2005-2007 CHIKV epidemics

To our knowledge, up until 2005 there was no direct epidemiological evidence for example, isolation of a virus from *Ae. albopictus* specimens collected in nature, which could support a role of the species in CHIKV transmission. From this perspective the 2005-2007 epidemics of CHIKV were very unusual because of strong evidence that incriminated *Ae. albopictus* as primary vector of the virus at least in several geographic regions. The first evidence of the involvement of *Ae. albopictus* as a CHIKV vector came from early studies of the 2005-2006 outbreak in Reunion Island. The species was

abundant during the epidemic, and other potential vectors, specifically *Ae. aegypti,* were relatively scarce with a very limited distribution due to intensive DDT treatments for malaria control in the 1950s (P. Reiter, personal communication). The decline of *Ae. aegypti* allowed *Ae. albopictus* to infest unoccupied breeding sites (Salvan and Mouchet, 1994). Also CHIKV RNA was detected in *Ae. albopictus* mosquitoes collected on the island during the epidemic (X. de Lamballerie, personal communication). A later study demonstrated virus isolation from field collected females (Delatte et al., 2008). It also was suggested that the local *Ae. albopictus* population may be more anthropophilic than in other parts of the world (Reiter et al., 2006).

Entomological investigations of the 2006-2007 CHIKV outbreak in Kerala India recorded high population densities of *Ae. albopictus* in affected areas. Numerous *Ae. albopictus* breeding sites were found in rainwater that collects in the hemispherical containers fitted to the trunks of rubber trees for latex collection in the region (National Informatics Centre, 2008; NVBDCP, 2008). *Ae. albopictus* was found to constitute up to 92% of the total mosquito larvae/pupae collected in districts of Alappuzha and Kottaya in June 2007 whereas only 0–3.6% of the total larvae/pupae were *Ae. aegypti*. In July 2007 *Ae. albopictus* constituted 58–76% of the larvae/pupae collected in Alappuzha, Kottayam and Pathanamthitta districts (VCRC, unpublished reports). These reports show that *Ae. albopictus* was the predominant mosquito species in the region during the 2007 chikungunya outbreak (Kumar et al., 2008).

Ae. albopictus was also the vector responsible for CHIKV transmission in Italy in 2007. It is believed that the virus was introduced into the region of Romagna by an Indian immigrant who returned from his home country in late June 2007 during the viremic asymptomatic stage of the infection (Sambri et al., 2008). Extremely large populations of *Ae. albopictus* in the area where this patient spent the acute phase of CHIKV infection, probably were responsible for initiation of the local transmission cycle. Pools of *Ae. albopictus* mosquito collected in August of 2007 in Ravenna province were found CHIKV-positive by RT-PCR analysis, whereas pools of *Cx. pipiens* and *Ae. caspius* mosquitoes collected in the same region did not contain CHIKV RNA (Bonilauri et al., 2008) (http://www.promedmail.org, archive number: 20070907.2957). In the following study the virus was isolated and characterized from both blood and mosquito samples

(Angelini et al., 2008; Bordi et al., 2008), confirming for the first time local transmission of CHIKV by *Ae. albopictus* outside of the tropical areas.

Entomological investigation of the 2007 CHIKV outbreak in Libreville (Gabon) incriminated *Ae. albopictus* as the main vector of the virus. *Ae. albopictus* was significantly more abundant in the region as compared to *Ae. aegypti* and the presence of viral RNA was detected only in pools of females belonging to the former species. Results of RT-PCR analysis were corroborated by direct viral isolations. No virus isolations were made from other mosquito species including *Ae. aegypti, Cx. quinquefasciatus*, *Mansonia uniformis* and *An. gambiae* (Pages et al., 2009).

1.4.6 E1-A226V mutation in genome of CHIKV transmitted by *Ae. albopictus* mosquitoes

Comparative studies of the CHIKV genome sequences during 2005-2006 CHIKV outbreak on Reunion Island identified the unique amino acid mutation E1-A226V in the three isolates collected after November 28 of 2005, although, this mutation was absent in the three earlier strains collected during this outbreak. Also, this mutation was absent in all sequenced CHIKV strains isolated prior to beginning of the outbreak. The rest of the sequences of early and late isolates from the 2005-2006 Reunion outbreak were almost 100% identical, indicating that this mutation probably occurred during microevolution of the virus on the island. A more detailed investigation revealed that none out of 20 isolates collected in the region between March and June of 2005 have this mutation, however, 67 out of 74 (90.5%) isolates collected between September 2005 and March 2006 were found to bear this substitution (Schuffenecker et al., 2006).

The circumstantial evidence presented by Schuffenecker et al. (2006) led to the suggestion that acquisition of this particular mutation could provide some selective advantage to the CHIKV transmitted by *Ae. albopictus* mosquitoes. At this time the detailed molecular biological investigation of the role of E1-A226V mutations in adaptation of CHIKV to *Ae. albopictus* was required to test this hypothesis which was accomplished by Tsetsarkin K. A. as a part of this dissertation project. This will be discussed in following chapters.

However, the 2005-2006 CHIKV outbreak on Reunion Island was just the first example of the appearance of this E1-A226V mutation in the CHIKV strains transmitted

by *Ae. albopictus,* which soon thereafter was followed by a series of other similar outbreaks. As discussed in the previous section, *Ae. albopictus* was considered to be the main CHIKV vector in Kerala, India in 2007. Interestingly, strains collected in India in 2006 all have alanine at E1-226, however, strains from 2007 from Kerala all bear the E1-A226V mutation (Kumar et al., 2008). Phylogenetic analysis indicated that CHIKV strains responsible for this outbreak belong to the ECSA clade, and, although they are closely related to strains isolated in 2005-2006 in Reunion Island with which they share a common ancestor, they form an independent branch on the phylogeny that is supported by high bootstrap values. These results indicate that the Indian acquisition of E1-A226V mutation happened independently from the Reunion outbreak (Arankalle et al., 2007; Cherian et al., 2009; Dash et al., 2007; de Lamballerie et al., 2008). Similarly, CHIKV isolates from the 2007 outbreak in Italy all have the same E1-A226V mutation (Bordi et al., 2008; de Lamballerie et al., 2008; Rezza et al., 2007). Phylogenetic studies showed that the Italian strain of CHIKV evolved directly from Indian strains of the 2006-2007 outbreak, and therefore currently it is unclear if E1-A226V mutation appeared independently in the Italian strain from an ancestor with alanine at E1-226, or the imported strain already possessed this mutation. During the Gabonese outbreak of 2007 the same E1-A226V mutation was discovered in CHIKV transmitted by *Ae. albopictus* (de Lamballerie et al., 2008; Pages et al., 2009; Vazeille et al., 2008). The strains involved in this outbreak are phylogenetically distinct from the Indian Ocean clade, indicating independent acquisition of this particular mutation (de Lamballerie et al., 2008; Peyrefitte et al., 2008; Peyrefitte et al., 2007).

These examples of the convergent evolution in the unrelated CHIKV lineages toward E1-A226V mutation additionally support the hypothesis of the role of this mutation in viral adaptation to *Ae. albopictus* mosquitoes.

1.4.7 Role of the mutations at E1-226 in other alphaviruses

Early studies with SFV and SINV showed that cell entry and exit of alphaviruses are dependent on the presence of cholesterol in cell membranes (Lu et al., 1999; Marquardt et al., 1993; Phalen and Kielian, 1991). Serial passage of mutagenized SFV in C6/36 cells, which have been previously depleted from cholesterol and then enriched by chlorocholestene (a cholesterol analogue that is normally nonfusogenic and contains

chloride at the 3β position), allowed selection of a virus population which was ~ 2-log more infectious for chlorocholestene-enriched cells compared to that of wt virus. Limited dilution of the adapted virus stock on cholesterol-depleted C6/36 cells allowed isolation of three clonal populations, named srf-1, srf-2, srf-3. Interestingly, these viruses grow to 3-4 log higher titers not only in chlorocholestene-enriched C6/36, but also in C6/36 cells depleted of cholesterol and which were not subsequently enriched by chlorocholestene. Sequencing analysis of the srf-1, srf-2 and srf-3 viruses identified a single amino acid substitution (proline to serine at E1-226) present in all of these viruses. Introduction of this mutation into an i.c. of SFV resulted in a virus which was 2-logs more infectious to, grew more efficiently in, and was 3-log more efficient in fusion to cholesterol-depleted C6/36 as compared with wt virus. Clone-derived virus with the E1-P226S mutation was also significantly more efficient in exit from cholesterol-depleted C6/36 cells as compared with wt virus. However, these characteristics were indistinguishable for srf-3 and clone-derived virus with the E1-P226S mutation in cholesterol depleted C6/36 cells, and for wt, srf-3 and clone-derived virus with the E1-P226S mutation in standard C6/36 cells. These data indicated that mutations at position E1-226 can modulate the cholesterol requirements of alphaviruses (Vashishtha et al., 1998).

The role of mutations within the E1-226 region in lipid requirements of alphaviruses for entry and exit was also demonstrated for SINV. SINV is very distantly related to SFV and shares only 40% sequence identity at the amino acid level. However, similar to SFV, the fusion and exit of SINV is dependent on the presence of cholesterol in cell membranes. SINV has alanine at E1-226 and its substitution to serine was not sufficient to confer a cholesterol-independent phenotype. However, simultaneous substitution of the three amino acids at E1- 226, 227, 228 to those present in the srf-3 cholesterol-independent mutant of SFV, resulted in the virus that shares same cholesterol-independent characteristics specific for SFV srf-3 virus (Lu et al., 1999).

The role of the E1-P226S mutation, which confers a cholesterol-independent phenotype of SFV in cell culture, was also investigated in *Ae. albopictus*. Mosquitoes, as all insects, do not possess the enzymatic pathways required for cholesterol production. However they require cholesterol for successful reproduction and development. The needed cholesterol is therefore obtained auxitrophically from dietary sources (Canavoso et al., 2001; Clayton, 1964), although, studies have shown that adult mosquitoes may be

maintained without dietary cholesterol for a considerable period of time (Clayton, 1964; Golberg and De Meillon, 1948).

The replication of wt and srf-3 mutant SFV was compared in intrathoracicaly injected *Ae. albopictus*, revealing that the srf-3 virus grew more efficiently, particularly at a low multiplicity of infection (Ahn et al., 1999). Also, this study showed that propagation of srf-3 and wt SFV in mosquitoes did not affect differential cholesterol requirements attributed to these viruses. These data demonstrated that the polymorphism at E1-226 can directly modulate fitness of alphaviruses in the vertebrate vector, which is associated with differential cholesterol requirements for cell entry and fusion.

1.5 SPECIFIC AIMS OF THE PROJECT

Due to the widespread distribution of *Ae. albopictus* in the United States and elsewhere (Gratz, 2004), it is important to determine the genetic aspects of CHIKV adaptation to this vector and the exact effector mechanisms of these genetic changes. This information will provide the means for assessment and prediction of the potential for CHIKV and other alphaviruses to invade new geographic regions. This knowledge will aid in the development of effective strategies to control the diseases caused by alphaviruses. The specific aims of the project are:

Specific Aim 1: To investigate effects of the E1-A226V mutation on CHIKV fitness in *Ae. albopictus* and *Ae. aegypti* mosquitoes.

1a: To construct and characterize in cell culture, *Ae aegypti* and *Ae. albopictus* mosquitoes, infectious cDNA clones (i.c.) of CHIKV using a recent isolate (strain LR2006 OPY1) from La Réunion Island.

1b: Using i.c. of the LR2006 OPY1 and 37997 strains of CHIKV, investigate the effect of the E1-A226V mutation on CHIKV midgut infectivity, viral dissemination to mosquito secondary organs, and transmission to suckling mice by *Ae. aegypti* and *Ae. albopictus* mosquitoes.

HYPOTHESIS: *The E1-A226V mutation increases fitness of CHIKV in Ae. albopictus but not in Ae. aegypti mosquitoes.* The understanding of how a single amino acid substitution can influence vector specificity will provide a plausible explanation of how CHIKV caused an epidemic in a region lacking the typical vector.

Specific Aim 2: To investigate the detailed relationship between cholesterol dependence of CHIKV and efficient CHIKV midgut infectivity for *Ae. albopictus*.

2a: Using a full length i.c. of the LR2006 OPY1 strain of CHIKV (pCHIKV-LR i.c.) the following amino acids (K, H, D, N, S, T, G, A, V, L, I, P, F, M) will be introduced at position E1-226, and resultant viruses will be characterized in cell culture for growth, and stability. Since a mutation at this position has previously been associated with viral dependence on cholesterol, this characteristic will also be studied. Equivalent viruses expressing GFP will be generated, and used to rapidly evaluate infectivity for *Ae. albopictus* using Oral Infectious Dose 50% (OID_{50}) assays.

2b: The OPY1 strain of CHIKV will be adapted for growth in cholesterol-depleted C6/36 cells. Mutations which confer CHIKV cholesterol independency will be introduced into the LR-GFP i.c. and analyzed using OID_{50} assays for their effect on *Ae. albopictus* midgut infectivity.

HYPOTHESIS: *CHIKV dependence on cholesterol is associated with enhanced Ae. albopictus midgut infectivity.* Comprehensive comparison of amino acid requirements at position E1-226 for CHIKV infectivity of *Ae. albopictus* and cholesterol dependency will provide essential clues about the underlying mechanisms of action of the E1-A226V mutation.

Specific Aim 3: To characterize the mutations in the E2 protein of CHIKV that affect the role of the E1-A226V mutation on CHIKV infectivity for *Ae. albopictus* midgut cells, and investigate the effects of these mutations on CHIKV interaction with putative cell surface receptors in virus overload protein binding assay (VOPBA).

3a: Using GFP-expressing i.c. of CHIKV strains LR2006 OPY1 and Ag41855, identify specific mutations in the E2 protein that modulate the effects of the E1-A226V mutation on *Ae. albopictus* midgut infectivity.

3b: Investigate the effect of mutations in the E2 protein (identified in aim 3a experiments), on CHIKV infectivity for midgut cells of *Ae. aegypti*.

3c: Using non-GFP-expressing CHIKV (strain LR2006 OPY1) with mutations that confer high, intermediate and low *Ae. albopictus* and *Ae. aegypti* midgut infectivity,

compare their ability to interact with putative cell surface receptors derived from midgut epithelial cells of *Ae. albopictus* and *Ae. aegypti* in VOPBA.

HYPOTHESIS*: Mutations in E2 protein differentially affect CHIKV infectivity for Ae. albopictus and Ae. aegypti midgut cells, which correlates with efficient CHIKV interaction with putative cell surface receptor from midgut epithelial cells*. Characterization of mutations in the E2 protein, which modulate the effect of the E1-A226V mutation on CHIKV infectivity for *Ae. albopictus,* is essential for understanding of the mechanism of CHIKV adaptation to a new vector. Additionally this information will be used in studies aimed at identification of cell surface receptors for CHIKV from midgut epithelial cells.

CHAPTER 2: CONSTRUCTION AND CHARACTERIZATION OF INFECTIOUS CLONES OF LA REUNION ISOLATE OF CHIKUNGUNYA VIRUS

ABSTRACT

The recent epidemics caused by Chikungunya virus (CHIKV) on island in the Indian Ocean and in India has focused attention on this reemerging virus and has highlighted the need for the development of new tools to study vector-virus-host interactions. Therefore, this chapter is dedicated to the development of infectious cDNA infectious clones (i.c.) based on a recent epidemic viral strain LR2006 OPY1 from the Island of La Réunion. The viruses produced from i.c. were characterized in cell culture and in *Ae. aegypti* and *Ae. albopictus* mosquitoes. Comparison of the growth kinetics and infection rates of the parental virus LR2006 OPY1, and of a virus produced from full-length i.c. (CHIKV-LR) indicates that the infectious clone has retained the viral phenotype of the original isolate. Infectious clones that express enhanced green fluorescent protein (eGFP) from either a 3' or 5'sub-genomic promoter were also produced and characterized in cell culture and in *Aedes* mosquitoes. The virus produced from pCHIKV-LR 5'GFP infected *Ae. aegypti* and *Ae. albopictus* mosquitoes at a rate similar to that observed for both the original virus, and virus produced from the pCHIKV-LR i.c. The virus produced from pCHIKV-LR 3'GFP i.c. only infected *Ae. albopictus* mosquitoes, but failed to infect *Ae. aegypti* and was less stable during serial passage in C6/36 and BHK-21 cells as compared to CHIKV-LR 5'GFP virus. The development of these infectious clones based on a strain obtained from the recent outbreak in the Indian Ocean has enabled further studies of the molecular determinants of infection and transmission by *Ae. aegypti* and *Ae. albopictus* mosquitoes which will be discussed in the following chapters.

Substantial parts of this chapter have been reported previously: (Tsetsarkin et al., 2006).

INTRODUCTION

Since its first isolation in Tanzania in 1953 (Mason and Haddow, 1957; Ross, 1956b) CHIKV has caused numerous epidemics in Africa and Asia. Recent large-scale epidemics of chikungunya which began in Kenya in 2004 and subsequently spread into the islands of the Indian Ocean, India, Sri Lanka, Indonesia and Italy, were the largest in the documented history. It has been postulated that the genetic changes in the virus might have contributed to the scale of these epidemics (Schuffenecker et al., 2006), therefore the development of infectious cDNA clone of the epidemic strain of CHIKV is necessary for future research aimed at investigations of viral determinants associated with unusual pattern of these epidemics.

Human infections with CHIKV are frequently associated with extremely painful arthralgia in many parts of the body, and patients may develop a stooped posture, hence the name chikungunya which is derived from a Makonde phrase describing the contorted posture of people with the disease. Other signs and symptoms include: chills, flushed face, nausea, vomiting, headache, photopohobia, lymphadenopathy, rash and in some cases bleeding from the nose. The incubation period is estimated to be about two to four days; onset is sudden and acute illness lasts for three to five days followed by recovery in five to seven days. Occasionally the arthritis can last for months or years. Prior to the outbreak in the Indian Ocean, fatalities had not been associated with the disease. However, at least 238 deaths may have been directly or indirectly caused by the disease (Charrel et al., 2007b; Simon et al., 2007).

Previously, the full-length cDNA i.c. of the 37997 strain of CHIKV has been developed by the degree applicant and was subsequently characterized by (Vanlandingham et al., 2005b). Based upon experiments in cell culture and in *Ae. aegypti* mosquitoes, virus produced from the i.c. was indistinguishable from the parental virus which was used for i.c. construction. Two additional variants, which differed in the placement of the subgenomic promoter and the gene encoding eGFP (5'CHIKV EGFP and 3'CHIKV EGFP), were constructed and analyzed in mosquitoes. The infectious rates for these viruses in *Ae. aegypti* were comparable with parental virus. eGFP expression was detected in the midgut, salivary glands, and nervous tissue. Dissemination rates of these new viral vectors exceeded those of previous alphavirus expression systems, thus

expanding the repertoire and potential for gene expression studies on this important vector species. The full length clone of the 37997 strain of CHIKV was also extensively used in our studies to identify the genetic determinants of *An. gambiae* infectivity of ONNV and CHIKV (Vanlandingham et al., 2006).

The 37997 strain is a member of the West African phylogroup, which is the outlier among CHIKV strains, and only very distantly related to the strains in the ECSA clade involved in the 2004-2007 CHIKV epidemics (Schuffenecker et al., 2006; Figure 1.9; Tsetsarkin unpublished data). The nucleotide and amino acid sequences of the strains within West African and ECSA clades differ by ~14% and ~7% respectively, which limits applications of the clones for genetic studies of the viral determinants of CHIKV re-emergence.

CHIKV is typically a zoonotic virus in Africa with a transmission cycle that principally involves primates and *Aedes* mosquitoes. Humans can develop relatively high viremias, and similar to dengue and yellow fever viruses, epidemics can be sustained strictly via human-mosquito transmission. In Africa, a number of different species of mosquitoes including: *Ae. furcifer-taylori*, *Ae. africanus*, *Ae. luteocephalus* and *Ae. aegypti*, can act as vectors; however, in Asia the large urban epidemics have primarily involved *Ae. aegypti* (Jupp and McIntosh, 1988) and *Ae. albopictus* probably played a secondary role at least in some areas. An interesting feature of the 2004-2007 CHIKV epidemics is the involvement of *Ae. albopictus* mosquitoes as the principal vector - at least in some areas. This mosquito was proven to be a primary CHIKV vector on Reunion Island in the 2005-2006 epidemic (Delatte et al., 2008; de Lamballerie, personal communication, 2007), in the 2006-2007 CHIKV outbreak in Kerala, India (National Informatics Centre, 2008; NVBDCP, 2008), in the 2007 CHIKV outbreak in Italy (Angelini et al., 2008; Bonilauri et al., 2008; Bordi et al., 2008; http://www.promedmail.org, archive number: 20070907.2957) and in the 2007 CHIKV outbreak in Libreville (Gabon) (Pages et al., 2009).

In this chapter the development of the infectious clones based on La Réunion 2006 CHIKV isolate will be discussed. The viruses derived from these clones were characterized in cell culture and in the typical (*Ae. aegypti*) and epidemic (*Ae. albopictus*) mosquito vectors. These clones represent a valuable tool to characterize the role of viral genes in studies of vertebrate host/virus/mosquito vector infections.

MATERIALS AND METHODS

Virus

The LR2006 OPY1 strain of CHIKV was obtained from the World Reference Center for Arboviruses at the University of Texas Medical Branch, Galveston, TX. This strain was originally isolated from serum of a febrile patient from La Réunion Island. The strain was passed five times on Vero cell culture and once in suckling mice. Stock virus, which later will be referred to as "parental virus," was produced following a single passage in C6/36 cells, grown at 28ºC in Leibovitz L-15 media with 10% FBS and stored at -80ºC until needed.

Sequencing of LR2006 OPY1 strain

RNA extraction RNA was extracted using the QIAamp Viral RNA Purification kit (Qiagen, Valencia, CA) following standard protocols established by the manufacturer. Five hundred sixty microliters of Buffer AVL were added per 140 µl of sample, and the samples were pulse-vortexed and incubated at room temperature for 10 min. The samples were centrifuged briefly, and 560 µl ethanol (EtOH) were added. Samples were pulse-vortexed, and added to the column in 630 µl aliquots, then centrifuged for 1 min at 8K rpm. Five hundred microliters of Buffer AW1 were added to each column, the columns were centrifuged for 1 min at 8K rpm, and the flow-through was discarded. Five hundred microliters of Buffer AW2 were added to each column, the columns were centrifuged for 1 min at 13K rpm, the flow-through was discarded, and the column was again centrifuged for 1 min at 13K rpm. The column was transferred to a 1.5 ml collection tube. To elute the RNA, 50 µl of H_2O were pipetted directly onto the membrane, let stand for 1 min, and the samples were centrifuged for 1 min at 13K rpm. RNA was stored at -80^{o}C until used.

RT-PCR Complementary DNA (cDNA) was produced from RNA by a reverse transcription reaction as follows: 5 µl RNA, 50 ng random hexanucleotide primers, 5 µl water, and 10mM dNTP were mixed, incubated at 65ºC for 5 min and placed on ice for 2 min. Four microliters of 5X buffer, 4 µl of 100 mM DTT, 10U of (1 µl) RNAse inhibitor,

and 1 µl Superscript II (Invitrogen, Carlsbad, CA) were added to each reaction, and the tubes were incubated at 25°C for 10 min then 42°C for 1 hr. cDNA was amplified in a reaction consisting of 5 µl 10X buffer, 5 µl 1.5 mM dNTP, 150 ng forward primer, 150 ng reverse primer, 3 µl cDNA, and 5 U (1 µl) *Taq* DNA polymerase (New England Biolabs) using 35 cycles at 94ºC, 20 sec; 55ºC, 20sec; 72ºC, 2 min; with a final extension at 70ºC for 5 min. Alternatively amplification of cloned cDNA fragments from plasmid DNA was performed using 25 cycles at 94ºC, 20 sec; 55ºC, 20sec; 72ºC, 2 min; with a final extension at 72ºC for 5 min. Primers for PCR reactions (Table 2.1) were designed based on the sequences of CHIKV strains 37997, S27 and Ag41855, which have been previously sequenced (Khan et al., 2002; Vanlandingham et al., 2005b; Tsetsarkin K. unpublished data).

The 3' terminal part of the genome was amplified using the 3' RACE method (Frohman, 1993). Viral RNA was reverse transcribed using 50 ng of Qt primer and then amplified using outer primers Chik-F3 and Qo using 30 cycles at 94ºC, 20 sec; 55ºC, 20sec; 72ºC, 1 min. One microliter of this PCR was used as a template for second round of PCR from inner primers 41855-Hind-R and Qi using the same conditions as for first PCR (Table 2.1).

The 5' terminal part of the genome was amplified using the FirstChoice® RLM-RACE kit (Ambion, Austin, TX) following the manufacturer's instructions. Initially viral RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) in the reaction consisting of 5 µl of RNA, 2 µl of 10X buffer, 11 µl of water and 2 µl of CIP for 1 h at 37 ºC. The reaction was terminated by acid phenol/chloroform extraction followed by EtOH precipitation. RNA was resuspended in 11 µl of water and 5' caps were digested by treatment of dephosphorylated RNA with Tobaco Acid pyrophosphatase (TAP) in the reaction containing 5 µl of RNA, 1 µl of 10X buffer, 2 µl of TAP and 2 µl of water incubated at 37 ºC for 1 h. 5'RACE RNA adapter was ligated to TAP treated RNA in the reaction consisting of 2 µl of RNA, 1 µl RNA adapter, 1 µl 10X buffer, 2 µl RNA ligase, 4 µl of water for 1 h at 37 ºC. This RNA was reverse transcribed as described above. cDNA was amplified from outer primers 41855-Ngo-R and 5'RACE outer primer followed by PCR using inner primers Chik-ns-R5 and 5'RACE inner primer (Table 2.1).

Gel extraction of Amplicons Amplicons were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Amplicons with DNA markers

(80% glycerol, 0.1M EDTA, 0.3% bromphenol blue) were run on a 1% agarose gel, and the DNA bands were visualized using ethidium bromide under UV light. For gel extraction, the desired band was excised, weighed, and placed into a tube with 3 volumes of Buffer QG. The tube was incubated at 50^{o}C for 10 min, and 1 volume of isopropanol was added. The mixture was then transferred to a QIAquick spin column and centrifuged for 1 min at 13K rpm. The flow-through was discarded, and 500 µl of Buffer QG were added. The column was centrifuged for 1 min, the flow-through was discarded, and the column was washed with 750 µl of Buffer PE by centrifugation for 1 min at 13K rpm. The flow-through was discarded, and the column was again centrifuged for 1 min at 13K rpm and placed in a 1.5 ml collection tube. To elute the amplicons, 30 µl of water were pipetted directly onto the membrane, the column was incubated at room temperature for 1 min and centrifuged for 1 min at 13K rpm.

Sequencing Agarose gel-purified amplicons were sequenced directly using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequencing reaction consisted of 4 µl of DNA (~100 ng), 1.5 µl of 5X buffer, 1 µl of BigDye Terminator v3.1, 1 µl of specific primer (30 ng) and 2.5 µl of water. The following cycling conditions were used: 96 ^{o}C 1 min, followed by 25 cycles of 96 ^{o}C 20 sec, 50 ^{o}C 5 sec, 60 ^{o}C, 4 min. Sequencing reactions were purified using Performa® DTR Gel Filtration Cartridges (Edge BioSystems Gaithersburg, MD) following the manufacturer's instructions. Sequencing was performed in an ABI PRISM model 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and sequences were edited and assembled in VectorNTI v10.3 (Invitrogen, Carlsbad, CA).

Infectious clone production

The CHIKV full-length infectious clone (pCHIKV-LR i.c.) was produced using standard molecular biology techniques (Sambrook et al., 1989). PCR fragments were cloned individually or in tandem into a full length i.c. using a modified pSinRep5 plasmid backbone (Invitrogen, Carlsbad, CA). The Sp6 promoter sequence was introduced upstream of the 5' end of the CHIKV cDNA sequence, and the viral poly A_{40} tail and *Not*I linearization site were added to the 3' end. To confirm that no mutations were introduced during the cloning procedures at least two clones of the intermediate and final plasmids were sequenced completely.

RT-PCR cDNA was produced as described above, and amplified in a reaction consisting of 5 μl 10X buffer, 5 μl 1.5 mM dNTP, 150 ng forward primer, 150 ng reverse primer, 3 μl cDNA, and 2.5U (1 μl) *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using 35 cycles at 94ºC, 20 sec; 55ºC, 20sec; 70ºC, 2 min; with a final extension at 70ºC for 5 min. Alternatively amplification of cloned cDNA fragments from plasmid DNA was performed using 25 cycles at 94ºC, 20 sec; 55ºC, 20sec; 72ºC, 2 min; with a final extension at 70ºC for 5 min.

Ligation of DNA fragments After restriction endonuclease enzyme digestions, the amplicons were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Fragments for all experiments were ligated with T4 DNA ligase (Stratagene, La Jolla, CA). Each ligation reaction consisted of 2 μl 10X reaction buffer, 3 μl (approximately 100 ng) of digested and phosphorylated vector DNA, 3-5 μl (100-200 ng) of digested cDNA fragment(s) to be inserted depending on cloning strategy, 0.7 μl T4 DNA ligase and ultrapure water to 20 μl. The tubes were incubated at 14-16 ^{o}C for 1-24 hr, and 15 μl of the ligation reaction were used to transform MC1061 cells.

Transformation of E.coli MC1061 competent cells Plasmids used in the construction of the clones were transformed into *E. coli* MC1061 cells as follows: MC1061 cells were grown to a density of 0.6-0.8 OD_{600} in 50 ml 2XYT medium (16 g tryptone, 8 g yeast extract, 5 g NaCl per 1 L water) and placed on ice for 5 min. Bacteria were pelleted in 3 ml aliquots (per sample) by centrifugation for 10 min at 4^{o}C, 3K rpm. Cells were then washed in 5 ml of cold buffer B1 (0.01M MOPS, 0.01 RbCl, pH 7.0), centrifuged as above, washed in 5 ml cold buffer B2 (0.1M MOPS, 0.05M $CaCl_2$, 0.01 RbCl, pH 6.5), incubated 15 min on ice and centrifuged as above. Each 3 ml sample of bacteria was resuspended in 200 μl B2 and used in the transformation. Three microliters of dimethyl sulfoxide (DMSO) and < 20 μl plasmid were added to an aliquot of freshly prepared competent cells, mixed, and incubated on ice for 30 min. Cells were heat shocked at 43.5^{o}C for 45 sec, incubated on ice another 3 min, and 3 ml of 2XYT medium were added to each tube. Cells were incubated on a shaker at 37 ^{o}C for 1h and then pelleted by centrifugation for 10 min at 4^{o}C, 3K rpm, the medium was aspirated, cells were resuspended in 50 μl of 2XYT and plated on 100 cm^2 Petri dish containing 2XYT agar with ampicillin 50 μg/ml.

Amplification of plasmids Transformed MC1061 cells containing the desired plasmids were grown overnight on 2XYT agar (16 g tryptone, 8 g yeast extract, 5 g NaCl, and 16 g agar per 1 L water; contents were mixed, autoclaved 20 min on the liquid cycle, and plates were poured once the agar mixture had partially cooled and ampicillin was added to final concentration 50 μg/ml), and individual colonies were picked and grown for 16 hours in 2XYT medium. Intermediate and final plasmids to be sequenced were extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) following manufacturer's protocol.

For purification of the final plasmids, the cesium-chloride purification method was used. Preparation of DNA by this technique serves to purify the DNA from the bacteria in which plasmids are propagated and helps to eliminate other biological macromolecules that can prevent efficient digestion by restriction endonucleases. One bacterial colony of *E. coli* containing the desired plasmid was inoculated into 250 ml of 2XYT broth and the bacteria were allowed to grow overnight at 37°C in a shaking incubator. The following morning, the bacteria were pelleted at 6,500 rpm at 4°C for 10 min. The supernatant was discarded and the bacteria were suspended in 8 ml of BF1 (12.5 ml of 1 M Tris, pH 7.5, 10 ml of 5 M NaCl and 10 ml of 0.5 M EDTA) by shaking. Next, 16 ml of BF2 (20 ml 1M NaOH,10 ml 10% SDS and 70 ml water) was added to the cells and the mixture was intensively shaken for a few seconds to homogenize the solution and to lyse the cells to release the plasmid DNA. After 10 min incubation on ice, 12 ml of the lysis neutralization solution BF3 (150 g KAc and 100 ml glacial acetic acid in 500 ml water) was added to the mixture and intensively shaken for a few seconds to homogenize the solution. After 15 min on ice, the tubes were centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant was transferred to a 50 ml conical tube. Isopropanol was added to the top of the tube and the contents were mixed and then stored at -20°C for 15 min to precipitate the DNA. Next, the nucleic acids were pelleted at 3,000 rpm for 10 min at 4°C and then supernatant was discarded and the residual isopropanol was evaporated by inversion of the tube on a paper towel for 1 minute. The DNA pellet was then dissolved in 2 ml TE (2.5 ml 1M Tris, 0.5 ml of 0.5 M EDTA in 250 ml water) buffer. The solution was then transferred to a 50 ml round bottom tube and 2 ml 5M lithium chloride was added to precipitate the RNA. After vortexing and 10 min incubation, the solution was centrifuged at 18,000 rpm for 10 min at 4°C to pellet the

RNA. The supernatant containing the DNA was transferred to a 15 ml conical tube and 8 ml of ethanol was added. After incubation for 60 min at -20°C the DNA was pelleted at 3,000 rpm for 10 min at 4°C. The supernatant was aspirated and the pellet was dried on a paper towel and then dissolved in 1 ml of TE. Next, 4.8 g of cesium chloride, then 1 ml TE and 40 µl ethidium bromide (10 mg/ml), then the plasmid solution was added to a 15 ml tube and TE was added to achieve a total weight of 9.1 g. The solution was then transferred to an ultracentrifuge tube with a 3 ml syringe and needle. The sealed tube was centrifuged at 70,000 rpm at 20 °C for ≥16 hours and the lower band consisting of the plasmid DNA was aspirated with a 1 ml syringe with a needle and then mixed with 1ml of TE and 3.5 ml of ethanol. After incubation at -20 °C for 1 hour the plasmid was pelleted at 3,000 rpm for 10 min at 4 °C, the supernatant was removed and the pellet was dissolved in 0.4 ml of TE for 30-40 min at room temperature. Next, 0.4 ml of phenol-chloroform was added to the DNA and the solution was mixed and then centrifuged for 5 min at 13,000 rpm. The upper phase was removed and placed in a new eppendorf tube with 0.1 ml of 5M NaCl and 1 ml of ethanol and then incubated at -20 °C for 15 min. The DNA was pelleted by centrifugation at 13,000 rpm for 5 min and the supernatant was aspirated. Next, 0.5 ml of 70% EtOH was added and the tube was centrifuged at 13,000 rpm for 10 min. The supernatant was aspirated and the plasmid pellet was dissolved in TE to a final concentration of 1 mg/ml.

RNA transfections and infectious center assays

Infectious viruses from the pCHIKV-LR clones (CHIKV-LR, CHIKV-LR 5'GFP, and CHIKV-LR-3'GFP) were produced by linearization with *Not*I followed by *in vitro* transcription from the SP6 promoter using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). One microgram of linearized plasmid DNA was incubated with 10X reaction buffer, 2X NTP/CAP, 10X GTP, and SP6 mix enzyme (1 µl per 10 µl reaction) for 1h at 37 ^{o}C. The yield and integrity of synthesized RNA were analyzed by agarose gel electrophoresis in the presence of 0.25 µg/ml of ethidium bromide.

Ten µg of RNA were electroporated into $1x10^7$ BHK-21 (Baby hamster kidney) cells as previously described (Higgs et al., 1997; Vanlandingham et al., 2005). Approximately $1x10^7$ BHK cells at ~80% confluence in a 150 cm^2 flask were trypsinized and resuspended in 10 ml of cold L-15 media in a 50 ml conical vial. Cells were

centrifuged at 2,500 rpm, at 4°C for 5 min and the media was removed. Cells were then washed by resuspension three times in 10 ml cold PBS in a 15 ml conical tube and centrifugation at 2,500 rpm, 4°C for 5 min. The PBS was removed, the cells were resuspended in 400 µl of cold PBS, and the RNA was added. Cells and RNA were transferred to a 0.2 cm gap cuvette and pulsed using a Gene Pulser Xcell electroporation system (Biorad, Hercules, CA) on BHK preset conditions. Six hundred microliters of L-15 growth medium was immediately added to the cells, and the cells were transferred to a 75 cm^2 flask with 15 ml L-15 after a 5 min recovery period on ice. Electroporated cells were transferred to the Biosafety Level-3 laboratory, incubated 2 days at 37°C, monitored daily. At 24 and 48h post-electroporation 10 ml of tissue culture supernatant were harvested, titrated, and stored in aliquots at -80ºC. Immediately following electroporation, ten-fold dilutions of electroporated BHK-21 were seeded in six-well tissue culture plates containing $1x10^6$ naïve Vero cells per well for infectious center assays. Following an incubation for 2h at 37°C, cells were overlaid with 2 ml of 0.5% agarose containing L-15 supplemented with 3.3% FBS. BHK-21 cells were incubated for 2 day at 37°C (until plaques developed). Plates were stained with crystal violet.

In vitro growth of virus

Vertebrate and mosquito cells were maintained at 37°C and 28°C, respectively, in Leibovitz L-15 medium with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Confluent monolayers of Vero (Green monkey (*Cercopithecus aethiops*) kidney) and C6/36 (*Ae. albopictus*) cells were infected with each virus at a multiplicity of infection (MOI) of 0.1 by rocking 1h at 25ºC in 25 cm^2 flasks. Cells were washed with 5 ml of L-15 medium three times and 5.5 ml of L-15 was added per flask. At 0 hours post infection (hpi) sample of 0.5 ml was removed and additional 0.5 ml samples were collected at 12, 24, 28, 72, and 96 hpi, the total volume maintained constant by the addition of 0.5 ml of fresh medium at each time point, all samples were stored at –80ºC. Data represents virus production for a standardized monolayer area ($25cm^2$).

Titrations

Viral samples harvested from cell culture and mosquitoes were quantified as tissue culture infectious dose 50 endpoint titers ($Log_{10}TCID_{50}$/ml) using a standard procedure (Higgs et al., 1997). 100 µl samples of cell culture supernatant/mosquito triturate were pipetted into wells of the first column of a 96-well plate, serially diluted in

a 10-fold series across the plate, seeded with Vero cells and incubated at 37ºC for seven days. Prior to titration, each mosquito was triturated in 1 ml of L-15 medium and centrifuged for 5 min at 10,000 rpm.

Stability assay

Stability of eGFP expression from double subgenomic viruses was evaluated as previously described (Brault et al., 2004a). Monolayers of BHK-21 and C6/36 cells in 25 cm^2 flasks were initially infected with CHIKV-LR 5'GFP, and CHIKV-LR 3'GFP at an MOI 0.1 and maintained in 6 ml of L-15. At 2 dpi, 2 µl of tissue culture supernatant from BHK-21 and 10 µl of supernatant from C6/36 cells were used to infect 25 cm^2 flasks of fresh BHK-21 and C6/36 cell cultures. These serial passages were performed ten times for BHK-21 cells and eight times for C6/36 cells. At the end of each passage the percentage of cells in the flask expressing eGFP was estimated by viewing the cells through a fluorescence microscope. Additionally, cell culture supernatants from each even passage were titrated by standard plaque assay. The plates were first analyzed under fluorescence microscope to determine titer of eGFP-expressing foci followed by staining with neutral red to determine the titer of plaque forming units.

Virus infections of mosquitoes

Each of the four viruses to be characterized was fed to four to five-day-old female *Ae. aegypti* (white-eyed Higgs variant of the Rexville D strain) and *Ae. albopictus* (Galveston strain) mosquitoes using an artificial infectious blood meal. (The white-eyed strain used in our experiments was selected based on eye-color, which facilitates visualization of GFP in the brain. We have no data to suggest that relatively high susceptibility of this strain to infection by flaviviruses confers an equivalent high susceptibility to infection with alphaviruses. Several attempts to obtain *Ae. aegypti* and *Ae. albopictus* from Reunion island have been unsuccessful, due to reasons beyond our control). The blood meal was produced using stock virus grown on C6/36 cells harvested at 2 dpi. mixed with equal volumes of defibrinated sheep blood (Colorado Serum Company, Denver, CO). Mosquitoes were infected as previously described (Vanlandingham et al., 2005a; Vanlandingham et al., 2005b) using an isolation glove box located in a Biosafety Level 3 insectary. Infectious blood was heated to 37ºC and placed in a Hemotek feeding apparatus (Discovery Workshops, Accrington, Lancashire, United Kingdom) and mosquitoes were allowed to feed for 45 min. Unfed females were

discarded and fully engorged females were counted and placed into new cartons. Three to 16 mosquitoes were removed for titration at 0, 1, 3, 7, and 14 dpi. and were stored at -80ºC. Day 0 samples were collected immediately following feeding and were used to determine the titer of virus imbibed. Five to 10 *Ae. aegypti* or *Ae. albopictus*, infected with either CHIKV-LR 5'GFP or CHIKV-LR-3'GFP, were cold anaesthetized and midguts and salivary glands were dissected for analysis of eGFP expression at 7 and 14 dpi. as previously described (Vanlandingham et al., 2005b). Dissemination rates were calculated as a ratio between eGFP positive salivary glands over positive midguts.

RESULTS

Sequencing analyses

The entire nucleotide sequence of LR2006 OPY1 strain of CHIKV was determined and compared to the low passage LR2006 OPY1 coding sequence available in GenBank (gi|90654092). Sequences and position of the primers in the CHIKV genome which were used in this sequencing project are summarized in Table 2.1. The determined sequence of LR2006 OPY1 differed at seven positions (1053 G → A (nsP1-V326M), 4168 A→ G (nsP-D1354G), 5050 T→ G (nsP-I1658R), 5645 T→A (nsP-opal codon1857R), 6608 A→ G, 6614 C→ A, 6623 A→ G) as compared with the low passage LR2006 OPY1 coding sequence available in GenBank (gi|90654092). Additionally this sequence (gi|90654092) had an insertion of a T in the third position of the viral genome and a deletion of 44 nucleotides beginning at position 11,696 in the 3' untranslated region and another deletion of the last five nucleotides adjacent to the poly A tail. We contacted Dr. Charrel (Unite des Virus Emergents, Faculte de Medecine, Marseille, France) who sequenced the low passage LR2006 OPY1 and asked him to check chromatograms of his sequencing reactions for polymorphisms at indicated positions. Positions 1053, 4168 and 5050 contained polymorphic signals which were subsequently changed in the modified sequence of LR2006 OPY1 strain (GenBank accession number; DQ443544); differences at positions 6608, 6614, 6623 and mutations in 5' and 3' NCR were not confirmed in the low passage variant and were changed to that determined in our analysis (DQ443544). Only the 5645 T→A (nsP-opal codon1857R) substitution was absent in the sequence of the low passage strain. The presence of this stop codon in our strain, we believe, is attributed to the difference in the passage history of the strains. Our LR2006 OPY1 strain

was additionally propagated twice in Vero cells, once in suckling mice and once in C6/36 cell as compared to the low passage strain sequenced by Dr. Charrel.

Name	Sequence	Position
Rep5-R2	5'-CACGGAAATGTTGAATACTC	NA
SinRep5-Sacv-F	5'-TATAGTCCTGTCGGGTTTC	NA
Qt	5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCT$_{17}$	NA
Qo	5'-CCAGTGAGCAGAGTGACG	NA
Qi	5'-TTAGAGGACTCGAGCTCAAGC	NA
5' RACE Adapter	5'-GCUGAUGGCGAUGAAUGAACACUGCGU UUGCUGGCUUUGAUGAAA	NA
5' RACE Outer Primer	5'-GCTGATGGCGATGAATGAACACTG	NA
5' RACE Inner Primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	NA
Chik-Sp6-F2	5'-CTAGCATCGATTTAGGTGACACTATAG ATGGCTGCGTGAGACACACGT	NA
Chik-ns-F5	5'-ATTGGGCAGATGAGCAGGTACT (Ross)	669-690
Chik-ns-R5	5'-GTAAAGCGTTGACCCGACTGAGA (37997)	898-820
41855-Ngo-R	5'-GGCGTGACTTCTGTAGCA (Ag41855)	1115-1131
41855-Ngo-F	5'-GTCATTCTCGGTGTGCACAT (Ag41855)	1054-1073
Chik-ns-F6	5'-TCCCAGCCGAATTTGACAGC (37997)	1408-1427
Chik-nsF3	5'-TCAACTATAATCACAACATCTGCA (Ag41855)	2562-2585
Chik-nsR3	5'-TGGCTTGTTGTACTCATTTG (37997)	2679-2698
41855-BglII-F	5'-GGAAGTGGAACATCAACA (Ag41855)	3411-3428
Chik-nsR4	5'-GCGTTGACTACACACTCTTC (37997)	4121-4140
41855-nsF1	5'-TGCCGTTACGCCATGACTC (Ag41855)	4865-4883
Chik-ns-R2	5'-TTGACTTTCTGCACTCCTTC (37997)	4967-4986
41855-Eag-R	5'-TCTCCCTCGCCTTCTTCTG (Ag41855)	5281-5299
41855-Hind-R2	5'-TAACTCGTCGTCCGTGTCT (Ag41855)	5626-5644
41855-nsF2	5'-AGTCAGTACGCCAGTCAGTG (Ag41855)	5709-5728
41855-nsF5	5'-ATATCTAGACATGGTGGAC (Ag41855)	6106-6124
41855-nsF3	5'-ATACTGGGAAGAATTTGCTG (Ag41855)	6373-6392
41855-nsR1	5'-TATCAAAGGAGGCTATGTC (Ag41855)	6776-6794
41855-nsR2	5'-CCTAAATCCTCTAACAGCA (Ag41855)	6831-6849
RSU-nsR1	5'-TGAGATCAGCTGGGCAAGT (Ag41855)	7686-7704
41855-BglI-F	5'-GTAATGAAACCAGCACACGT (Ag41855)	7966-7985
41855-Mlu-R	5'-AGCTCCTCCTAAGACTATG (Ag41855)	8244-8269
41855-stF2	5'-GTAGCACTAGAACGCATCAG (Ag41855)	8635-8654
41855-Xho-F	5'-ATCAAGAAGAGTGGGTGAC (Ag41855)	9458-9476
41855-Xho-R	5'-ATACTTATACGGCTCGTTG (Ag41855)	9537-9556
41855-stF1	5'-ACTGCTTCTGCGACGCTGA (Ag41855)	10271-10289
41855-stR1	5'-TCAACTTCTATCTCAGCTTC (Ag41855)	11014-11033
Chik-F3	5'-GGTGCAGAAGATTACGGGAG (37997)	11220-11239
41855-Xmn-R	5'-ACCTACATCTCTTCCGTTTG (Ag41855)	11672-11691
41855-Hind-R	5'-AGTTCGGTATGCTATGCCT (Ag41855)	11733-11751
Chik-pA-R	5'-TCCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTGAAATATTAAAAACAAAATAAC	NA

Table 2.1 Primers used for sequencing of LR2006-OPY1 strain of CHIKV and construction of the full length i.c.

In parenthesis are names of strains used for primer design. Position is given according to LR2006-OPY1 genome. NA- not applicable.

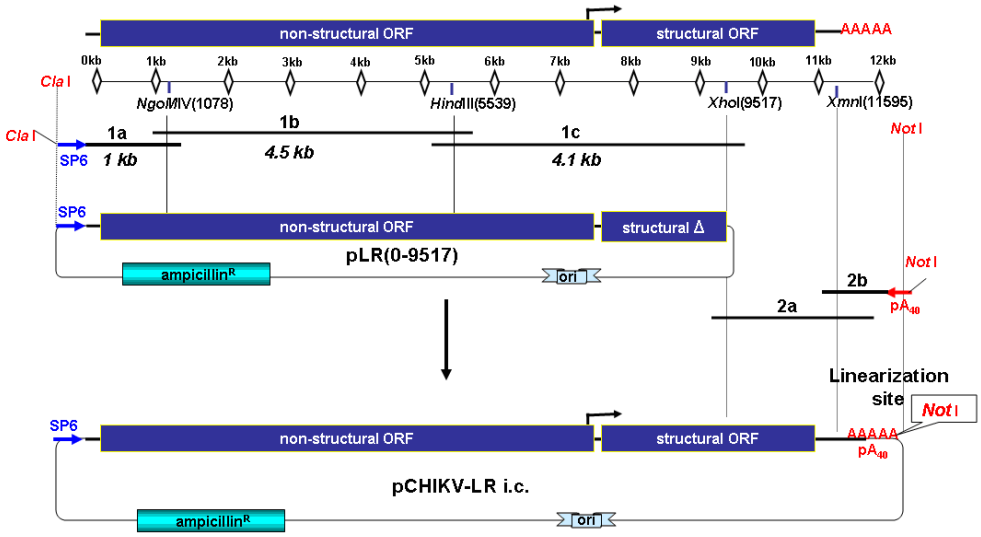


Figure 2.1 Construction of full-length cDNA clone of LR2006 OPY1 strain of CHIKV.

Construction of full-length cDNA clone of LR2006 OPY1 strain of CHIKV

The strategy used for construction of full length clone of CHIKV is depicted in Figure 2.1. The modified version of the pSinRep5 plasmid (Invitrogen, Carlsbad, CA) which has an additional *Cla*I site introduced at 9330 nt in front of the Sp6 promoter (Tsetsarkin K. unpublished data), was used as a backbone for the clone construction. In the first step, three overlapping PCR fragments (1a, 1b, 1c) which cover 9556 nt of the 5' end of the viral genome were generated: fragment 1a was amplified using primers Chik-Sp6-F2 and 41855-Ngo-R; 1b using Chik-ns-F5 and 41855-Hind-R2, and 1c, using 41855-nsF1 and 41855-Xho-R (Table 2.1). Fragment 1a was digested with *Cla*I and *NgoM*IV (1078); fragment a2 - with NgoMIV (1078) and *Hind*III (5539); fragment a3 with *Hind*III (5539) and *XhoI* (9517) restriction endonucleases. *Cla*I, *NgoM*IV, *Hind*III, *XhoI* produce different sticky ends in DNA after the endonuclease reaction, which allows the correct orientation of these fragments in a ligation reaction. Fragments were purified in 1% agarose gels, and cloned simultaneously into pSinRep5-Cla plasmid by *Cla*I and *Xho*I sites. Clones with the desired insert were identified by screening of the individual bacterial colonies using PCR with primers 41855-nsF1 and 41855-Hind-R2. The PCR positive bacterial colonies were grown in 2xYT, plasmid was purified using a QIAprep Spin Miniprep Kit. The resultant plasmid was designated as pLR(0-9517). Integrity of insertion was verified by digestion with *Cla*I, *NgoM*IV, *Hind*III and *XhoI* rectrictases and

by sequencing of the entire PCR-generated region of the plasmid. One synonymous mutation at position 7738 C→A (Capsid-P57P) was found in pLR(0-9517) compared with the sequence of the parental virus. This most likely resulted from errors of *Pfu* DNA polymerase. Since this substitution does not cause a change of amino acid it could serve as a convenient genetic marker in competition experiments of the virus generated from i.c. versus parental virus.

In the final step, two overlapping PCR fragments (2a, 2b), which cover the entire 3' region of the viral genome starting at position 9458 nt, were generated: fragment 2a was amplified using primers 41855-Xho-F and 41855-Xmn-R; 2b using Chik-F3 and Chik-pA-R (Table 2.1). Amplicon 2a was digested with *Xho*I and *Xmn*I restrictases; fragment 2b was digested with *Xmn*I and *Not*I. Fragments 2a, 2b were cloned into the *Xho*I and *Not*I sites of pLR(0-9517). The resultant plasmid designated as pCHIKV-LR i.c., contained the full length cDNA of CHIKV strain LR2006 OPY1 between an Sp6 promoter and *Not*I linearization site. The PCR-generated region of the clone was completely sequenced. There were no mutations compared to the sequence of parental virus. Plasmid was purified using cesium chloride gradient centrifugation, which was used in all following experiments. The sequence of pCHIKV-LR i.c. was deposited into GenBank (EU224268.1).

Characterization of the virus produced from pCHIKV-LR i.c. in cell culture

Template used for *in vitro* transcription	Specific infectivity (pfu/µg of RNA)	Virus titers[a]	
		24h	48h
CHIKV-LR i.c.	8.0×10^5	6.95	7.95
CHIKV-LR 5'GFP	4.8×10^5	6.52	6.52
CHIKV-LR 3'GFP	3.2×10^5	8.52	7.95

Table 2.2 Specific infectivity of RNAs *in vitro* transcribed from CHIKV-LR i.c., CHIKV-LR 5'GFP and CHIKV-LR 3'GFP infectious clones.

[a] – Virus titer from cell culture supernatant at 24 and 48h post-electroporation expessed as $Log_{10}TCID_{50}$/ml.

The quality of the pCHIKV-LR i.c. was analyzed by two methods: specific infectivity of the RNA, following *in vitro* transcription of the pCHIKV-LR i.c. plasmid; and viral titers of rescued virus, following electroporation of the RNA into BHK-21 cells

(Table 2.2). The specific infectivity value was found to be in the range of 10^5-10^6 pfu/µg of RNA, all plaques were similar in size, and viruses grew to a high titer as early as 1 d.p.e. The virus harvested after electroporation of pCHIKV-LR i.c. was named CHIKV-LR. The fact that 100% of cells transfected with RNA were capable of initiating the viral replication cycle, which ultimately led to the production of mature fully infectious viruses, indicates that *in vitro* transcribed RNA does not contain aberrant mutations and the virus harvested after electroporation genetically is identical to the parental virus used for clone construction.

Replication of the parental virus and virus produced from pCHIKV-LR i.c. was analyzed in Vero and C6/36 cell culture (Figure 2.2). Growth kinetics of both viruses were nearly identical in both cell lines, indicating that the CHIKV-LR retained the phenotype of the LR2006 OPY strain at least in cell culture. The maximum titer of 7.52 $Log_{10}TCID_{50}$/ml in Vero and 8.52 $Log_{10}TCID_{50}$/ml in C6/36 cell culture was reached after 24 and 48h, respectively (Figure 2.2).

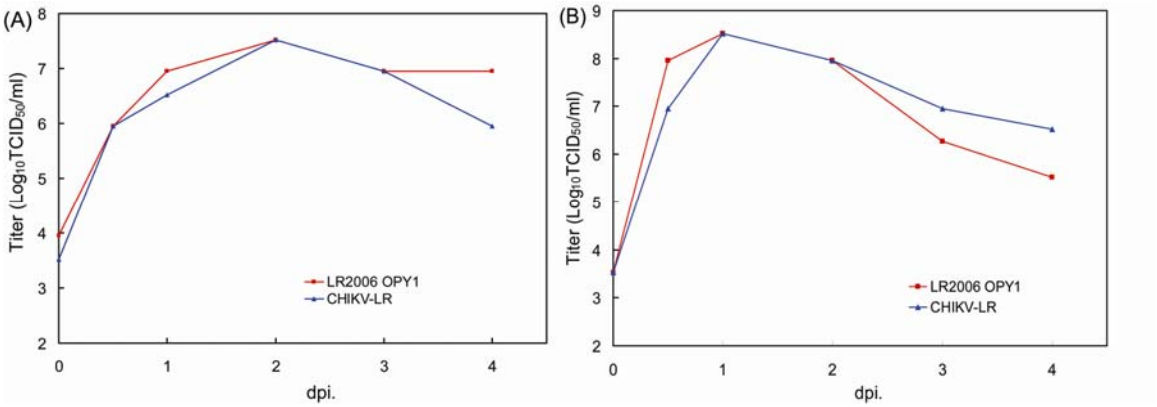


Figure 2.2 *In vitro* growth curves of parental virus (LR2006 OPY1) and viruses produced from pCHIKV-LR i.c. (CHIKV-LR) in (A) Vero and (B) C6/36 cell culture.
Cell were infected at an MOI of 0.1.

Characterization of the virus produced from pCHIKV-LR i.c. in *Ae. aegypti* and *Ae. albopictus* mosquitoes

Infection rates of the parental virus and the virus derived from the full length CHIKV-LR i.c. were compared in *Ae. aegypti* and *Ae. albopictus* mosquitoes. Both viruses were orally presented to *Ae. aegypti* and *Ae. albopictus* and on the indicated days post infection, 6-8 mosquitoes were triturated and the homogenate was analyzed for the presence of the virus by titrating on Vero cells. The average blood meal titers of LR2006

OPY1 and CHIKV-LR in *Ae. aegypti* were the same, 7.24 ± 0.4 $Log_{10}TCID_{50}$/ml (Table 2.3). For *Ae. albopictus*, the blood meal titers of CHIKV-LR and virus derived from pCHIKV-LR i.c. were 7.24 ± 0.4 $Log_{10}TCID_{50}$/ml and 6.95 ± 0.0 $Log_{10}TCID_{50}$/ml, respectively. Viruses were found to be infectious to both species of mosquitoes, and the differences in infection rates were not statistically significant between these two viruses at any time point analyzed (Table 2.3).

Mosquito	Day p.i.	Infection rate Infected / Total (%)		p-value [a]
		LR2006 OPY1	CHIKV-LR	
Ae. aegypti	0	6/6 (100)	6/6 (100)	1
	1	6/6 (100)	6/6 (100)	1
	3	5/5 (100)	7/8 (88)	0.615385
	7	11/15 (73)	14/18 (78)	0.541292
	14	14/18 (78)	6/10 (60)	0.283957
Ae. albopictus	0	6/6 (100)	6/6(100)	1
	1	5/5 (100)	4/4(100)	1
	3	4/4 (100)	6/6(100)	1
	7	17/18 (94)	15/16 (94)	0.742857
	14	17/18 (94)	15/16 (94)	0.742857

Table 2.3 Infection rates of *Ae. aegypti* and *Ae. albopictus* mosquitoes orally infected with LR2006 OPY1 and CHIKV-LR. (Summary of the two experiments).

Titer of LR2006 OPY1 virus in the blood meals fed to *Ae. aegypti* and *Ae. albopictus* was 7.24 ± 0.4 $Log_{10}TCID_{50}$/ml.Titers of CHIKV-LR viruses in the blood meal fed to *Ae. aegypti* and *Ae. albopictus* were 7.24 ± 0.4 $Log_{10}TCID_{50}$/ml and 6.95± 0.0 $Log_{10}TCID_{50}$/ml, respectively.

[a] p-value was calculated using one-tail Fisher's exact test.

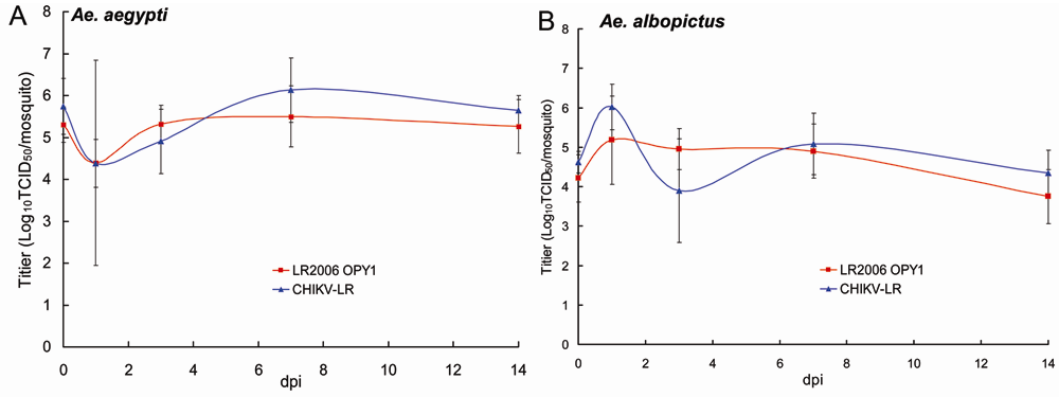


Figure 2.3 Replication of LR2006 OPY1 and CHIKV-LR in *Ae. aegypti* (A) and *Ae. albopictus* (B) mosquito body after oral infection.

Replication of these viruses in the mosquito body after oral infection was also compared (Figure 2.3). There were no statistical differences (Student's t-test) in virus titers at any time point analyzed, indicating that virus produced from pCHIKV-LR i.c. had retained the phenotype of LR2006 OPY strain in both CHIKV vectors: *Ae. aegypti* and *Ae. albopictus*.

Construction of double subgenomic i.c. expressing eGFP based on pCHIK-LR ic.

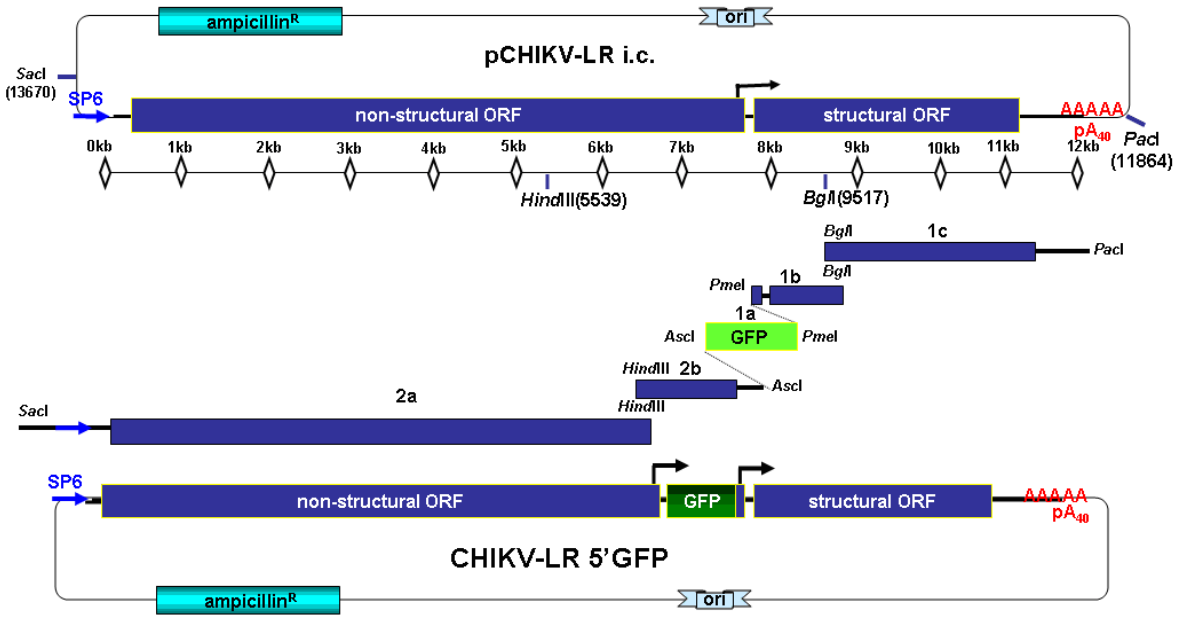


Figure 2.4 Construction of the pCHIKV-5'GFP.

To facilitate future studies of viral determinants of vector specificity, two additional double subgenomic infectious clones, which expressed eGFP, were developed (Figure 2.5 A). These clones differ by the placement of the eGFP gene and additional subgenomic promoter sequence in the viral genome. In pCHIKV-LR 5'GFP, these sequences were introduced at a 5' position relative to the structural part of the CHIKV genome followed by subgenomic promoter driving the expression of structural proteins. In pCHIKV-LR 3'GFP the second subgenomic promoter controlling the expression of eGFP gene was placed at a 3' position to the structural genes (Figure 2.5A).

pCHIKV-LR 5'GFP was constructed in two steps as depicted in Figure 2.4. In the first step, three DNA fragments were cloned simultaneously by *Asc*I and *Pac*I sites into the modified 5' pCHIKic EGFP plasmid described elsewhere (Vanlandingham et al., 2005b).

The first fragment (1a) was generated from 5' pCHIKic EGFP using primer set GFP-Asc-F and GFP-Pme-R (Table 2.4) to amplify the region encoding the eGFP gene. This fragment was digested with *Asc*I and *Pme*I restrictases. The second PCR reaction used primer set Chik-LR-Pme-F and 41855-MluR to generate fragment 2b and was digested with *Pme*I and *Bgl*I restrictases. The third fragment of 3844 nt was produced by digestion of pCHIKV-LR i.c. with *Bgl*I and *Pac*I restrictases. The resultant plasmid was named pCHIKV-LR-GFP-X1 and the entire PCR-generated region of this plasmid was sequenced. No mutations were found as compared to the sequences of pCHIKV-LR i.c. and 5' pCHIKV ic EGFP. For the second step, two DNA fragments (2a and 2b) were cloned simultaneously into pCHIKV-LR-GFP-X1 by *Sac*I and *Asc*I sites. Fragment 2a of 5625 nt was generated by digestion of pCHIKV-LR i.c. with *Sac*I and *Hind*III restrictases. Fragment 2b was amplified by RCR from pCHIKV-LR using 41855-nsF1 and Chik-LR-Asc-R primers set (Table 2.1 and 2.4). The resultant plasmid was named pCHIKV-LR 5'GFP, purified using CsCl gradient centrifugation. PCR-generated region of the plasmid was sequenced. The sequence of pCHIKV-LR 5'GFP was deposited into GenBank (EU224269.1).

Name	Sequence
GFP-Asc-F	5'-CCTTC**GGCGCGCC**ATGGTGAGCAAGGGCGAG
GFP-Pme-R	5'-CCTTCGTTTAAACTACTTGTACAGCTCGTCCA
Chik-LR-Pme-F	5'- CCTTCGTTTAAACCATGGCCACCTTTGCAAG
Chik-LR-Asc-R	5'-AGATGGCGCGCCTGATTAGTGTTTAGATAC- 3
LR-St-Sac-R	5' CAGTCGAGCTCAAGTTAGTGCCTGCTGA
LR-Sg-Sac-F	5'-CAGTCGAGCTCAGTTGTGGTAATGTCCAT
LR-3UTR-Pme-F	5'-CCTTCGTTTAAAACTTGACAATTAAGTATG

Table 2.4 Specific primers used for pCHIKV-LR 5'GFP and pCHIKV-LR 3'GFP construction.

pCHIKV-LR 3'GFP was constructed by one-step cloning. Three DNA fragments were generated by PCR and digested with appropriate restrictases: fragment 1 was amplified from pCHIKV-LR using 41855-XhoF and LR-St-Sac-R primers (Table 2.1 and 2.4) set and digested with *Xho*I and *Sac*I. Fragment 2 was PCR amplified from pCHIKV-LR 5'GFP using primers LR-Sg-Sac-F and GFP-Pme-R and digested with *Sac*I and *Pme*I. Fragment 3 was amplified from pCHIKV-LR using LR-3UTR-Pme-F and Rep5-R2 primers (Table 2.1 and 2.4) followed by digestion with *Pme*I and *Pac*I. These

fragments were simultaneously ligated and cloned by *Xho*I and *Pac*I sites into pCHIKV-LR. The resultant plasmid designated as pCHIKV-LR 3'GFP, was purified using CsCl gradient centrifugation and the PCR generated region of the plasmid was sequenced. No mutations were found in the sequence of the plasmid as compared to pCHIKV-LR.

Characterization of the virus produced from pCHIKV-LR 5'GFP and pCHIKV-LR 3'GFP in cell culture

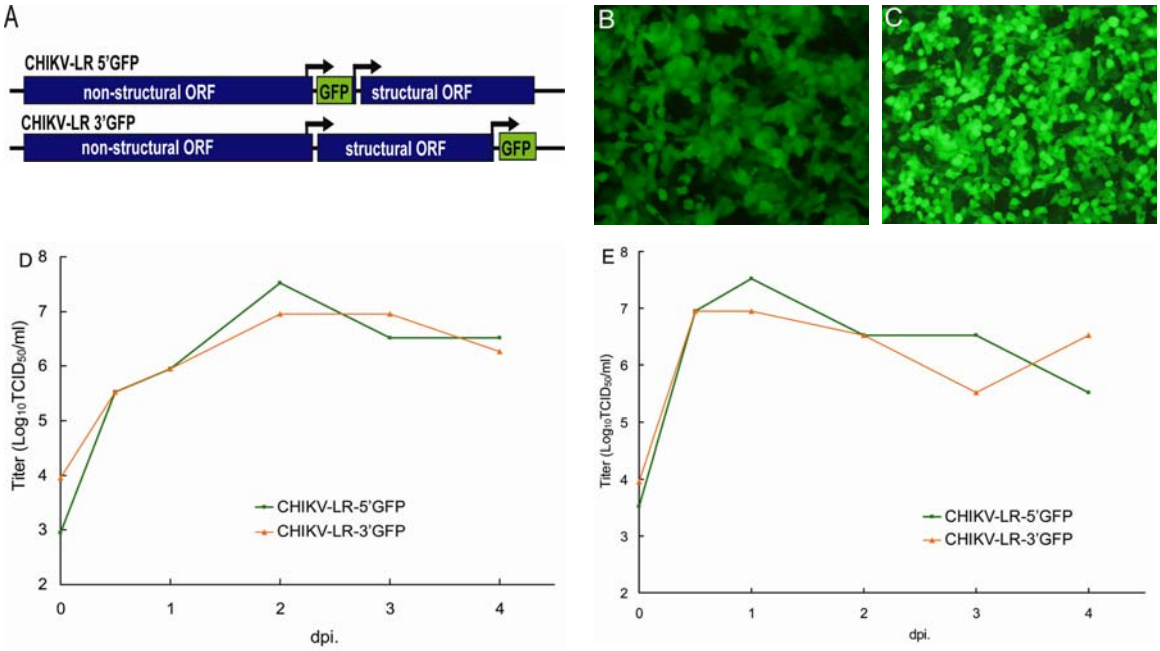


Figure 2.5 Characterization of the virus produced from pCHIKV-LR 5'GFP and pCHIKV-LR 3'GFP in cell culture.
A Schematic representation of viral genomes. B and C eGFP expression from CHIKV-LR 5'GFP (B) and CHIKV-LR 3'GFP (C) in BHK cells 15h after electroporation. D and E *in vitro* growth curves of CHIKV-LR 5'GFP and CHIKV-LR 3'GFP in (D) Vero and (E) C6/36 cell culture.

Specific infectivity and viral titer *in vitro* were used to determine the quality of the double subgenomic promoter (ds) of infectious clones that express eGFP (Table 2.2). Specific infectivities of RNA produced from both the CHIKV-LR 5'GFP and CHIKV-LR 3'GFP infectious clones were similar to each other and were slightly lower than the specific infectivity of the RNA produced from pCHIKV-LR i.c. This indicates that no lethal mutations were introduced into the viral genome during construction of these plasmids. eGFP expression in BHK-21 cell cultures transfected with RNA derived from both ds eGFP-expressing constructs was detected at 3-4 h post-electroporation and

reached its maximum intensity at 15-24h post-electroporation. The level of eGFP expression was considerably higher for the CHIKV-LR 3'GFP construct than for the 5' construct (Figure 2.5 B and C). Growth kinetics of both viruses expressing eGFP were analyzed in Vero and C6/36 cell culture. In both cell lines, viruses produced similar growth curves, reaching peaks at 2 dpi. for Vero and 1 dpi. for C6/36 cells. Interestingly, both eGFP-expressing viruses grew less efficiently on Vero cells as compared to LR2006 OPY1 and CHIKV-LR. The growth kinetics of the CHIKV-LR 5'GFP and CHIKV-LR 3'GFP on C6/36 cells indicated a more prominent attenuated pattern. The peak titer of both viruses was at least one $Log_{10}TCID_{50}$/ml lower as compared to LR2006 OPY1 and CHIKV-LR (Figure 2.5 D, E and Figure 2.2).

Stability of eGFP expression during serial passaging of CHIKV-LR 5'GFP and CHIKV-LR 3'GFP viruses in cell culture

To assess the stability of the expression of the gene of interest (GOI), eGFP, under the control of the second sub-genomic promoter, both CHIKV-LR 5'GFP and CHIKV-LR 3'GFP viruses were serially passed in a vertebrate (BHK-21) and an invertebrate (C6/36) cell line. Previously, it has been shown that alphavirus expression vectors can be unstable and can lose their ability to express a GOI during serial passages in the cell culture (Brault et al., 2004a; Pugachev et al., 1995). For each passage, the percentage of the cells expressing eGFP was determined (Table 2.5). In BHK-21 cells recombinant viruses were relatively stable and retained the ability to drive eGFP expression in at least 85% of the cells after six serial passages. The CHIKV-LR 5'GFP was more stable and demonstrated slower kinetics of eGFP loss as compared to CHIKV-LR 3'GFP. After ten passages in BHK-21, the CHIK-LR 5'GFP and CHIK-LR 3'GFP

Virus	Percentage of cells expressing eGFP									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
BHK cells										
CHIKV-LR 5'GFP	100	100	99	98	95	90	90	85	80	50
CHIKV-LR 3'GFP	100	99	99	95	80	85	65	50	30	5
C6/36 cells										
CHIKV-LR 5'GFP	100	95	95	90	87	85	83	80	ND	ND
CHIKV-LR 3'GFP	100	90	90	70	50	10	2	<1	ND	ND

Table 2.5 Stability of eGFP expression induced by CHIKV-LR 5'GFP and CHIKV-LR 3'GFP viruses after serial passages in BHK-21 and C6/36 cell culture.

P1-10 passage number

viruses retained the ability to express eGFP in approximately 50% and 5% of the cells, respectively (Table 2.5). The same pattern was observed during the serial passage of both viruses in C6/36 cells; after six passages only 10% of C6/36 cells infected with virus derived from pCHIKV-LR 3'GFP were able to express eGFP while 85% of the cells infected with virus derived from CHIKV-LR 5'GFP were positive for eGFP (Table 2.5). Similar results were obtained in experiments where we compared titers of plaque forming units (pfu) to the titer of eGFP foci forming units (ffu) of viruses collected after each passage (Figure 2.6). For CHIKV-LR 3'GFP in BHK-21, the difference between fluorescent titer and pfu was less then 0.5 Log_{10}pfu/ml during the first eight passages and reached a difference of 2 Log_{10}pfu/ml at passage ten. In C6/36 cell culture a 2 Log_{10}pfu/ml difference was reached at passage six whereas for CHIKV-LR 5'GFP this difference was less than 0.5 Log_{10}pfu/ml (Figure 2.6).

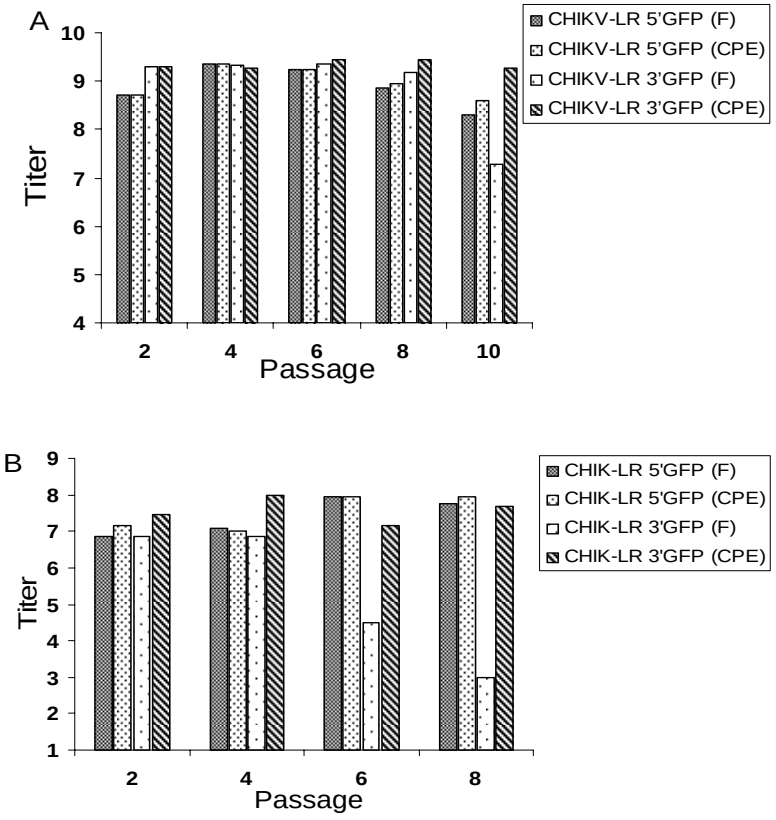


Figure 2.6 Titers of CHIKV-LR 5'GFP and CHIKV-LR 3'GFP following serial passages in (A) BHK-21 and (B) C6/36 cell lines.

F - titer of eGFP positive foci (Log_{10}/ml)
CPE - titer of plaque forming units (Log_{10}/ml)

Characterization of the viruses produced from pCHIKV-LR 5'GFP and pCHIKV-LR 3'GFP in *Ae. aegypti* and *Ae. albopictus* mosquitoes

Viruses derived from the 5' and 3' CHIKV-LR GFP ic were compared in orally infected *Ae. aegypti* and *Ae. albopictus* mosquitoes (Table 2.6). The titers of the blood meals were 5.52 $Log_{10}TCID_{50}$/ml for CHIKV-LR 5' GFP and 6.27 $Log_{10}TCID_{50}$/ml for CHIKV-LR 3'GFP. Viruses derived from pCHIKV-LR 5'GFP infected 63% and 100% of the *Ae. aegypti* and *Ae. albopictus* mosquitoes examined at 14 dpi. Infection rates of CHIKV-LR 5'GFP were not statistically different from parental virus (LR2006 OPY1) and from CHIKV-LR ($p>0.1$ Fisher's Exact Test) in both mosquito species. In contrast, CHIKV-LR 3'GFP failed to infect *Ae. aegypti* (0/8), and infected only 63% (5/8) of *Ae. albopictus*. This is significantly less efficient ($p<0.01$ Fisher's exact test) as compared to infectious rates of LR2006 OPY1 and CHIKV-LR in *Ae. aegypti*, and close to significance for *Ae. albopictus* mosquitoes ($p<0.1$ Fisher's Exact Test)(Table 2.6).

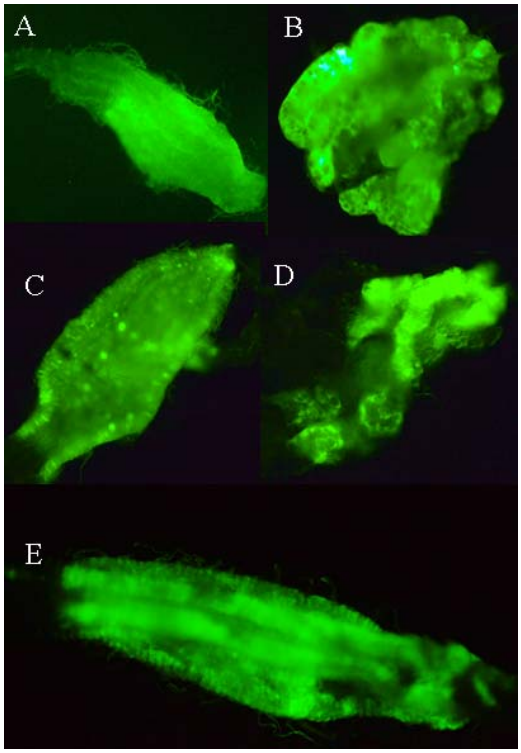
Ae. aegypti and *Ae. albopictus* mosquitoes were also examined by eGFP expression in the midguts and salivary glands (Figure 2.7). Virus derived from the pCHIKV-LR 5'GFP ic infected 100% of midguts and disseminated in 88% of the *Ae. aegypti* and *Ae. albopictus* examined at 14 dpi. (Table 2.7). Virus derived from the CHIKV-LR 3'GFP i.c. did not infect *Ae. aegypti* mosquitoes at 7 or 14 dpi. Although, this virus did infect 90% of the *Ae. albopictus* mosquito midguts, it disseminated into only one of nine (11%) salivary glands at 14 dpi. which is significantly lower compared to dissemination rates for CHIKV-LR 5'GFP ($p = 0.003$ Fisher's Exact Test) (Table 2.7).

Mosquito	dpi.	CHIKV-LR 5'GFP		CHIKV-LR 3'GFP	
		Infected / Total (%)	Titer[a] ± S.D.	Infected / Total (%)	Titer[a] ± S.D.
Ae. aegypti	7	8/8 (100)	5.25 ± 0.39	0/8	0
	14	5/8 (63)	4.78 ± 0.74	0/8	0
Ae. albopictus	7	8/8 (100)	4.36 ± 0.51	7/8 (88)	0.99 ± 1.49
	14	7/7 (100)	3.81 ± 0.53	5/8 (63)	4.69 ± 0.24

Table 2.6 Infection rates and average titers of CHIKV-LR 5'GFP and CHIKV-LR 3'GFP in *Ae. aegypti* and *Ae. albopictus* mosquitoes.

Titer of CHIKV-LR 5'GFP blood meals fed to *Ae. aegypti* and *Ae. albopictus* – 5.52 $Log_{10}TCID_{50}$/ml. Titer of CHIKV-LR 3'GFP blood meal fed to *Ae. aegypti* and *Ae. albopictus* – 6.27 $Log_{10}TCID_{50}$/ml.
[a] Titer reported as $Log_{10}TCID_{50}$/ml ± standard deviation.

Figure 2.7 eGFP expression in *Ae. aegypti* (A and B), and *Ae. albopictus* (C, D, E) mosquitoes infected with viruses derived from pCHIKV-LR 5'GFP (A, B, C, D) or pCHIKV-LR 3'GFP (E) infectious clones on day 14 p.i.
A, C, E - mosquito midguts. B, D mosquito salivary glands.



DISCUSSION

The cDNA of CHIKV LR2006 OPY1 strain was fully sequenced during the construction of the infectious clones and compared to the sequence of low passage LR2006 OPY1 available in GenBank (gi|90654092). Analysis of these sequences revealed that positions 1053, 4168 and 5050 contained polymorphic signals in the early strain, but these positions were represented by a single nucleotide variant in the virus sequenced in our study, suggesting that the original CHIKV LR2006 OPY1 contained mixture of at least two different mutants and that one of the strains outcompeted the other during the additional passes in Vero, C6/36 cells and suckling mice presiding sequence of our variant of LR2006 OPY1. Only 5645 T→A (nsP-opal codon1857R) substitution was absent in the sequence of the low passage strain. The presence of this sequence in our strain, we believe, is also attributed to the difference in the passage history of the strains. It is possible that the mutation was incorporated into the virus genome as a result of viral adaptation to cell culture conditions.

The specific infectivity of the RNA produced from pCHIK-LR ic was $8x10^5$ pfu/μg of RNA (Table 2.2) which is close to upper limits of the detection and is similar to

the specific infectivity of the other well-characterized alphavirus clones (Liljestrom et al., 1991; Rice et al., 1987). The growth kinetics of virus produced from this infectious clone were similar to original virus in both cell culture (Figure. 2.2) and in *Ae. aegypti* and *Ae. albopictus* mosquitoes (Figure 2.3; Table 2.2), indicating that CHIK-LR retained the viral phenotypes of the original isolate. Infections rates of both of these viruses were near 100% in *Ae. albopictus* mosquitoes; whereas only 60-78% of *Ae. aegypti* were infected (Table 2). This is similar to previously reported infection rates of CHIKV infection in mosquitoes (Turell et al., 1992). Interestingly, in previous experiments using CHIKV strain 37997, the infection rate of CHIKV was found to be 100% for *Ae. aegypti* (Vanlandingham et al., 2005b; Vanlandingham et al., 2006). One possible explanation for this discrepancy is that Vanlandingham et al. (2005) used higher blood meal virus titers for infecting *Ae. aegypti*. Differences may also be due to a change of protocol for the production of the infectious blood meal from Vero to C6/36 cell culture. Thus, it has been shown that biological properties of the virus can vary depending on the origin of the infectious particles, possibly due to differences in the glycosylation pattern (Hsieh and Robbins, 1984; Klimstra et al., 2003). It is also possible that genetic differences between 37997 and LR2006 OPY1 strains determine CHIKV infectivity for *Ae. aegypti*.

Mosquito	Day p.i.	CHIK-LR 5'GFP		CHIK-LR 3'GFP	
		Positive midguts/ Total (%)	Positive SG/ Positive midguts (%)	Positive midguts/ Total (%)	Positive SG/ Positive midguts (%)
Ae. aegypti	7	5/8 (63)	5/5 (100)	0/8 (0)	0
	14	8/8 (100)	7/8 (88)	0/8 (0)	0
Ae. albopictus	7	8/8 (100)	4/8 (50)	9/10 (90)	0/9 (0)
	14	8/8 (100)	7/8 (88)	9/10 (90)	1/9 (11)

Table 2.7 Expression of eGFP in *Ae. aegypti* and *Ae. albopictus* mosquitoes orally infected with CHIKV-LR 5'GFP and CHIKV-LR 3'GFP.

Titer of CHIKV-LR 5'GFP blood meals fed to *Ae. aegypti* and *Ae. albopictus* – 5.52 $Log_{10}TCID_{50}$/ml. Titer of CHIKV-LR 3'GFP blood meal fed to *Ae. aegypti* and *Ae. albopictus* – 6.27 $Log_{10}TCID_{50}$/ml.

Two additional ds infectious clones, CHIKV-LR 5'GFP and CHIKV-LR 3'GFP, were developed and characterized in cell culture (Figure 2.5) and in *Ae. aegypti* and *Ae. albopictus* (Figure. 2.7; Table 2.6; Table 2.7). The specific infectivity for the RNA produced from these clones was similar to those obtained from virus derived from

pCHIKV-LR i.c., which indicates that the introduced genetic changes are not lethal (Table 2.2). The level of eGFP expressed by BHK-21 cells transfected with CHIKV-LR 3'GFP RNA was much higher than eGFP expressed using the CHIKV-LR 5'GFP construct (Figure. 2.5 B and C). Higher levels of eGFP expression in alphavirus infectious clones constructed using the 3' configuration have been reported previously (Bredenbeek and Rice, 1992; Pugachev et al., 1995; Vanlandingham et al., 2005b). The growth of both viruses derived from the 3' or 5' infectious clones in Vero and C6/36 cells was attenuated as compared to the growth of both LR2006-OPY1 and virus produced from pCHIKV-LR i.c. This suggests that these viruses might be unstable and can lose the ability to express the eGFP following serial passages in cell culture (Table 2.5;Figure 2.6). The phenomena of instability of ds viruses has been described for several alphaviruses including SINV and ONNV (Brault et al., 2004a; Higgs et al., 1995; Pierro et al., 2003; Pugachev et al., 1995). Consistently, 5' constructs have been shown to be more stable as compared to 3' constructs. In our work we also observed that during serial passage, eGFP expression from both ds viruses decreases with passage number and that CHIKV-LR 5'GFP is more stable in both cell lines as compared to CHIKV-LR 3'GFP (Table 2.5; Figure 2.6). This is most likely due to the placement of the second subgenomic promoter and the GOI in the center of the genome, which leads to a lower probability of recombination events around this region, which would produce live virus that had lost its capacity to express eGFP.

Interestingly, since the multiplicity of infection was similar for both CHIKV-LR 5'GFP and CHIKV-LR 3'GFP, our data also indicate that the CHIKV-LR 3'GFP is more unstable in C6/36 cell culture than in BHK-21 culture (Table 2.5; Figure 2.6). It is possible that the region of the viral genome where the second subgenomic promoter was introduced contains sites for binding of some cellular proteins that are important for viral replication. Kuhn et al. (1990) demonstrated that deletion of parts of the 3' non-coding region (NCR) of Sindbis virus had much stronger effects on virus replication in mosquito cells as compared to avian cell line (Kuhn et al., 1990).

Infection rates of *Ae. aegypti* and *Ae. albopictus* mosquitoes orally infected with virus derived from CHIKV-LR 5'GFP statistically were not different from those observed for parental virus used for i.c. construction and virus derived from pCHIK-LR i.c., indicating that the introduction of the eGFP gene into the viral genome does not

significantly impair the viral infectivity phenotype (Table 2.3; Table 2.6). Analysis of the eGFP expression in mosquito tissues revealed that virus derived from CHIKV-LR 5'GFP can efficiently infect midgut epithelial cells, was capable to escape from the midgut and disseminate to the salivary gland of both mosquitoes species (Table 2.7; Figure 2.7). The infection rates of CHIKV-LR 5'GFP estimated by the presence of eGFP in *Ae. aegypti* and *Ae. albopictus* mosquito midgut epithelium were not significantly different from the infection rates of LR2006 OPY1, CHIK-LR and CHIKV-LR 5'GFP viruses estimated by the presence of infectious viruses in mosquito bodies collected at 7 and 14 dpi., determined by titration in Vero cells. This indicates that the presence of eGFP in the mosquitoe midguts could be used as a convenient marker for estimation of CHIKV infectivity phenotype in its principle mosquito vectors.

Interestingly, eGFP expression in *Ae. aegypti*, where eGFP was localized primarily in the posterior parts of the midgut, was different from expression in *Ae. albopictus*, where eGFP was found throughout the midgut epithelial cells (Figure 2.6). This indicates that cellular permissiveness during initial viral infection is not homogenously distributed in the *Ae. aegypti* midgut. In the case of *Ae. aegypti* similar observations have been reported for the MRE 1001 strain of Sindbis virus (Pierro et al., 2003).

The pattern of eGFP expression in *Ae. aegypti* and *Ae. albopictus* mosquitoes infected with virus derived from CHIKV-LR 3'GFP was markedly different compared with virus derived from CHIKV-LR 5'GFP. Although, virus derived from CHIKV-LR 3'GFP was able to infect *Ae. albopictus* midguts, no eGFP fluorescence was observed in the mosquito salivary glands (Table 2.7). This most likely reflects the instability of CHIKV-LR 3'GFP construct; only viruses that lacked eGFP were able to disseminate to the salivary glands. Thus, when mosquito bodies were titrated, eGFP fluorescence was not detected in every dilution that showed cytopathic effects (data not shown). Similar results have suggested instability of another alphavirus infectious clone, MRE/3'2J/GFP, in *Ae. aegypti* (Pierro et al., 2003).

CHIKV-LR 3'GFP was unable to infect *Ae. aegypti* by the oral route of infection (Table 2.6; Table 2.7), although in our previous study (Vanlandingham et al., 2005b) 3' CHIKV EGFP virus was shown to be infectious for this mosquito. A plausible explanation of this discrepancy is that that the LR2006 OPY strain of CHIKV might have

sites recognized by mosquito proteins that are important for viral replication in the region where the eGFP gene was introduced. Since the 37997 and LR2006 OPY strains have only 80-85% sequence identity among ORFs and even less in the 3' NCR, it is conceivable the 3' CHIKV EGFP would behave differently in the same mosquito species.

The development of three new CHIKV infectious clones based on the epidemic LR2006 OPY strain obtained from La Réunion have already proven to be invaluable tools to study molecular determinants of infection in mosquitoes and pathogenicity in vertebrate hosts, and may provide data for the development of novel methods to control this reemerging virus.

CONCLUSIONS

Results of this study indicate:

1 – Virus produced from full-length i.c. of the recent epidemic CHIKV viral strain LR2006 OPY1 from the Island of La Réunion has retained the phenotype of the original isolate in both cell culture and in *Ae. aegypti* and *Ae. albopictus* mosquitoes.

2 – CHIKV-LR 5'GFP was more stable and demonstrated slower kinetics of eGFP loss as compared with CHIKV-LR 3'GFP in both C6/36 and BHK-21 cells.

3 – The CHIKV-LR 5'GFP infected *Ae. aegypti* and *Ae. albopictus* mosquitoes at a similar rate to the original virus and to the virus produced from the full length infectious clone.

4 – The CHIKV-LR 3'GFP only infected *Ae. albopictus* mosquitoes, but failed to disseminate into mosquito salivary glands.

5 – Overall, pCHIKV-LR i.c. and pCHIK-LR 5'GFP infectious clones are suitable for investigation of genetic factors influencing CHIKV fitness in cell culture and in the mosquito vector.

CHAPTER 3: ROLE OF THE E1-A226V MUTATION IN INFECTION, DISSEMINATION AND TRANSMISSION OF CHIKV BY *AE.ALBOPICTUS* AND *AE. AEGYPTI* MOSQUITOES

ABSTRACT

CHIKV is an emerging arbovirus associated with several recent large-scale epidemics. The 2005-2006 epidemic on Reunion island that resulted in approximately 266,000 human cases was associated with a strain of CHIKV with a mutation in the envelope protein gene (E1-A226V). An unusual feature of this outbreak was the involvement of *Ae. albopictus* as a principle vector of CHIKV. To test the hypothesis that the E1-A226V mutation in the epidemic CHIKV (strain LR2006 OPY1) might influence fitness for different vector species, the viral infectivity, dissemination, and transmission of CHIKV were compared in *Ae. albopictus*, the species implicated in the epidemic, and the recognized typical vector *Ae. aegypti.* Using viral infectious clones of Reunion and a West African strain of CHIKV, into which either the E1-226 A or V residue was engineered, we demonstrated that the E1-A226V mutation was directly responsible for a significant increase in CHIKV infectivity for *Ae. albopictus*, and led to more efficient viral dissemination into mosquito secondary organs and transmission to suckling mice. This mutation caused a marginal decrease in the ability of CHIKV to infect the *Ae. aegypti* midgut, but had no effect on viral dissemination, and was associated with a slight increase in transmission by *Ae. aegypti* to suckling mice in competition experiments.

The observation that a single amino acid substitution can influence vector specificity provides a plausible explanation of how this mutant virus caused an epidemic in a region lacking the typical vector. This has important implications with respect to how viruses may establish a transmission cycle when introduced into a new area.

Substantial parts of this chapter have been reported previously: (Tsetsarkin et al., 2007).

INTRODUCTION

The recent large scale epidemic of the mosquito-borne *Alphavirus*, Chikungunya virus (CHIKV), began in Kenya in 2004 and spread to several Indian Ocean islands including the Comoros, Mauritius, the Seychelles, Madagascar, Mayotte and Reunion. On Reunion island alone there were approximately 266,000 cases (34% of the total island population) (Chastel, 2005; Consigny et al., 2006; Enserink, 2006; Higgs, 2006; Ligon, 2006; Paganin et al., 2006). In the continuing Indian epidemic there have been at least 1.4M cases reported (Charrel et al., 2007a; Pialoux et al., 2007; Ravi, 2006; Saxena et al., 2006) with continued expansion in Sri Lanka and Indonesia. CHIKV had not been reported to cause fatalities in prior outbreaks; however, during the outbreak on Reunion island, CHIKV was associated with at least 260 deaths (Charrel et al., 2007b; Simon et al., 2007). The strain of CHIKV responsible for the Indian Ocean island epidemic has been well-characterized in cell culture and mosquito models (Ozden et al., 2007; Sourisseau et al., 2007; Tsetsarkin et al., 2006), however the underlying genetic basis of the atypical phenotype of this CHIKV strain remains unknown.

CHIKV is transmitted by *Aedes* species mosquitoes, primarily *Ae. aegypti*. However, the 2005-2006 CHIKV epidemic on Reunion island was unusual because the vector responsible for transmission between humans was apparently the Asian tiger mosquito, *Aedes albopictus* (Enserink, 2006; Reiter et al., 2006). This conclusion is based on several factors. This species is known to be susceptible to CHIKV infection and although infectious virus was not isolated from *Ae. albopictus* during the epidemic, CHIKV RNA was detected (X. de Lamballerie personal communication). Furthermore, the species is anthropophilic, was abundant during the epidemic, and other potential vectors specifically *Ae. aegypti* were relatively scarce with a very limited distribution (P. Reiter, personal communication). A later study demonstrated virus isolation from field-collected *Ae. albopictus* females (Delatte et al., 2008). It also was suggested that the local *Ae. albopictus* population may be more anthropophilic than in other parts of the world (Reiter et al., 2006).

Ae. albopictus is abundant and widely distributed in urban areas of Europe and the United States of America (Aranda et al., 2006; Gratz, 2004; Knudsen et al., 1996; Romi et al., 2006; Schaffner and Karch, 2000; Schaffner et al., 2004). CHIKV infections have been reported in many travelers returning to the U.S. and Europe (CDC, 2007b;

Cordel et al., 2006; Krastinova et al., 2006; Lanciotti et al., 2007; Simon et al., 2007) causing concern that the virus could be introduced and become established in these areas (Chastel, 2005; Depoortere and Coulombier, 2006; Service, 2007). In August and September of 2007, a CHIKV-*Ae. albopictus* transmission cycle was reported for the first time in Europe, with an estimated 254 human cases occurring in Italy (ECDC, 2007; Enserink, 2007).

Analysis of CHIKV genome microevolution during the 2005-2006 Indian Ocean epidemic identified an alanine to valine mutation at position 226 in the E1 envelope glycoprotein (E1-A226V) among viral isolates obtained during the outbreak (Schuffenecker et al., 2006). The reason for this and its significance was unclear, but it was hypothesized that the E1-A226V mutation might influence infectivity of CHIKV for mosquito vectors (Charrel et al., 2007b; Schuffenecker et al., 2006). Interestingly, earlier studies identified that a P→S mutation in the same position of the E1 glycoprotein of Semliki Forest virus (SFV – a member of *Alphavirus* genus) is responsible for the modulation of the viral requirement for cholesterol in the target membrane (Vashishtha et al., 1998). It also was shown that the presence of this mutation results in more efficient growth of SFV in *Ae. albopictus* mosquitoes (Ahn et al., 1999).

To test the hypothesis that the E1-A226V mutation might influence the fitness of CHIKV in mosquito vectors we compared the effect of this mutation on CHIKV mosquito infectivity, the ability to disseminate into heads and salivary glands, and the relative fitness in competition assays for transmission by *Ae. albopictus* and *Ae. aegypti* to suckling mice. Here we report findings that a single nucleotide change, which arose during the epidemic, significantly increases fitness of the virus in *Ae. albopictus*, but has no effect on viral fitness in *Ae. aegypti* mosquitoes. This change likely enhanced CHIKV transmission by an atypical vector and contributed to the maintenance and scale of the epidemic.

MATERIALS AND METHODS

Viruses and plasmids

The viruses and plasmids encoding full length infectious clones of the LR2006 OPY1 strain pCHIK-LR ic (GenBank accession number EU224268) and GFP-expressing

full length clone pCHIK-LR 5'GFP (GenBank accession number EU224269) have been previously described (Chapter 2; Tsetsarkin et al., 2006; Vanlandingham et al., 2005b). To provide a logical nomenclature that can be easily interpreted by the reader, abbreviations for the different plasmids and viruses include the viral origin, marker gene (if any) and relevant aa at a given position in the sequence. For example, the plasmid for the CHIKV from La Reunion (LR) with the valine mutation at position E1226, expressing GFP from a 5' promoter (pCHIK-LR 5'GFP), was renamed pLR-GFP-226V. The plasmids p37997-226A (pCHIK-37997ic, GenBank accession number EU224270) encoding the full length infectious clones of the West African 37997 strain of CHIKV and a eGFP-expressing full length clone p37997-GFP-226A (pCHIK-37997-5GFP, GenBank accession number EU224271) were derived from previously described plasmids pCHIKic and 5'CHIK EGFP (Vanlandingham et al., 2005b) by introducing CHIKV-encoding cDNA into a modified pSinRep5 (Invitrogen) at positions 8055-9930. Viruses derived from 37997-226A and 37997-GFP-226A are identical to viruses derived form pCHIKic and 5'CHIK EGFP. Plasmids were constructed and propagated using conventional PCR-based cloning methods (Chapter 2; Sambrook et al., 1989). The entire PCR-generated regions of all constructs were verified by sequence analysis.

Introduction of point mutations. For studies comparing the relative fitness of the mutant (E1-226V) virus and the pre-epidemic genotype (E1-226A), a synonymous mutation (6454C) was introduced into the pCHIK-LR ic, to add an *Apa*I restriction site into the coding sequence of CHIK-LR ic. The plasmid was constructed by simultaneous ligation and cloning of three DNA fragments by *Age*I and *Xho*I sites into pCHIKV-LR i.c. Fragment 1 was generated by PCR from pCHIKV-LR i.c. using 41855-nsF1 and Chik-LR C6454-R primers set followed by digestion with *Age*I and *Apa*I restrictases. Fragment 2 was amplified from pCHIKV-LR i.c. with primers Chik-LR C6454-F and RSU-R1 and digested with *Apa*I and *BsrG*I restrictases (Table 2.1; 3.1). Fragment 3 (2829nt) was generated by digestion of pCHIKV-LR i.c. with *BsrG*I and *Xho*I. The plasmid was sequenced from 41855-nsF1 and 41855-nsF2primers. The resulting plasmid was named LR-ApaI-226V.

For studies of mosquito infectivity, the mutation encoding pre-epidemic E1-226A residue was introduced into the backbone of pLR-GFP-226V. The plasmid was constructed by simultaneous cloning of 2 DNA fragments into pLR-GFP-226V.

Fragment 1was generated using the fusion PCR method. Initially, two PCR reactions were run on pCHIKV-LR i.c. under the following conditions: 20 cycles at 94ºC, 20 sec; 55ºC, 20sec; 70ºC, 2 min; with a final extension at 70ºC for 5 min. The first reaction (PCR1) was run using the 41855-XhoF and146R primers set; for the second reaction (PCR2) 146F and 41855-XmnR primers were used. 0.5 µl of each amplicon generated by PCR1 and PCR 2 were used as a template for PCR3 using primers 41855-XhoF and 41855-XmnR and the cycling conditions identical to those used for PCR1 and PCR 2 (Table 2.1; 3.1). The resultant amplicon containing a single point mutation T10670C (E1-V226A) was digested by *Xho*I and *Kpn*I restrictases. Fragment 2 (961 nt.) was generated by digestion of pCHIKV-LR i.c. with *Kpn*I and *Pac*I restrictases. Fragments 1 and 2 were cloned by *Xho*I and *Pac*I into pLR-GFP-226V. The resultant plasmid was designated as pLR-GFP-226A and the PCR generated region was completely sequenced using primers 41855-XhoF and 41855-St-F1 (Table 2.1).

The pre-epidemic E1-226A residue was also introduced into pCHIKV-LR i.c., which does not contain eGFP. The pLR-GFP-226A was digested with *Xho*I and *Not*I restrictases to generate a fragment of 2320 nt. This fragment was ligated and cloned by *Xho*I and *Not*I sites into pCHIKV-LR i.c. The resultant plasmid was designated pLR-226A. To confirm the introduction of the E1-226A sequence, the plasmid was sequenced from the 41855-St-F1 primer (Table 2.1).

The post epidemic mutation E1-A226V was also introduced into plasmid p37997-226A and p37997-GFP-226A to generate plasmids designated p37997-226V and p37997-

Name	Sequence
Chik-LR C6454-R	5’-CCAC**G**GGCCCTTTTAGTTTAGTAACA
Chik-LR C6454-F	5’-CCACGGGCC**C**AAAGCAGCAGCGCTA
146R	5’-CACGTGTACCGTACCC**G**CAGCCGGTCTCTGCAG
146F	5’-CTGCAGAGACCGGCTG**C**GGGTACGGTACACGTG
CHIK-E2T-F	5’-CAGTC**GAGCTC**ACACCAGGAGCCACTGTTC
160-F	5’-ACTACAGAGGCCAGCAG**T**AGGCACGGTACATGTACCA
160-R	TGGTACATGTACCGTGCCT**A**CTGCTGGCCTCTGTAGT
Chik-F4	5’-GAAACAACATTACCGTAGC

Table 3.1 Specific primers for introduction of the point mutations into pCHIK-LR ic, pLR-GFP-226V, p37997-226A and p37997-GFP-226A constructs.

GFP-226V respectively. The p37997-226V plasmid was constructed by simultaneous cloning of 2 DNA fragments into p37997-226A. Fragment 1 (2647 nt.) was generated by digestion of p37997-GFP-226A with *NgoM*IV and *Apa*I restrictases. Fragment 2 was amplified using fusion PCR technique. Initially, two PCRs were run on p37997-GFP-226A from 2 primers sets: CHIK-E2T-F and 160-R (PCR1) and 160-F and Rep5R2 (PCR2) (Table 2.1; 3.1). Fusion PCR was run using amplicons generated from PCR1 and PCR2 as a template and used the CHIK-E2T-F and Rep5R2 primer set. The fragment 2 was digested with *Apa*I and *Pac*I restrictases. Fragments 1 and 2 were simultaneously cloned, using the *NgoM*IV and *Pac*I sites, into p37997-226A. PCR-generated region of p37997-226V was sequenced from CHIK-E2T-F, Chik-F4 and Chik-F3 (Table 2.1; 3.1).

The eGFP gene was introduced into plasmid p37997-226V to generate p37997-GFP-226V. One DNA fragment (3554 nt.) was produced by digestion of p37997-GFP-226A with *BspE*I and *NgoM*IV restrictases. This fragment was cloned using the *BspE*I and *NgoM*IV sites into p37997-226V. The resultant p37997-GFP-226V plasmid was sequenced from Chik-F4.

All plasmids were purified by centrifugation in CsCl gradients, linearized with *Not*I and *in vitro* transcribed from the minimal SP6 promoter using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) following the manufacturer's instructions. The yield and integrity of synthesized RNA were analyzed by agarose gel electrophoresis in the presence of 0.25 μg/ml of ethidium bromide. RNA (10 μg) was transfected into $1x10^7$ BHK-21 cells by electroporation as previously described (31). Cells were transferred to 25 cm^2 tissue culture flasks with 10 ml of Leibovitz L-15 (L-15) medium, and supernatants were collected at 24 and 48 h post-electroporation and stored at -80ºC. In parallel, $1x10^5$ electroporated BHK-21 cells were serially ten-fold diluted and seeded into six-well plates for infectious centers assay as previously described (Chapter 2; Tsetsarkin et al., 2006).

Cells and mosquitoes

BHK-21 (baby hamster kidney) cells were maintained at 37ºC in L-15 medium supplemented with 10% fetal bovine serum (FBS), 100U penicillin, and 100 μg/ml streptomycin. C6/36 cells (*Ae. albopictus*) were grown in the same medium at 28ºC. *Ae. aegypti* (white-eyed Higgs variant of the Rexville D strain) and *Ae. albopictus* (Galveston strain) were reared at 27ºC and 80% relative humidity under a 16h light: 8h dark

photoperiod, as previously described (Tsetsarkin et al., 2006). Adults were kept in paper cartons supplied with 10% sucrose on cotton balls. To promote egg production females were fed on anaesthetized hamsters once per week.

Rexville D strain of *Ae. aegypti* mosquitoes were originally selected for susceptibility to flavivirus infection (Miller and Mitchell, 1991a). Since there are no known consequences of this original selection with respect to susceptibility to CHIKV, a white eyed variant of (Wendell et al., 2000) the strain that facilitates detection of GFP was used in our experiments.

In vitro growth of CHIKV

CHIKV growth curves were determined by infecting BHK-21 and C6/36 cells at a multiplicity of infection (MOI) of 1.0 by rocking for 1 h at 25°C. The cells were washed three times with L-15 medium and 5.5 ml of fresh L-15 supplied with 10 % FBS was added to the flask. At the indicated times post-infection, 0.5 ml of medium was removed and stored at -80ºC until titrated. The volume of medium was then restored by adding 0.5 ml of appropriate medium.

Titrations

Viral titers from mosquito samples and from tissue culture supernatant were determined using Vero cells and expressed as tissue culture infectious dose 50 percent endpoint titers ($Log_{10}TCID_{50}$) as previously described (Higgs et al., 1997). Additionally, for viral competition experiments, titers of LR-Apa-226V and LR-226A viruses were determined using standard plaque assay on Vero cells as previously described (Lemm et al., 1990).

Oral infection of mosquitoes

Ae. aegypti and *Ae. albopictus* were infected in an Arthropod Containment Level 3 insectary as described previously (Chapter 2; McElroy et al., 2006; Vanlandingham et al., 2005b). To make infectious blood meals for the viruses lacking eGFP, viral stocks derived from electroporated BHK-21 cells were mixed with an equal volume of defibrinated sheep blood and supplemented with 3 mM ATP as a phago-stimulant. To produce infectious blood meals for the eGFP-expressing viruses, the viruses were additionally passed on BHK-21 cells. The cells were infected at an MOI ≈ 1.0 with virus derived from electroporation. At 2 dpi., cell culture supernatants were mixed with an

equal volume of defibrinated sheep blood and presented to four-five days old female mosquitoes that had been starved for 24h, using a Hemotek membrane feeding system (Discovery Workshops, Accrington, United Kingdom) and hamster skin membrane. Mosquitoes were allowed to feed for 45 min, and engorged mosquitoes [stage ≥3+ (Pilitt and Jones, 1972)] were sorted and counted into a cage for maintenance. Blood meals and three to four mosquitoes were immediately removed for titration and/or RNA extraction. Depending on the purpose of the experiments, mosquitoes were collected at different days post-infection and either titrated to determine viral titer, dissected for analysis of eGFP expression in the midguts or salivary glands (Tsetsarkin et al., 2006), or used for RNA extraction in competition experiments.

To estimate the Oral Infectious Dose 50% values (OID_{50}), serial ten-fold dilutions of viruses were made in L-15 medium followed by mixing the samples with defibrinated sheep blood. Mosquitoes were dissected at 7 dpi. and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one focus of eGFP expressing cells was present in the midgut. The experiments were performed twice for each virus. OID_{50} values and confidence intervals were calculated using PriProbit (Version 1.63). To compare oral infectivity of non-eGFP-expressing viruses, 16 to 24 mosquitoes from each viral dilution were collected at 7 dpi., individually triturated in 1ml of L-15 media and titrated as previously described (Vanlandingham et al., 2005b). A mosquito was considered infected if it contained more than 0.94 $Log_{10}TCID_{50}$ infection units (limit of detection). The experiments were performed once or twice for each virus. OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63). The SAS equivalent method was used to calculate the fiducial limits (confidence intervals), assuming normal function distribution and an "all or nothing" response parameter. The difference between two OID_{50} values was considered statistically significant ($p<0.05$) if 95% fiducial limits did not overlap.

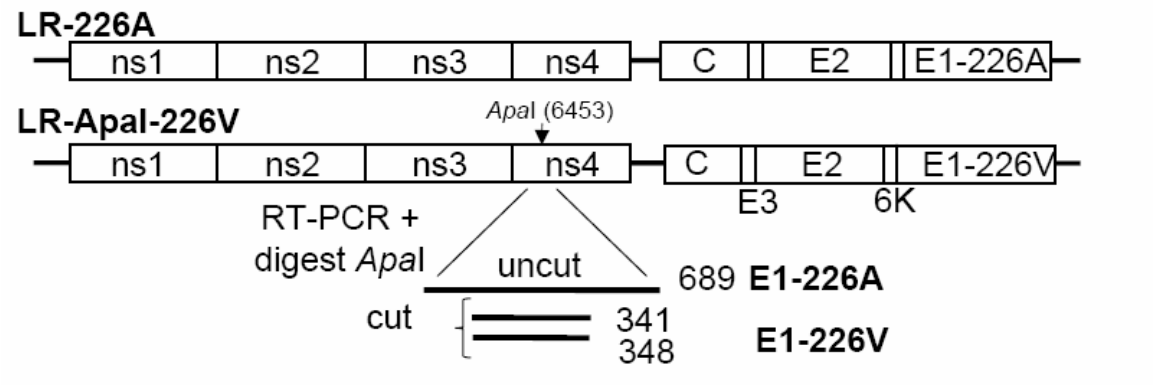


Figure 3.1 Schematic representation of viral competition experiment.

Viral competition experiments

Competition assays were designed similar to those described previously in mice (Pfeiffer and Kirkegaard, 2005), with minor modifications (Figure 3.1). Both *Ae. aegypti* and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 plaque-forming units (pfu)/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. Midguts were collected at 7 dpi. and analyzed in pools of 8-10, and heads were collected at 12 dpi and analyzed in pools of 5. RNA was extracted from the tissue pools using TRIzol reagent (Invitrogen Carlsbad, CA) followed by additional purification using a Viral RNA mini kit (QIAGEN, Valencia, CA). RNAs from blood meal samples were extracted using Viral RNA Mini Kit followed by treatment with DNAse (Ambion, Austin, TX) to destroy any residual plasmid DNA contaminant in the viral samples. RNA was reversed transcribed from random hexamer primers using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was amplified from 41855ns-F5 (5'- ATATCTAGACATGGTGGAC) and 41855ns-R1 (5'- TATCAAAGGAGGCTATGTC) primers using Taq DNA polymerase (New England Biolabs, Ipswich, MA). PCR products were purified using Zymo clean columns (Zymo Research, Orange, CA) and were quantified by spectrophotometry. Equal amounts of PCR products were digested with *Apa*I, separated in 2% agarose gels that were stained using ethidium bromide. Thus the LR-Apa-226V and LR-226A viruses could be distinguished by size on an agarose gel (Figure 3.1). Gel images were analyzed using TotaLab (Version 2.01). The relative fitness of LR-Apa-226V and LR-226A viruses was

calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio of 226V and 226A in the blood meal.

Virus competition in an animal transmission model

Ae. aegypti and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V or LR-226A virus. At 13 dpi, 10-15 mosquitoes were placed in separate paper cartons and starved for 24 h. The next day the mosquitoes in each carton were presented with individual 2-3 day old suckling mice (Swiss Webster). Feeding continued until 2-3 mosquitoes per carton were fully engorged. In a parallel experiment six 2-3 days old suckling mice were subcutaneously injected with a 20 µl inoculum containing ≈ 25 pfu of LR-Apa-226V and ≈ 25 pfu of LR-226A viruses. Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (≈50 µl) was collected and immediately mixed with 450 µl of TRIzol reagent for RNA extraction. The RNA was processed as described above. All animal manipulations were conducted in accordance with federal laws, regulations, and in compliance with National Institutes of Health and University of Texas Medical Branch Institutional Animal Care and Use Committee guidelines and with the Association for Assessment and Accreditation of Laboratory Animal Care standards.

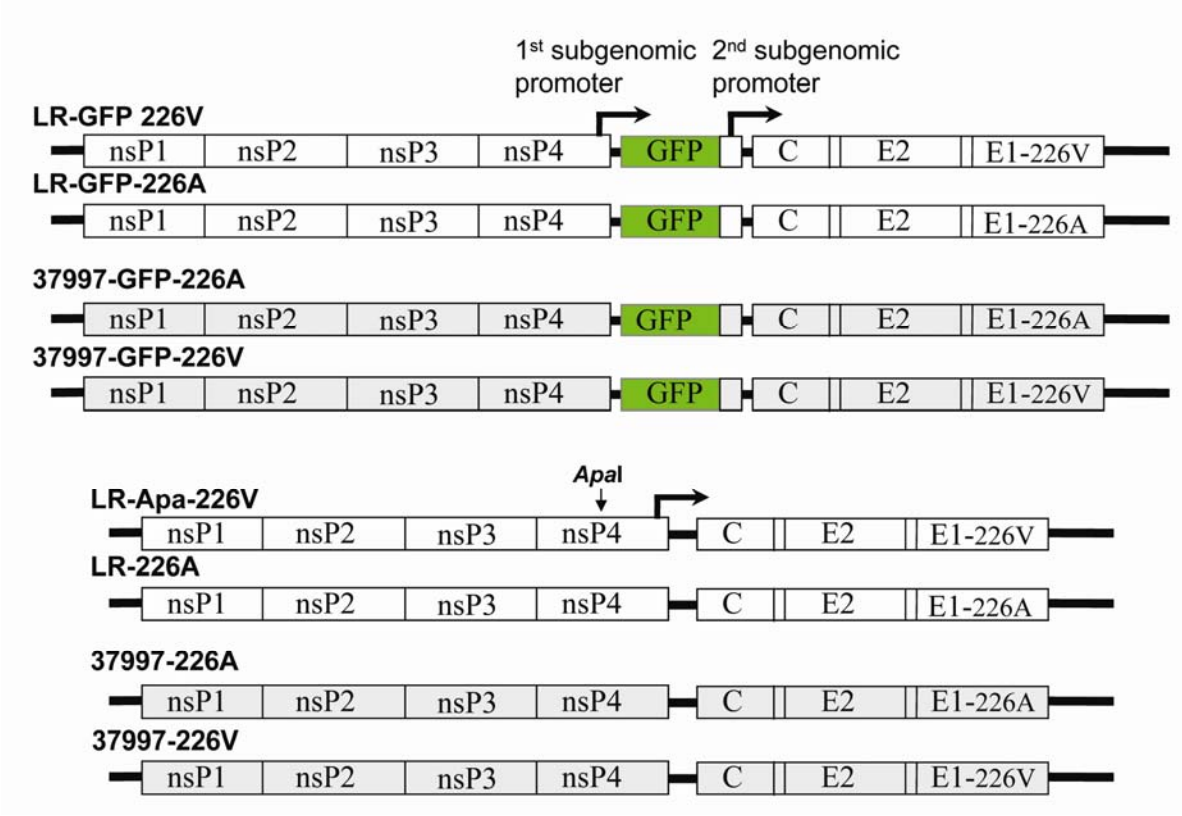


Figure 3.2 Schematic representation of the viruses used in this study.

RESULTS

Effect of E1-A226V mutation on fitness of CHIKV in *Ae. albopictus* mosquitoes

To test the hypothesis that the E1-A226V mutation altered CHIKV infectivity for *Ae. albopictus* mosquitoes, CHIKV infectious clones derived from an epidemic Reunion island human isolate were used (Chapter 2; Tsetsarkin et al., 2006), including one clone (LR-GFP-226V) expressing enhanced green fluorescent protein (eGFP). Clones were further engineered to express E1 protein containing an alanine at position E1-226 (LR-GFP-226A) representing the CHIKV genotype prevalent prior to the outbreak gaining momentum (Figure 3.2). RNAs produced from both clones (LR-GFP-226V and LR-GFP-226A) have comparable specific infectivity values, produced similar viral titers following transfection into BHK-21 cells (Table 3.2) and have similar growth kinetics in mosquito (C6/36) and mammalian (BHK-21) cells lines (Figure 3.3 A; B).

Template used for *in vitro* transcription.	E1-226 [a]	Specific infectivity (pfu/µg of RNA)	Virus titer[b] 24h	48h
pLR-ApaI-226V	V	9.1 x 10^5	6.95	7.52
pLR-226A	A	8.3 x 10^5	6.52	7.52
pCHIK-LR ic	V	8.0 x10^5	6.95	7.95
pLR-GFP-226V	V	4.8 x 10^5	6,52	6.52
pLR-GFP-226A	A	3.3 x 10^5	6.95	6.95
p37997-226A	A	5.0 x 10^5	7.52	7.52
p37997-226V	V	9.0 x 10^5	7.95	7.95
p37997-GFP-226A	A	5.0 x 10^5	7.52	7.52
p37997-GFP-226V	V	5.0 x 10^5	6.95	7.52

Table 3.2 Specific infectivity and virus titers after electroporation.

a – amino acids at position E1-226.
b – Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml.

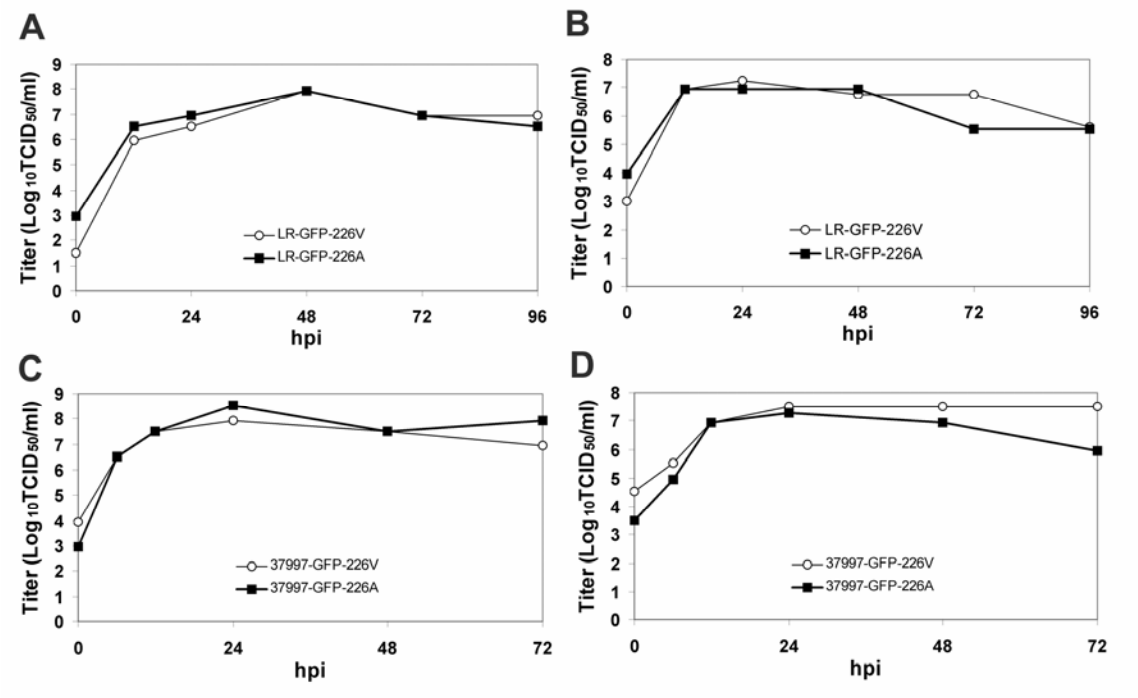


Figure 3.3 Growth of the eGFP expressing viruses in BHK-21 (A, C) and C6/36 (B, D) cells.
Cells were infected at an MOI of 0.1.

The relative infectivity of LR-GFP-226V and LR-GFP-226A viruses was analyzed in female *Ae. albopictus* mosquitoes orally exposed to serial ten-fold dilutions of CHIKV (LR-GFP-226 V or A) (Figure 3.4). To determine whether infection rates correlate with blood meal titer, midguts dissected from mosquitoes at 7 days post-infection (dpi) were analyzed for foci of eGFP-expressing cells by fluorescence microscopy (Figure. 3.5 A and Table 3.3).

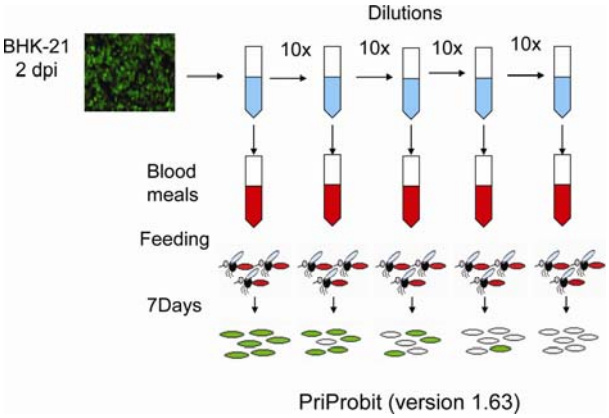


Figure 3.4 Experimental design of OID_{50} experiments.

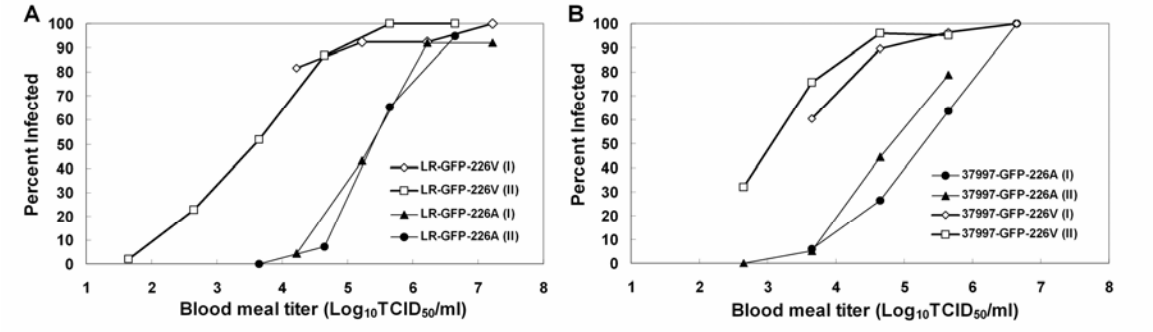


Figure 3.5 Effect of E1-A226V mutation on CHIKV-GFP viruses *Ae. albopictus* midgut infectivity.
Serial ten-fold dilutions of viruses in the backbone of LR2006 OPY1 (LR-GFP-226V and LR-GFP-226A) (A) and 37997 (37997-GFP-226A and 37997-GFP-226V) (B) strains of CHIKV were made to produce blood meals. The experiments were performed twice for each virus (I and II).

In two independent experiments, LR-GFP-226V virus was approximately 100-fold more infectious to *Ae. albopictus* than LR-GFP-226A virus ($p<0.01$). To test if the infectivity phenotype was directly linked to the mutation, the complementary reverse mutation, E1-A226V, was introduced into an infectious clone of a West-African CHIKV strain, 37997-GFP (37997-GFP-226A) (Figure 3.2). The LR2006 OPY and 37997 strains of CHIKV are distantly related, with only 85% nucleotide sequence identity. The parental 37997-GFP-226A and the 37997-GFP-226V viruses were indistinguishable in cell culture experiments (Table 3.2; Figure 3.3 C, D); however, *in vivo* experiments in *Ae. albopictus*

Backbone	E1 226[a]	Exp[b]	N[c]	OID_{50}[d]	C_{95}[d]	p-value
LR-GFP	V	1	98	2.89	0.22-3.75	p<0.01
	A		101	5.42	5.11-5.72	
	V	2	171	3.52	3.24-3.80	p<0.01
	A		93	5.48	5.24-5.71	
37997-GFP	A	1	131	5.17	4.93-5.42	p<0.01
	V		138	3.32	3.34-3.73	
	A	2	129	4.90	4.65-5.16	p<0.01
	V		136	3.06	2.72-3.34	

Table 3.3 Effect of E1-A226V mutation on CHIKV infectivity for *Ae. albopictus* mosquitoes.

a– amino acid at position E1-226
b – experiment number
c – number of mosquitoes used to estimate OID_{50} value
d – Oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml. OID_{50} values and confidence intervals were calculated using PriProbit (Version 1.63).

mosquitoes revealed that the introduction of the E1-A226V mutation led to a significant decrease in the OID_{50} for the 37997-GFP-226V virus as compared to 37997-GFP-226A ($p<0.01$) to an extent similar to that observed for LR-GFP-226V virus (Figure. 3.5 B; Table 3.3). These data conclusively demonstrate that the single point mutation E1-A226V is therefore sufficient to significantly increase the infectivity of CHIKV for the midgut cells of *Ae. albopictus* mosquitoes, and might therefore be directly responsible for adaptation of the virus to this vector species.

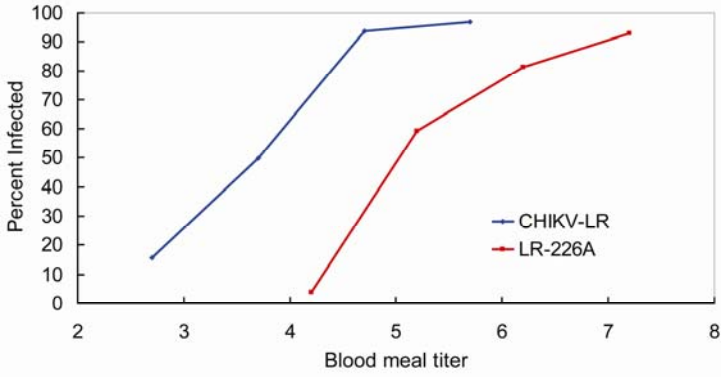


Figure 3.6 Effect of E1-A226V mutation on infectivity of non-eGFP expressing CHIKV for *Ae. albopictus* mosquitoes.

Although eGFP-expressing CHIKV, have similar infection properties in mosquitoes as wild-type viruses (Tsetsarkin et al.,

2006; Vanlandingham et al., 2005b), to address potential concerns that eGFP expression might influence results of infectivity experiments, the OID_{50} values were additionally determined for non-eGFP viruses which have valine or alanine at E1-226 (Figure 3.6 Table 3.3). The OID_{50} value of CHIKV-LR in *Ae. albopictus* mosquitoes was found to be significantly lower as compared to the OID_{50} value of LR-226A ($p<0.01$). More importantly the oral infectivity of eGFP expressing viruses with alanine or valine at E1-226 was almost identical (statistically not different $p>0.1$) to the non-eGFP viruses which have appropriate amino acid at E1-226. This indicates that infectivity values determined based on the presence of eGFP expression in the midgut cells in *Ae. albopictus* could serve as an indicative of general infectivity of the CHIKV for this mosquito species.

Virus	E1 226[a]	N m	OID_{50}	C_{95}	p-value
CHIKV-LR	V	126	3.62	3.32-3.88	p<0.01
LR-226A	A	103	5.29	4.97-5.57	

Table 3.4 Effect of E1-A226V mutation on infectivity of non-eGFP expressing CHIKV for *Ae. albopictus* mosquitoes.

To further evaluate effect of the epidemic E1-A226V mutation on CHIKV fitness in *Ae. albopictus*, viral competition experiments were performed. LR-ApaI-226V was derived from the previously described CHIK-LR i.c., by the introduction of a silent marker mutation, A6454C, in order to add an *Apa*I restriction site into the coding sequence. To our knowledge this genome region of any alphavirus does not contain any *cis*-acting elements affecting the replication rate of positive, negative, and subgenomic RNAs, or affecting

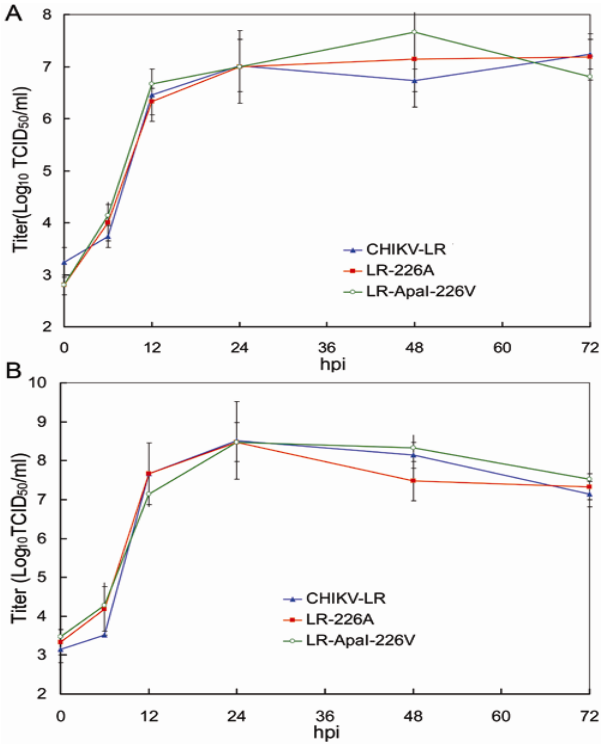


Figure 3.7 Growth of the CHIKV-LR , LR-ApaI-226V and LR-226A viruses in BHK-21(A) and C6/36 (B) cells.
Cells were infected at an MOI of 1.0.

the rate of packaging of genomic RNAs into viral particles. We found that this position is not conserved among different strain of CHIKV. Thus the CHIKV IND-73-MH5 strain (GenBank EF027141) has G at this position, whereas ONNV [strain SG650 GenBank (AF079456)], which is the virus most closely related to CHIKV at this position contains U, circumstantially suggesting that mutations at this position should not affect the fitness of the virus.

Mosquitoes	Day p.i.	CHIK-LR		LR-ApaI-226V	
		Infected / Total (%)	Titer±S.D	Infected / Total (%)	Titer±S.D
Ae. aegypti	7	14/18 (78)	6.13±0.78	7/10 (70)	5.90±0.43
	14	6/10 (60)	5.64±0.36	7/10 (70)	4.90±0.64
Ae. albopictus	7	15/16 (94)	5.08±0.78	10/10 (100)	4.91±0.31
	14	15/16 (94)	4.35±0.58	10/10 (100)	4.36±0.37

Table 3.5 Infection rates and average titers of CHIKV-LR and LR-ApaI-226V in orally infected *Ae. aegypti* and *Ae. albopictus.*

Ae. aegypti mosquitoes were orally presented with 7.24±0.4 $Log_{10}TCID_{50}$/ml of CHIKV-LR (summary of two experiments) and 6.52 $Log_{10}TCID_{50}$/ml of LR-ApaI-226V.
Ae. albopictus mosquitoes were orally presented with 7.24±0.4 $Log_{10}TCID_{50}$/ml of CHIKV-LR (summary of two experiments) and 7.52 $Log_{10}TCID_{50}$/ml LR-ApaI-226V.
Titers are reported as $Log_{10}TCID_{50}$/ml ± standard deviation

It was additionally shown that the A6454C mutation does not affect: the specific infectivity value, the viral titer after RNA transfection into BHK-21 cells value (Table 3.2), the viral growth kinetics in BHK-21 and C6/36 cells (Figure 3.7), infectivity for and viral titers in *Ae. aegypti* and *Ae. albopictus* mosquitoes (Table 3.6), or viral fitness for growth in BHK-21 and C6/36 cells as determined by competition assay (Figure 3.8). These data altogether indicate that the introduced mutation is indeed silent and does not affect the fitness of LR-ApaI-226V.

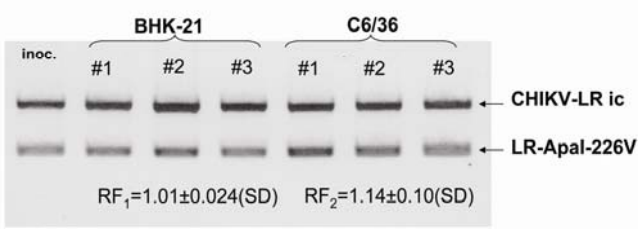


Figure3.8 Competition between CHIK-LR and LR-ApaI-226V for growth in BHK-21 and C6/36 cells.

Cells were infected with a 1:1 mixture of both viruses at an MOI of 0.001. 2 dpi cell culture supernatant was collected and samples processed as described. The experiment was repeated three times for each of the cell types.
Inoc. - initial ratio of CHIK-LR ic and LR-ApaI-226V in the inoculum used for infection of cells.
Relative fitness (RF) of CHIK-LR ic and LR-ApaI-226V was calculated as an average ratio between CHIK-LR and LR-ApaI-226V bands in the supernatant obtained from BHK-21 cells (RF_1) and C6/36 cells (RF_2), divided by the control ratio between CHIK-LR and LR-ApaI-226V in the inoculum.

For viral competition experiments LR-ApaI-226V virus (10^7 pfu) was mixed with an equal amount of LR-226A virus. Mixtures of LR-ApaI-226V and LR-226A viruses were orally presented to *Ae. albopictus* mosquitoes in a blood meal, and midguts were examined at 7 dpi. The relative amount of RNA derived from LR-ApaI-226V in the midgut cells increased 5.7±0.6 times as compared to the initial relative amount of LR-ApaI-226V RNA in the blood meal sample (Figure 3.9). These data further support our observation that the E1-A226V mutation enhances infectivity of CHIKV for *Ae. albopictus* mosquitoes.

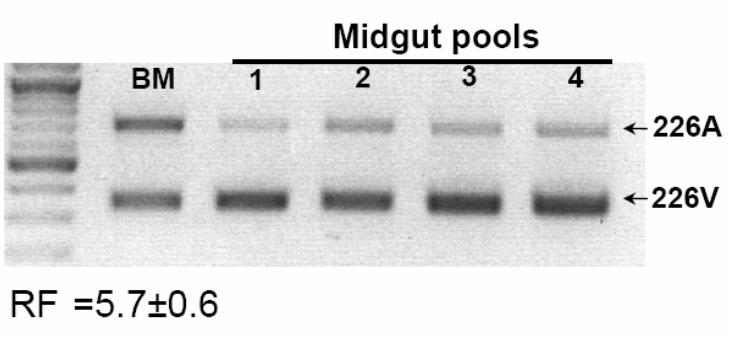


Figure 3.9 Competition between LR-ApaI-226V and LR-226A viruses for colonization of midgut cells of *Ae. albopictus* .

BM- ratio of the RNAs derived from LR-ApaI-226V and LR-226A in blood meal.
1-4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the 8-10 midguts per replica.
RF - relative fitness calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio between 226V and 226A in the blood meal

To determine if the enhanced midgut infectivity associated with the E1-A226V mutation may result in more efficient viral dissemination into secondary tissues, the kinetics of viral dissemination by LR-GFP-226V and LR-GFP-226A into salivary glands, and competition between LR-ApaI-226V and LR-226A for dissemination into mosquito heads were analyzed (Figure 3.10). LR-GFP-226V virus disseminated more rapidly into *Ae. albopictus* salivary glands at all time points, with a significant difference at 7 dpi ($p=0.044$, Fisher's exact test). Similarly, in three of four replicates of competition experiments, RNA from LR-ApaI-226V virus was dramatically more abundant in the heads of *Ae. albopictus* mosquitoes as compared to RNA from LR-226A (Figure 3.10 B lines 1,3,4), although in one replicate LR-ApaI-226V RNA was only slightly more abundant as compared to the initial viral RNA ratio (Figure 3.10 B line 2). The variability of the results may be due to random pooling of mosquito heads and/or reflect a bottleneck in infection or dissemination. Thus, replicate two may have included more heads negative

for LR-ApaI-226V relative to heads positive for LR-226A RNA. Another possibility is that at some point during viral dissemination from the midguts into mosquito heads, LR-226A may replicate more rapidly than LR-ApaI-226V.

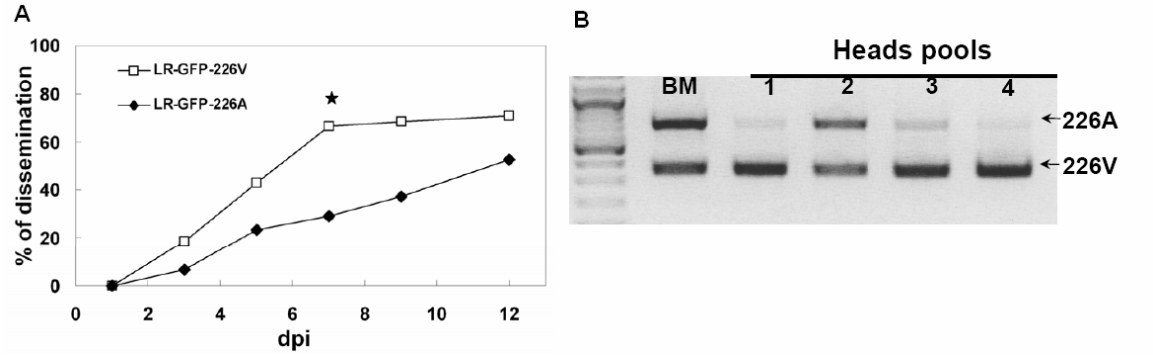


Figure 3.10 Effect of E1-A226V mutation on CHIKV dissemination into salivary glands and heads of *Ae. albopictus* mosquitoes.

A - *Ae. albopictus* mosquitoes were orally infected with LR-GFP-226V and LR-GFP-226A. At indicated time points 16-21 mosquitoes were dissected and salivary glands were analyzed for eGFP expression. Percent of dissemination was estimated as a ratio of the number of mosquitoes with eGFP positive salivary glands to the number of mosquitoes with eGFP positive midguts. Blood meal titers were 5.95 and 6.52 $Log_{10}TCID_{50}$/ml for LR-GFP-226V and LR-GFP-226A respectively.

B - Competition between LR-ApaI-226V and LR-226A for dissemination into heads of *Ae. albopictus* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus*. Viral RNAs were extracted from four pools of 5 heads collected at 12 dpi.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1-4 - ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the 5 pooled heads per replica.

To further investigate this relationship, *Ae. albopictus* mosquitoes were orally presented with either LR-ApaI-226V or LR-226A and whole mosquito body viral titers were compared at different time points pi. Surprisingly, no significant differences between viral titers were found, with the exception of 1 dpi; where the average LR-ApaI-226V titer was 0.5 $Log_{10}/TCID_{50}$/mosquito higher than that of the LR-226A (Figure 3.11A). This may be due to more efficient colonization of *Ae. albopictus* midguts by LR-ApaI-226V. The absence of significant differences in viral titers at later time points may be due to variation in viral titers among individual mosquitoes. Competition between LR-ApaI-226V and LR-226A was analyzed at different time points in order to investigate the relationship between replication of LR-ApaI-226V and LR-226A viruses in *Ae. albopictus* mosquitoes (Figure. 3.11B). As expected, the viral RNA from LR-ApaI-226V was predominant at the early time points of 1 and 3 dpi. Interestingly, between 3 and 5 dpi the viral RNA ratio shifted toward LR-226A virus indicating that at these time points, LR-226A replicates more efficiently in some mosquito tissues (Figure 3.11 B). This short period of time may have a slight effect on the overall outcome of competition for

dissemination into salivary glands because there is a reverse shift in the RNA ratio between days 5 and 7 toward the LR-ApaI-226V virus, which continues through 14 dpi. These data suggest that the E1-A226V mutation not only increases midgut infectivity but also is associated with more efficient viral dissemination from the midgut into secondary organs, suggesting that the E1-A226V mutation would increase transmissibility of CHIKV by *Ae. albopictus* mosquitoes.

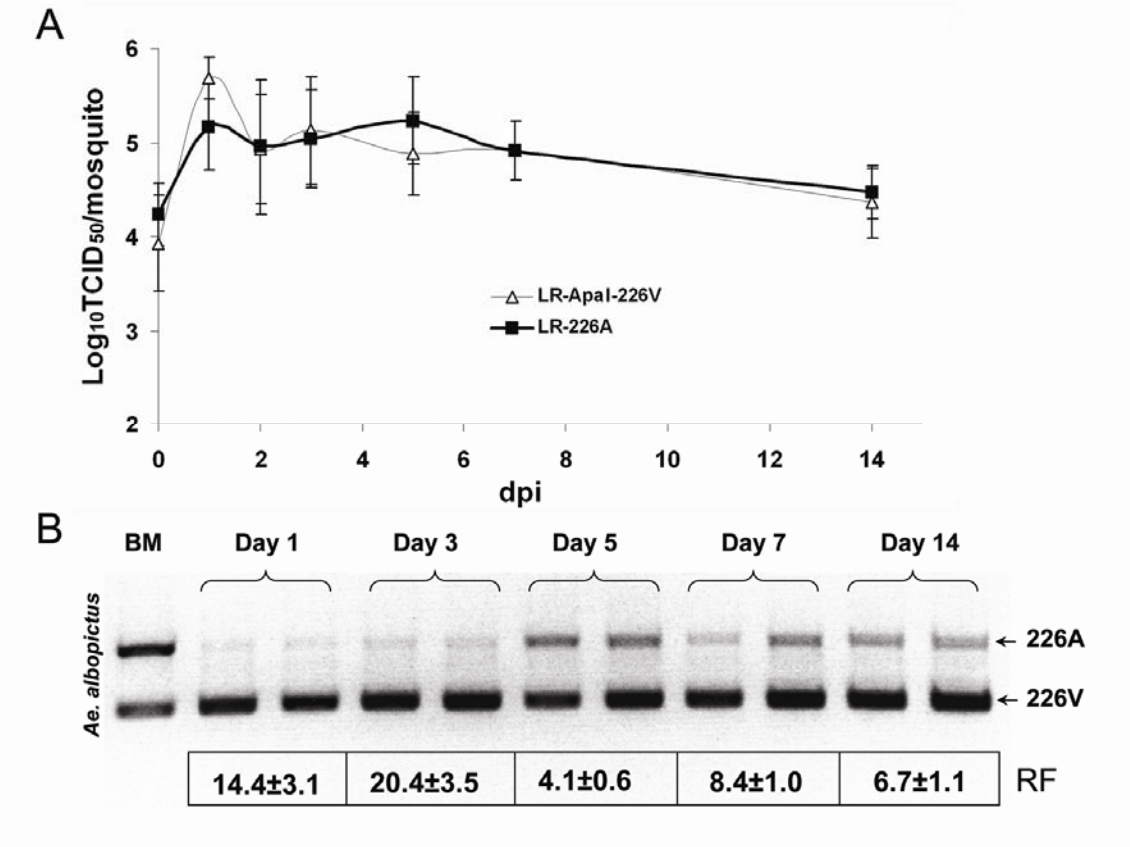


Figure 3.11 Effect of E1-A226V mutation on CHIKV kinetics of viral growth in bodies of *Ae. albopictus* mosquitoes.

A - Virus production in orally infected *Ae. albopictus* mosquitoes. Differences in viral titers were analyzed by Pairwise t-tests. Star indicates $p < 0.05$.

B – Competition between LR-ApaI-226V and LR-226A in bodies of *Ae. albopictus* mosquitoes. For each time point, viral RNA was extracted from a two pools of 10 mosquitoes.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples.

RF - relative fitness of LR-Apa-226V to LR-226A was calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio between 226V and 226A in the blood meal. Results expressed as average of two replicates ± standard deviation.

A competition assay between LR-ApaI-226V and LR-226A viruses was used to examine transmission of CHIKV by *Ae. albopictus* to suckling mice to assess the potential for the E1-A226V mutation to influence virus transmission. *Ae. albopictus* mosquitoes were orally presented with a mixture of LR-ApaI-226V and LR-226A viruses and at 14 dpi were allowed to feed on suckling mice. Mice were sacrificed and bled on day 3 following exposure and the presence of CHIKV RNA in the blood was analyzed by RT-PCR followed by restriction with *Apa*I (Figure 3.12). Blood obtained from 100% of experimental mice contained detectible amounts of viral RNA, indicating that virus was transmitted by *Ae. albopictus* mosquitoes to suckling mice. More importantly, in all six mice analyzed, RNA derived from LR-ApaI-226V was the predominant viral RNA species, indicating that under the conditions of competition for transmission, the E1-A226V mutation directly increases CHIKV transmission by *Ae. albopictus* mosquitoes. Interestingly, in the control experiment in which mice were subcutaneously inoculated with ≈ 50 pfu of 1:1 mixture of LR-ApaI-226V and LR-226A viruses, RNAs from both viruses were readily detected and no difference was observed in the viral RNA ratio at 3 dpi (Figure 3.12A). This demonstrates that in mice, E1-A226V is not associated with changes in viral fitness.

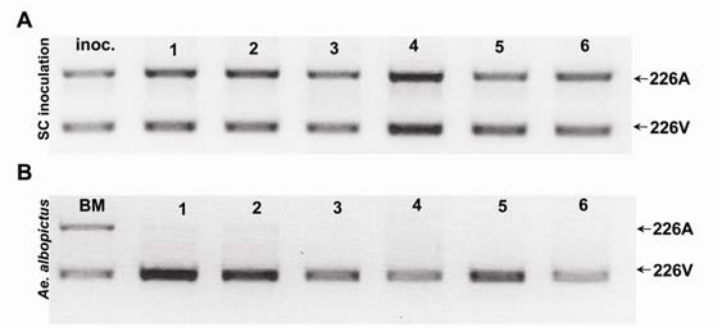


Figure 3.12 Effect of E1-A226V mutation on CHIKV transmission by *Ae. albopictus.*

A – Six 2-3 days old suckling mice were infected s.c. with a 20 µl mixture of ≈ 25 pfu LR-Apa-226V and ≈ 25 pfu of LR-226A viruses.
B - *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. At 14 dpi, the mosquitoes were presented with a 2-3 day old suckling mouse.
Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (≈50 µl) was collected and immediately mixed with 450 µl of TRIzol reagent for RNA extraction.
BM and inoculum - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples and inoculum for s.c infection. 1-6 - ratio of LR-ApaI-226V and LR-226A RNA in six individual mice.

Effect of E1-A226V mutation on fitness of CHIKV in *Ae. aegypti* mosquitoes.

Since the E1-A226V mutation confers a fitness advantage in *Ae. albopictus*, it is unknown why this mutation had not been observed previously, for example in South-east Asia, where CHIKV has circulated in native albopictus regions for decades. It is possible that this change might have a deleterious effect on viral fitness in the vertebrate host, although our data of direct competition between LR-ApaI-226V and LR-226A viruses in suckling mice (Figure 3.12), and analysis of CHIKV cellular tropism of four clinical isolates from Reunion (which have either A or V at position E1-226) (Sourisseau et al., 2007), suggest that this is unlikely. An alternative hypothesis is that the E1-A226V mutation might compromise the fitness of CHIKV or have neutral fitness effects in the mosquito species that served as a vector for CHIKV prior to its emergence on Reunion island. Since *Ae. aegypti* has generally been regarded as the main vector for CHIKV prior to the emergence on Reunion island, we analyzed the effect of the E1-A226V mutation on fitness of CHIKV in *Ae. aegypti*.

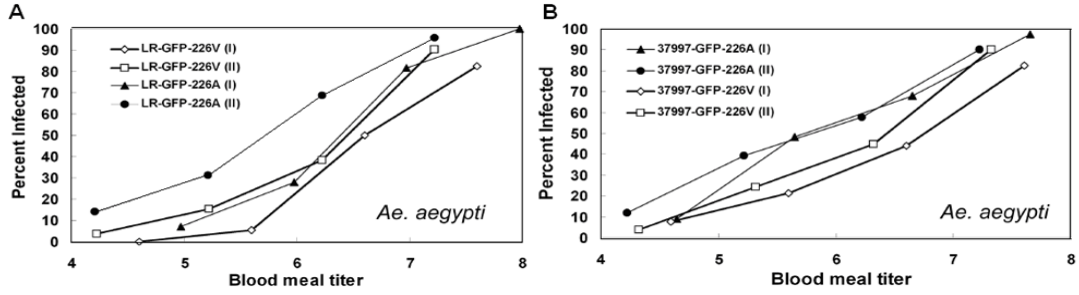


Figure 3.13 Effect of E1-A226V mutation on CHIKV-GFP viruses *Ae. aegypti* midgut infectivity.

Serial ten-fold dilutions of viruses in the backbone of LR2006 OPY1(LR-GFP-226V and LR-GFP-226A) (A) and 37997 (37997-GFP-226A and 37997-GFP-226V) (B) strains of CHIKV were made to produce blood meals. The experiments were performed twice for each virus (I and II). Blood meal titers expressed as $Log_{10}TCID_{50}$/ml

In contrast to the results obtained in *Ae. albopictus* mosquitoes, OID_{50} values of viruses containing the E1-226V in the backbone of the Reunion and 37997 strains of CHIKV ecpressing eGFP were approximately 0.5 $Log_{10}OID_{50}$/ml higher than the OID_{50} values of E1-226A viruses in all experiments using *Ae. aegypti*. These differences were statistically significant for one out of two replicates for each virus pair (Figure 3.13; Table 3.6). Infectivity of the non-eGFP expressing LR-226A virus was also slightly higher in *Ae. aegypti* as compared to CHIKV-LR (Table 3.6). A competition assay examining LR-ApaI-226V and LR-226A virus infection in *Ae. aegypti* midguts, demonstrated that LR-226A virus out-competed LR-ApaI-226V virus at 7 dpi in all four

Backbone	E1 226[a]	Exp[b]	N m[c]	OID_{50}[d]	C_{95}[d]	p value
LR-GFP	V	1	65	6.77	6.41-7.17	p>0.1
	A		103	6.28	6.01-6.55	
	V	2	171	6.26	5.95-6.63	p<0.05*
	A		93	5.62	5,11-6.01	
37997-GFP	A	1	161	5.86	5.62-6.11	p<0.01**
	V		162	6.59	6.31-6.93	
	A	2	136	5.83	5.46-6.13	p>0.1
	V		127	6.34	6.05-6.63	
CHIKV-LR	V	1	103	5.96	5.67-6.25	p>0.1
LR-226A	A		87	5.73	5.40-6.07	

Table 3.6 Effect of E1-A226V mutation on CHIKV infectivity for *Ae. albopictus* mosquitoes.

a– amino acid at position E1-226
b – experiment number
c – number of mosquitoes used to estimate OID_{50} value
d – Oral infectious dose 50 and 95% confidence intervals(C_{95}) are expressed as $Log_{10}TCID_{50}$/ml. OID_{50} values and confidence intervals were calculated using PriProbit (Version 1.63).

replicates using 8-10 midguts per replicate and that the amount of LR-226A RNA increased on average 3.1 times as compared to the initial blood meal RNA ratio (Figure 3.14). These data suggest that the E1-A226V mutation has a slight negative effect on CHIKV infectivity of *Ae. aegypti* midguts.

The effect of the E1-A226V mutation on the ability of CHIKV to disseminate into *Ae. aegypti* secondary organs was also analyzed (Figure 3.15). LR-GFP-226V and LR-GFP-226A viruses both have similar kinetics of dissemination into salivary glands following oral infection using titers 1-2 $Log_{10}TCID_{50}$/ml higher

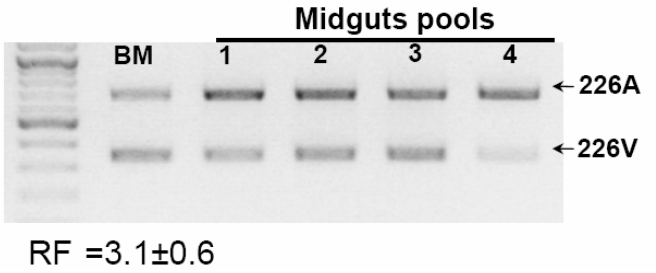


Figure 3.14 Competition between LR-ApaI-226V and LR-226A viruses for colonization of midgut cells of *Ae. aegypti*.

BM- ratio of the RNAs derived from LR-ApaI-226V and LR-226A in blood meal.
1-4 - ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the 8-10 midguts per replica.
RF - relative fitness calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio between 226V and 226A in the blood meal

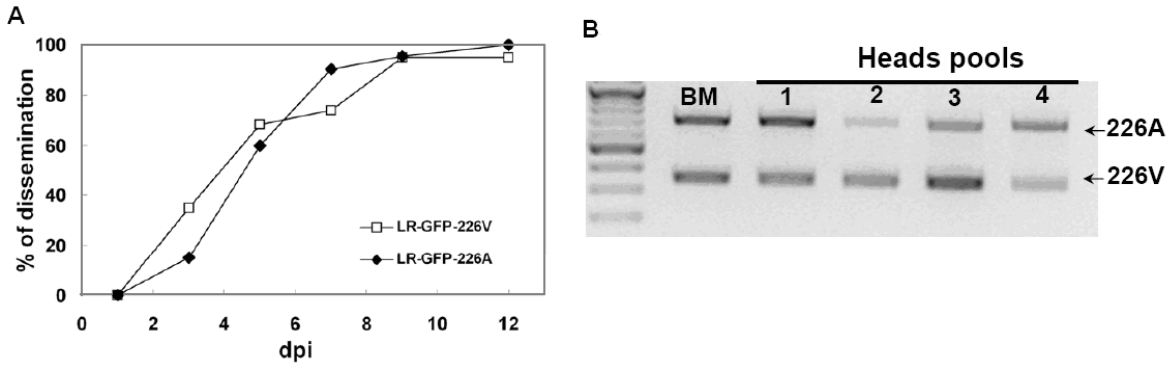


Figure 3.15 Effect of E1-A226V mutation on CHIKV dissemination into salivary glands and heads of *Ae. aegypti* mosquitoes.

A - *Ae. aegypti* mosquitoes were orally infected with LR-GFP-226V and LR-GFP-226A. At indicated time points 16-21 mosquitoes were dissected and salivary glands were analyzed for eGFP expression. Percent of dissemination was estimated as a ratio of the number of mosquitoes with eGFP positive salivary glands to the number of mosquitoes with eGFP positive midguts. Blood meal titers were 6.95 $Log_{10}TCID_{50}$/ml for both LR-GFP-226V and LR-GFP-226A viruses.
B - Competition between LR-ApaI-226V and LR-226A for dissemination into heads of *Ae. albopictus* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus*. Viral RNAs were extracted from four pools of 5 heads collected at 12 dpi.
BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1-4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the 5 pooled heads per replica.

than their OID_{50} value in *Ae. aegypti* . In a competition assay, both LR-ApaI-226V and LR-226A viruses disseminated similarly into the heads of *Ae. aegypti*. In two of four replicates, there was a slight increase in the relative amount of LR-226A RNA (Figure 3.15B lines 1, 4); whereas the other two replicates showed a decrease in LR-226A RNA (Figure 3.15 B, lines 2, 3), relative to the initial ratio of the RNA of LR-ApaI-226V and LR-226A viruses in the blood meal. These data indicate that, in contrast to *Ae .albopictus,* the E1-A226V mutation does not confer more efficient CHIKV dissemination into salivary glands and heads of *Ae. aegypti* mosquitoes.

Ae. aegypti mosquitoes were orally presented with either LR-ApaI-226V or LR-226A and whole mosquito body viral titers were compared at different time points pi. No significant differences between viral titers were found at any time points (Figure 3.16 A). Competition between LR-ApaI-226V and LR-226A for growth in *Ae. aegypti* mosquitoes bodies was also analyzed at different time points (Figure. 3.16 B). Results of this experiment indicate that relative amount of LR-ApaI-226V and LR-226A RNA remains unchanged at early time points (day1, day3) followed by increase in the relative amount of LR-226A RNA at day 5 and day 7. However at day14 both of the RNA species

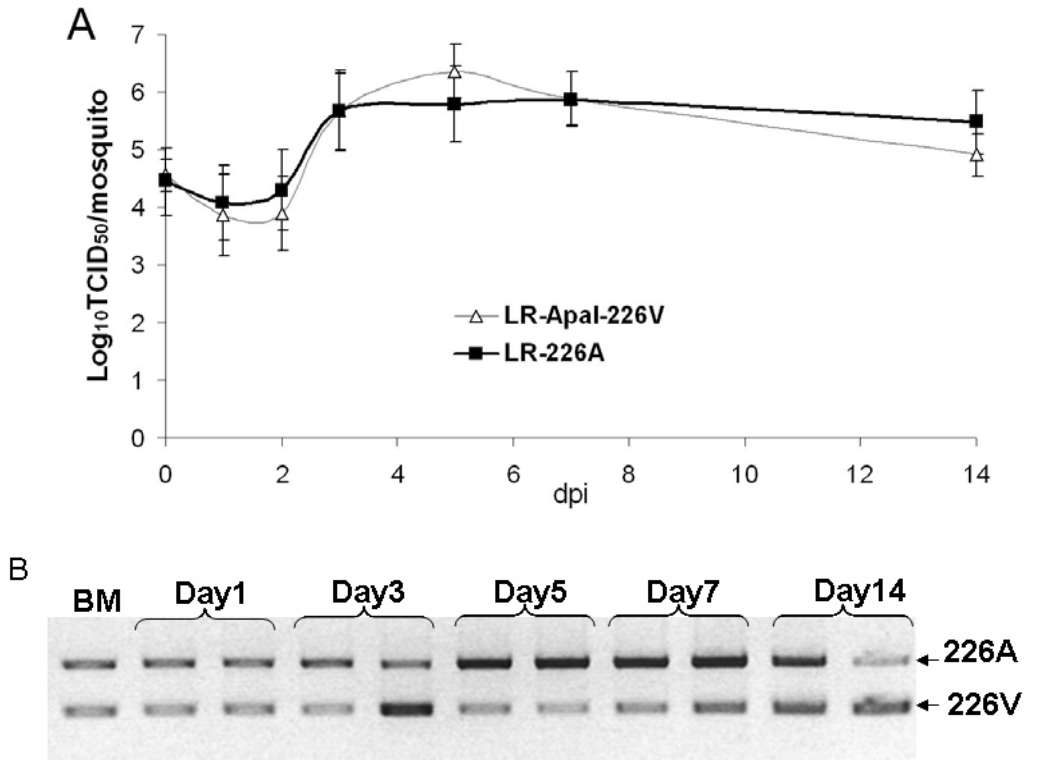


Figure 3.16 Effect of E1-A226V mutation on CHIKV kinetics of viral growth in bodies of *Ae. aegypti* mosquitoes.

A - Virus production in orally infected *Ae. aegypti* mosquitoes.
B – Competition between LR-ApaI-226V and LR-226A in bodies of *Ae. albopictus* mosquitoes. For each time point, viral RNA was extracted from a two pools of 10 mosquitoes.
BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples.

were expressed at relatively equal amount (Figure. 3.16 B). These results are substantially different as compared to those obtained in *Ae. albopictus* mosquitoes. A competition of LR-ApaI-226V and LR-226A viruses for transmission by *Ae. aegypti* to suckling mice was also analyzed (Figure 3.17). In contrast to transmission by *Ae. albopictus* mosquitoes, five out of six mice fed upon by *Ae. aegypti* contained comparable amounts of RNA derived from both viruses and only one out of six mice contained RNA derived exclusively from LR-ApaI-226V.

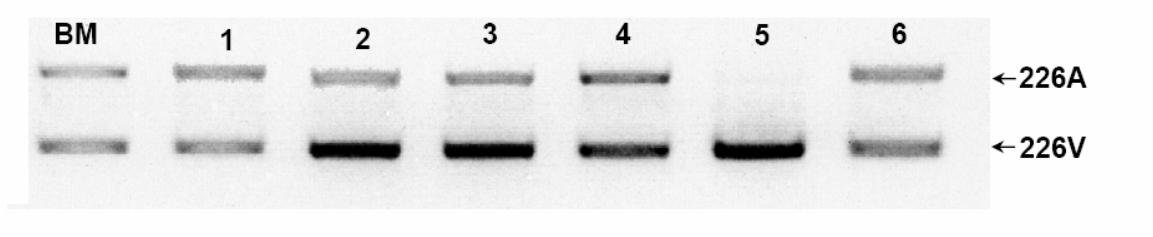


Figure 3.17 Effect of E1-A226V mutation on CHIKV transmission by *Ae. aegypti*.
Ae. aegypti mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. At 13 dpi, 10-15 mosquitoes were placed in separate paper cartons and starved for 24 h. The next day, the mosquitoes in each carton were presented with a 2-3 day old suckling mouse. Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (≈50 µl) was collected and immediately mixed with 450 µl of TRIzol reagent for RNA extraction.
BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1-6 ratio of LR-ApaI-226V and LR-226A RNA in six individual mice.

DISCUSSION

The CHIKV outbreak in Reunion Island is unique because it is the first well - documented report of an alphavirus outbreak for which *Ae. albopictus* was the main vector. Our data clearly indicate that an E1-A226V mutation in CHIKV results in increased fitness of CHIKV in *Ae. albopictus* mosquitoes with respect to midgut infectivity, dissemination to the salivary glands, and transmission to a vertebrate species. Also, results of mosquito infectivity experiments employing the 37997 strain of CHIKV into which E1-A226V mutation was introduced (Table 3.3, Figure 3.5) indicate that this mutation is sufficient to dramatically increase the ability of different strains of CHIKV to infect *Ae. albopictus* mosquitoes and that this substitution requires no additional adaptive mutations to gain intermolecular compatibility. These complimentary experimental data demonstrate that a single mutation is sufficient to modify viral infectivity for a specific vector species and as a consequence, can fuel an epidemic in a region that lacks the typical vector - *Ae. aegypti*. These observations provide the basis for an explanation of the observed rapid shift among CHIKV genotypes to viruses containing the E1-A226V mutation during the 2005-2006 Reunion outbreak (Schuffenecker et al., 2006).

Similar results were obtained by Vazeille et al. (2007) studying interactions of the Indian Ocean 05.115 and 06.21strains of CHIKV with *Ae. albopictus* mosquitoes collected from Reunion and Mayotte Islands in March 2006 (Vazeille et al., 2007). These two CHIKV strains were isolated in Reunion Island during the 2005-2006 outbreak and

their consensus sequences differ only by presence of either alanine or valine at E1-226. The 06.21 strain which has valine at E1-226 disseminated significantly more efficiently into *Ae. albopictus* salivary glands as compared to the 05.115 strain with alanine at this position. Interestingly, the 05.115 strain required ~ 2 Log_{10}pfu/ml higher blood meal titer to reach dissemination efficiency comparable to the 06.21 strain. This is strikingly similar to the effects of virus dose in blood meals on CHIKV infectivity for *Ae. albopictus* observed in our study. Furthermore, infection of *Ae. albopictus* with the 06.21 strain results in more efficient CHIKV RNA replication in mosquito bodies and midgut as compared with the 05.115 strain. This is in agreement with our result of competition between LR-Apa-226V and LR-226A for colonization of mosquito midguts and bodies (Figure 3.9 and 3.11). This indicates that the effects of the E1-A226V mutation on CHIKV fitness in *Ae. albopictus* observed in our study would be probably common for different geographical strains of this mosquito species.

The conclusion that the E1-A226V mutation provides a selective advantage for CHIKV strains transmitted by *Ae. albopictus* mosquitoes was additionally supported in the more recent comparative studies of the CHIKV genome sequences obtained during 2005-2007 CHIKV outbreak in different geographic locations (Arankalle et al., 2007; Cherian et al., 2009; Dash et al., 2007; de Lamballerie et al., 2008; Kumar et al., 2008; Peyrefitte et al., 2008; Peyrefitte et al., 2007). It was shown that this mutation appeared convergently at least on three separate occasions in the CHIKV strains transmitted exclusively by *Ae. albopictus* mosquitoes (For more details see Chapter 1.4). These studies, together with our data and the results presented by Vazeille et al. (2007) provide sufficient evidence to incriminate the E1-A226V mutation as the major genetic determinant of adaptation of CHIKV to *Ae. albopictus* mosquitoes.

Interestingly, the results of our investigation and data from previous studies (Tesh et al., 1976; Turell et al., 1992) indicate that prior to acquiring the E1-A226V mutation, CHIKV is capable of producing high enough viremia in humans to efficiently infect *Ae. albopictus* mosquitoes. One explanation of the evolutionary force which allowed CHIKV to be selected so rapidly into a CHIKV strain which was adapted to *Ae. albopictus*, is that the increased infectivity (lower OID_{50}) of CHIKV E1-A226V mutants for *Ae. albopictus* means that the human viremic thresholds required for *Ae. albopictus* infection would likely occur earlier and be sustained for longer. Several recent studies indicate that during

the course of human viremia, which last up to 6 days, CHIKV loads can reach up to $3.3x10^9$ RNA copies/ml of the blood (Carletti et al., 2007; Parola et al., 2006), which corresponds to 6-7 $Log_{10}TCID_{50}$/ml (Carletti et al., 2007). Earlier studies, which utilized a suckling mouse intracerebral lethal dose (SMICLD) titration protocol that is more sensitive than titration on Vero cells, also found that human viremia often exceeded 6 $Log_{10}SMICLD_{50}$/ 0.02 ml (Carey et al., 1969). Based on viremia studies in rhesus monkeys that can develop up to 7.5 Log/ml if assayed by suckling mice brain titration (Paul and Singh, 1968), and a maximum viremia of only 5.5 Log_{10}/ml based on Vero cell titration (Binn et al., 1967), we believe that viremias in humans would correlate to 6-7 $Log_{10}TCID_{50}$/ml. From these data we calculate that the maximum virus load which can be achieved in human blood is 1-2 $Log_{10}TCID_{50}$/ml higher than the OID_{50} for E1-226A viruses but 3-4 $Log_{10}TCID_{50}$/ml higher than the OID_{50} for E1-226V viruses. During the course of viremia there should therefore be a substantial time frame during which CHIKV blood load is high enough for E1-226V viruses to infect *Ae. albopictus,* but below the threshold for infection with E1-226A viruses. This increased opportunity for *Ae. albopictus* infection would perpetuate the selection and transmission of the mutant virus.

During transmission competition assays, only the E1-226V virus was transmitted to suckling mice by *Ae. albopictus*, although in these experiments, titers of E1-226V and E1-226A viruses were of a high enough magnitude to allow both of these viruses to efficiently infect this mosquitoes species (Figure 3.12). This indicates that there are additional mechanisms that could ensure evolutional success of the E1-A226V viruses transmitted by *Ae. albopictus*. It is possible that one of these mechanisms is associated with more efficient dissemination of the E1-226V as compared with E1-226A viruses. This could shorten the extrinsic incubation period (EIP) - the time from mosquito infection to transmission, and could have contributed to the evolutionary success of CHIKV during the Reunion outbreak because vectors infected with the LR-226V virus would transmit it more quickly than those infected with LR-226A virus. Additionally, with relatively short-lived vectors such as mosquitoes (Christophers, 1960), longer EIPs reduce transmission efficiency simply because fewer mosquitoes survive long enough to transmit the virus.

Our studies do not provide data to determine if dissemination efficiency of the E1-226V viruses into the salivary glands is a consequence of more efficient midgut infectivity or if these two phenomena are independent. In this regard, it will be of particular interest to investigate the effect of the E1-A226V mutation on CHIKV transmission by orally or intrathoracically infected *Ae. albopictus* mosquitoes.

Although the CHIKV E1-A226V mutation gives a selective advantage in *Ae. albopictus*, there was not a corresponding advantage in *Ae. aegypti.* The OID_{50} and midgut competition assay data indicate that E1-226V viruses were slightly less infectious for midgut cells of *Ae. aegypti* mosquitoes (Figure 3.12; Table 3.6; Figure 3.13). Additionally, in contrast to *Ae. albopictus*, E1-226V viruses do not have a detectable advantage for dissemination into the salivary glands and heads of *Ae. aegypti*. In transmission competition experiments from *Ae. aegypti* to suckling mice, the E1-226V conferred a slight competitive advantage over E1-226A (Figure 3.17). However, five out of six mice exposed to CHIKV-infected *Ae. aegypti* had equivalent amounts of both E1-226A and E1-226V viral RNAs. These results are markedly different compared to the results obtained in similar experiments using *Ae. albopictus* mosquitoes and further support the hypothesis that this E1-A226V was specifically selected as a result of adaptation of CHIKV to *Ae. albopictus* mosquitoes. To explain the small fitness advantage associated with the E1-A226V mutation, which was observed in transmission experiments, we hypothesize that, similarly to *Ae. albopictus*, E1-226A and E1-226V viruses colonize different *Ae. aegypti* organs at different efficiencies. E1-226A appears to colonize midgut cells of *Ae. aegypti* better than E1-226V viruses; however, following dissemination into salivary glands, the E1-226V virus gains an advantage for transmission to vertebrates.

The E1-A226V mutation was found to have a slightly negative effect on infectivity, a negligible effect on dissemination, but a slight positive effect on transmissibility of CHIKV by *Ae. aegypti* in the competition experiment. We suggest that these small (as compared with *Ae. albopictus*) differences associated with the E1-A226V mutation would be insufficient to have a significant effect on the evolution of CHIKV transmitted by *Ae. aegypti* and would not result in accumulation of this mutation in the regions where *Ae. aegypti* serves as a primary vector for CHIKV. This may explain the lack of emergence of the E1-226V genotype in previous outbreaks and the predominance

of E1-226A viruses during the 2006 CHIKV epidemic in India, in which *Ae. aegypti* is considered to be the main vector species (Arankalle et al., 2007). Adaptation of African strains of CHIKV from forest dwelling mosquitoes species to *Ae. aegypti* has never been shown to be associated with any particular mutations; therefore we believe that the same negative impact of E1-A226V would be seen in African mosquito vectors which were responsible for transmission of CHIKV strains ancestral to Reunion isolates.

Our data do not exclude the possibility that the E1-A226V mutation might have a negative effect on the evolution of CHIKV transmitted by *Ae. aegypti*. Since our dissemination and transmission studies were performed using blood meal titers that were 1-2 $Log_{10}TCID_{50}$/ml higher than $Log_{10}OID_{50}$/ml values, we suggest that the negative effect of decreased midgut infectivity of E1-A226V on virus transmissibility would be almost completely missed, simply because, under this condition, almost 100% of mosquitoes could become infected. In general, CHIKV requires significantly higher blood meal titers for infection of *Ae. aegypti* compared to *Ae. albopictus* (Tesh et al., 1976; Turell et al., 1992) (Table 3.5; 3.6), which suggests that the slight decrease in midgut infectivity of E1-226V viruses would have a more profound effect on the evolution of CHIKV transmitted by *Ae. aegypti*, compared to the effect of a small advantage in the ability to compete with E1-226A viruses for transmission to suckling mice. Therefore, if the E1-A226V mutation occurred in CHIKV transmitted by *Ae. aegypti* it would have a weak negative effect on viral fitness and would most likely not be preferentially selected. Additional experiments are required to evaluate this hypothesis.

Available data cannot exclude the possibility that E1-226A viruses may have an unknown beneficial effect on the fitness of CHIKV in vertebrate hosts over E1-226V viruses, and that the minor negative effect of E1-226A observed in transmission experiments with *Ae. aegypti* can be compensated for by more efficient viral replication in the vertebrate host, leading to an overall more efficient adaptation to the transmission cycle. However, comparison of the different effects of A or V residues at position E1-226 on CHIKV infectivity for, and transmission by *Ae. aegypti* and *Ae. albopictus* mosquitoes clearly suggests that polymorphisms at this position may determine the host range of the alphaviruses and may play an important role in adaptation of the viruses to a particular mosquito vector.

To our knowledge this is the first report describing the major genetic determinant of vector specificity for any alphaviruses outside of the E2 glycoprotein gene region. The molecular mechanisms explaining the effect of the E1-226V mutation in vector specificity are unknown. It has been previously shown that various mutations in the same region of the E1 protein of SFV and SINV can modulate the cholesterol dependence of these viruses (Lu et al., 1999; Vashishtha et al., 1998) and that SFV independence from cholesterol coincides with more rapid growth of the virus in *Ae. albopictus* (Ahn et al., 1999). In this regard we believe that it would be important to investigate effects of mutations at E1-226 of CHIKV on the cholesterol dependence for growth and fusogenic properties of this virus. Also it would be of interest to determine possible relationships between mutations, which modulate cholesterol-dependence of alphaviruses other than CHIKV and their infectivity for *Ae. aegypti* and *Ae. albopictus* mosquitoes and perhaps other epidemiologically important mosquito vectors.

CONCLUSIONS

Although previous laboratory studies have demonstrated susceptibility of *Ae. albopictus* to CHIKV infection (Tesh et al., 1976; Turell et al., 1992), our data demonstrate that the E1-A226V mutation promoted infection and accelerated dissemination of CHIKV in *Ae. albopictus* mosquitoes and conferred a selective advantage over infection of *Ae. aegypti.* Although the mutation did not increase the maximum viral titer attainable in the mosquitoes, the synergistic effects of increased infectivity and faster dissemination of the E1-A226V virus in *Ae. albopictus* would accelerate virus transmission to a naïve human population which would have contributed to initiating and sustaining the 2005-2006 CHIKV epidemic on Reunion island. That a single amino acid change can act through multiple phenotypic effects to create an epidemic situation has implications for other arthropod-transmitted viruses and the evolution of human infectious diseases (Wolfe et al., 2007).

CHAPTER 4: THE ROLE OF E1-A226V MUTATION ON CHIKUNGUNYA VIRUS DISSEMINATION IN, AND TRANSMISSION BY, ORALLY AND INTRATHORACICALLY INFECTED *AE.ALBOPICTUS* MOSQUITOES

ABSTRACT

The evolutionary success of the CHIKV strains bearing the E1-A226V mutation observed during epidemics maintained by *Ae. albopictus* led to the suggestion that this mutation might be responsible for CHIKV adaptation to this mosquito vector. Previously we showed that thes E1-A226V mutation is directly responsible for increased CHIKV infectivity for, dissemination in and transmission by orally infected *Ae. albopictus,* supporting epidemiological finding (Chapter 3). However, the precise role played by the E1-A226V mutation at the different steps of the infection process/transmission cycle remained unknown. It was hypothesized that the E1-A226V mutation exerts its effect primarily by increasing the ability of CHIKV to infect and replicate in *Ae. albopictus* midgut epithelial cells as compared to the pre-epidemic virus containing an alanine residue at this position. More efficient infectivity of the midgut cells leads to more efficient dissemination of the virus into salivary glands and more efficient transmission. To test this hypothesis we compared effects of the E1-A226V mutation on CHIKV dissemination in, and transmission by, orally and intrathoracically infected *Ae. albopictus* mosquitoes. Intrathoracically injected CHIKV into which either the E1-226 A or V residues were engineered, demonstrated similar kinetics of replication in *Ae. albopictus* bodies, and were indistinguishable in experiments of competition for replication in the mosquito body, dissemination into salivary glands, and transmission by *Ae. albopictus* to suckling mice. These results are drastically different compared to the results obtained in orally infected mosquitoes where the E1-A226V mutation conferred increases in CHIKV dissemination in and transmission by *Ae. albopictus.* Since in intrathoracically infected *Ae. albopictus* the E1-A226V mutation did not enhance dissemination/transmission rates, we concluded that the effect of this mutation occurs prior to virus escape from the midgut. These data, together with previous findings (Tsetsarkin et al., 2007; Vazeille et

al., 2007), support the hypothesis that the increased *Ae. albopictus* midgut infectivity resulting from the A226V mutation plays a primary role in enhanced viral transmission.

INTRODUCTION

Arbovirus transmission by its vector is a complex process involving infection and replication of the virus in midgut epithelial cells, dissemination from the midgut into the hemocoel, followed by spread of virus into mosquito secondary organs including the salivary glands. Efficient virus replication in the salivary glands and secretion of the virus into saliva are the final obligatory steps of the transmission process (Hardy et al., 1983; Higgs, 2004). In the previous chapter we showed that the E1-A226V mutation in CHIKV is directly responsible for an increase in virus infectivity for, dissemination in and transmission by orally infected *Ae. albopictus*. The kinetics of CHIKV growth in bodies of orally infected *Ae. albopictus* mosquitoes revealed that LR-ApaI-226V develops significantly higher titers in the mosquito bodies only very early after infection as compared to the LR-226V virus. However starting from day 3 the difference in the virus titers becomes statistically insignificant (Figure 3.11). This early replication phase coincides with the time when virus is mostly present in the midgut cells of *Ae. albopictus* prior to dissemination into the other organs (Figure 3.10). Vazeille et al. (2007), studying interactions of the 05.115 and 06.21 strains of CHIKV with *Ae. albopictus* mosquitoes collected from Reunion and Mayotte Islands, showed that *Ae. albopictus* infected with the 06.21strain (that contains the E1-226V residue) supports high level virus replication in mosquito bodies and midguts (Vazeille et al., 2007). However, mosquitoes infected with strain 05.115 (with the E1-226A residue) demonstrated high variation in the amount of viral RNA in the body; in most females the 05.115 strain replicated very inefficiently and in only a few females this virus strain replicated as efficiently as CHIKV strain 06.21. Similar variation in the amount of 05.115 RNA was detected in the mosquito midguts, whereas all midguts infected with the 06.21 strain were found to contain high amounts of viral RNA. Interestingly, the amount of viral RNA in the salivary glands was equal for both viruses. These observations allowed us to hypothesize that the E1-A226V mutation exerts its effect primarily by increasing the ability of CHIKV to infect and replicate in *Ae. albopictus* midgut epithelial cells which in turn leads to more efficient dissemination of the virus into salivary glands and more efficient transmission.

To test this hypothesis we compared the virus replication profile, dissemination and transmission rates for CHIKV with either alanine or valine at E1-226 in orally and intrathoracically infected *Ae. albopictus* mosquitoes. When intrathoracically injected into the mosquito hemocoel, CHIKV does not need to infect midgut cells and can directly infect secondary organs including the salivary glands. This allowed differentiation between the effects of the E1-A226V mutation on infection of the midgut cells from the effects of the mutation on replication and dissemination in the mosquito secondary organs.

MATERIALS AND METHODS

Viruses and plasmids

The construction of non-eGFP expressing viruses LR-ApaI-226V and LR-226A, and eGFP-expressing viruses LR-GFP-226V and LR-GFP-226A whose sequences are derived from the LR2006 OPY1 strain of CHIKV have been previously described (Chapter 2; Chapter 3; Tsetsarkin et al., 2006; Vanlandingham et al., 2005b).

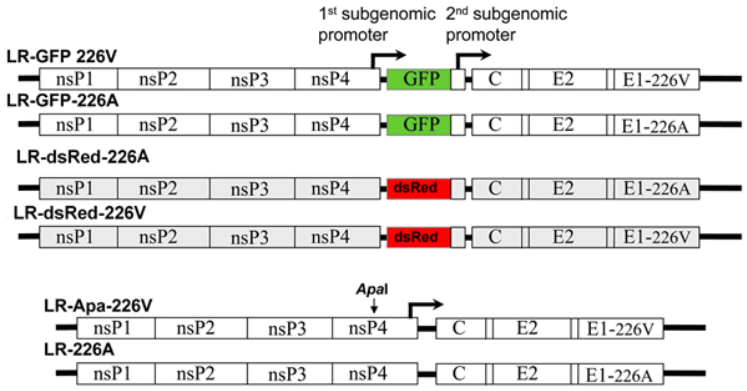


Figure 4.1 Schematic representation of the viruses used in this study.

For studies comparing the dissemination efficiency of CHIKV into salivary glands of *Ae. albopictus,* two additional viruses were constructed which express dsRed fluorescent protein under the control of the additional subgenomic promoter. In the first construct dsRed protein cDNA was introduced into backbone of CHIKV with the E1-226V residue. The dsRed gene was PCR amplified with dsRed-Asc-F (5'-AGATGGCGCGCCATGGCCTCCTCCGAGAACGT) and dsRed-Pme-R (5'-CATGGTTTAAACTACAGGAACAGGTGGTGGC) primers set using pDsRed2 (Clontech, Mountain View, CA) as a template. The amplicon was digested with *Asc*I and

*Pme*I restrictases, and cloned by *Asc*I and *Pme*I sites into pLR-GFP-226V. The resultant plasmid was designated as LR-dsRed-226V.

The second construct contained dsRed cDNA in the backbone of CHIKV with the E1-226A residue. This construct was produced using a similar strategy to that implemented for LR-dsRed-226V. dsRed cDNA was amplified using dsRed-Asc-F and dsRed-Pme-R primres, digested with *Asc*I and *Pme*I restrictases, and cloned using *Asc*I and *Pme*I sites into pLR-GFP-226A. The resultant plasmid was designated as LR-dsRed-226A.

Cells and mosquitoes

Cells and mosquitoes were maintained at conditions similar to those described in Chapter 2 and Chapter 3. To evaluate the ability of viruses to directly infect secondary tissues within *Ae. albopictus,* such as the salivary glands after the midgut is bypassed, various CHIKV clones were inoculated directly into the thoracic hemocoel individually or in combination. *Ae. albopictus* were immobilized by chilling and inoculated with 0.5 µl virus using glass needles fashioned from pulled capillary tubes (Higgs et al., 1997). Injected mosquitoes were returned to the carton and incubated at 28^{o}C, 80% humidity for up to 14 days and provided with sugar *ad libitum*.

For competition experiments between non-eGFP/(dsRed) viruses, 5 pfu of LR-ApaI-226V and 5 pfu of LR-226A viruses in 0.5 µl of L-15 media were injected into *Ae. albopictus* intrathoracically. RNA from 2 pools of 10 mosquitoes per pool was extracted at 7 dpi and was processed as previously described (Chapter 3; Materials and Methods). For transmission experiments, intrathoracically injected mosquitoes at 7 dpi. were allowed to feed on 2-day-old suckling mice. Mice were sacrificed at 3 dpi. RNA was extracted from the blood of individual mice using TRIzol reagent and processed as previously described (Chapter 3; Material and Methods).

Midguts and salivary glands from mosquitoes infected with eGFP and dsRed expressing viruses were dissected on indicated days post-infection and analyzed under a fluorescent microscope. An organ was considered infected if it contained at least one focus of cells expressing fluorescent protein.

RESULTS

To compare virus production in orally infected versus intrathoracically infected mosquitoes, 5 pfu of LR-ApaI-226V or LR-226A in 0.5 µl of L15 media were intrathoracically injected into *Ae. albopictus*. Both viruses demonstrated statistically indistinguishable growth kinetics in intrathoracically infected mosquitoes ($p>0.05$ by Pairwise Student`s t-tests), reaching maximum titers at 2 dpi (Figure 4.2). However, the growth of LR-ApaI-226V and LR-226A in orally infected *Ae. albopictus* was markedly different (Figure 3.11) where LR-ApaI-226V reached significantly higher titers at 1 dpi ($p<0.05$ by Pairwise Student`s t-tests). These data indicate that the E1-A226V mutation has no effect on CHIKV growth in *Ae. albopictus* mosquitoes tissues after the virus escapes from the mosquito midguts.

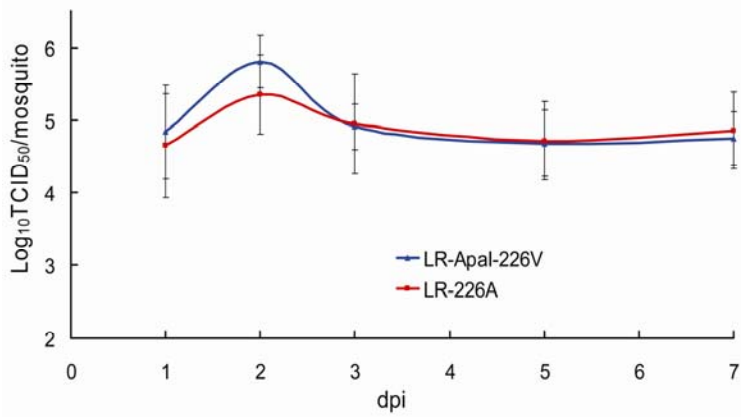


Figure 4.2 Growth of LR-ApaI-226V and LR-226A in bodies of intrathoracically infected *Ae. albopictus* mosquitoes.

5 pfu of LR-ApaI-226V or LR-226A in 0.5 µl of L15 media were intrathoracically injected into *Ae. albopictus*. On indicated days p.i 8 individual mosquitoes were titrated to estimate virus loads in mosquito body. dpi –days post infection.

Template used for *in vitro* transcription.	E1-226 [a]	Specific infectivity (pfu/µg of RNA)	Virus titer[b]	
			24h	48h
pLR-GFP-226V	V	4.8×10^5	6.52	6.52
pLR-dsRED-226V	V	5.0×10^5	6.52	7.52
pLR-GFP-226A	A	3.3×10^5	6.95	6.95
pLR-dsRED-226A	A	1.0×10^5	ND	6.52

Table 4.1 Specific infectivity and virus titers after electroporation.

a – amino acids at position of E1-226.
b – Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml.
ND – titer was not determined.

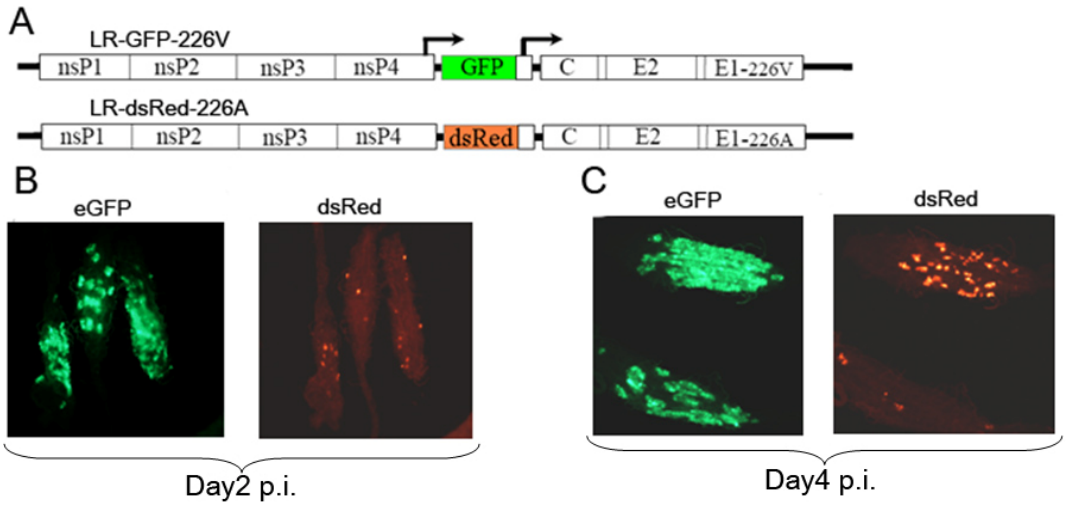


Figure 4.3 Competition of the LR-GFP-226V and LR-dsRed-226A in orally infected *Ae. albopictus.*

Blood meal contained 6.52 $Log_{10}TCID_{50}$/ml of 1:1 mixture of LR-GFP-226V and LR-dsRed-226A.
A - genome organization of LR-GFP-226V and LR-dsRed-226A.
B and C - eGFP and dsRed fluorescence in the midguts of *Ae. abopictus* at 2 and 4 dpi.

To further evaluate the role of the observed increased midgut infectivity associated with the E1-A226V mutation on CHIKV dissemination into the salivary glands (Chapter 3), competition experiments were conducted between the LR-GFP-226V and CHIKV expressing dsRed protein in the backbone of CHIKV with the E1-226A residue (LR-dsRed-226A). Substitution of eGFP with the dsRed protein allows for easy differentiation of the cells infected with viruses containing either E1-226V or E1-226A residues in the mosquito organs. Specific infectivity values and titers after electroporation for LR-dsRed-226A were comparable with LR-GFP-226V and LR-GFP-226A indicating the substitution of the eGFP with dsRed does not significantly affect CHIKV phenotypes in cell cultures (Table 4.1).

To test the hypothesis that efficient infectivity of the midgut cells by CHIKV with the E1-A226V mutation is associated with more efficient viral dissemination into salivary glands, equal amount of LR-GFP-226V and LR-dsRed-226A were mixed and orally or intrathoracically presented to *Ae. albopictus*. Dissemination of the viruses into salivary glands was monitored at 7 and 14 dpi for orally infected mosquitoes and at 2, 4, 7, and 14 dpi. for intrathoracically infected mosquitoes (Table 4.3).

When presented orally, both LR-GFP-226V and LR-dsRed-226A viruses were able to simultaneously infect almost all mosquitoes midguts, which was determined by presence of eGFP and dsRed expressing cells in the same midguts (Figure 4.3). No significant differences were observed between infectious rates for the 2 viruses determined at 2, 4, 7, and 14 dpi (Table 4.2). However, as it was expected the eGFP fluorescence was markedly more abundant within individual midguts as compared to dsRed fluorescence (Figure 4.3), indicating that CHIKV with E1-226V colonizes midgut cells of *Ae. albopictus* much more efficiently as compared to E1-226A virus. The results are consistent with results of oral infectivity of the CHIKV with E1-226V and E1-226A residues presented in Chapter 3 (Figure 3.5; Figure 3.6; Figure 3.9; Table 3.3; Table 3.4).

Days p.i.	Midgut infection rates. Infected / Total (%)		p value [a]
	LR-GFP-226V	LR-dsRed-226A	
2	9/9 (100%)	8/9(88,9%)	0.50
4	6/6 (100%)	6/6 (100%)	0.23
7	8/8 (100%)	7/8(87,5%)	1
14	8/8 (100%)	7/8 (87,5%)	0.499

Table 4.2 Infection rates of the LR-dsRed-226V and LR-GFP-226A in orally infected *Ae. albopictus.*

a - p value was calculated using Fisher's exact test.

The dissemination rates of the LR-GFP-226V and LR-dsRed-226A in orally infected *Ae. albopictus* were determined at 7 and 14 dpi. LR-GFP-226V was found in 6

Days p.i.	Salivary gland infection rates: SG Infected / Total (%)					
	Oral infection			Intrathoracic infection		
	LR-GFP-226V	LR-dsRed-226A	p value [a]	LR-GFP-226V	LR-dsRed-226A	p value [a]
2	ND	ND	ND	9/10 (90)	10/10 (100)	0.5
4	ND	ND	ND	10/10 (100)	10/10 (100)	1
7	6/16 (37.5)	1/16 (6.2)	0.041	10/10 (100)	10/10(100)	1
14	31/50 (62)	10/50 (20)	0.000018	10/10 (100)	10/10 (100)	1

Table 4.3 Comparison of the salivary gland infection rates in the orally and intrathoracically infected *Ae. albopictus*.

Blood meal contained 6.52 $Log_{10}TCID_{50}$/ml of 1:1 mixture of LR-GFP-226V and LR-dsRed-226A.
Inoculum for intrathoracic infection contained 1.95 $Log_{10}TCID_{50}$/mosquito of 1:1 mixture of LR-GFP-226V and LR-dsRed-226A viruses.
a - p value was calculated using Fisher's exact test.
ND – data is not determined

out of 16 (37.5%) salivary glands on day 7 and in 31 out of 50 (62%) on day 14. These values are significantly higher compared to dissemination rates of the LR-dsRed-226A virus (Table 4.3). These data are consistent with results of dissemination rates of the CHIKV with E1-226V and E1-226A residues presented in Chapter 3 (Figure 3.10). However, no statistically significant differences in dissemination rates between LR-GFP-226V and LR-dsRed-226A were detected in intrathoracically infected mosquitoes. Both viruses were simultaneously present in salivary gland cells of *Ae. albopictus* as early as 2 dpi (Table 4.3). No marked differences were observed in abundance and intensity of the eGFP and dsRed expression in salivary gland cells, however, the number of cells expressing both of these proteins gradually increased from day 2 to day 14 p.i. (data not shown). These data altogether indicate that the increase in dissemination rates observed for CHIKV with the E1-226V mutation resulted from more efficient midgut infection, but not from more efficient virus propagation in *Ae. albopictus* secondary organs such as salivary glands.

Introduction of dsRed protein into the CHIKV genome resulted in a virus that had similar phenotypes in cells culture as compared to eGFP-expressing viruses (Table 4.1),

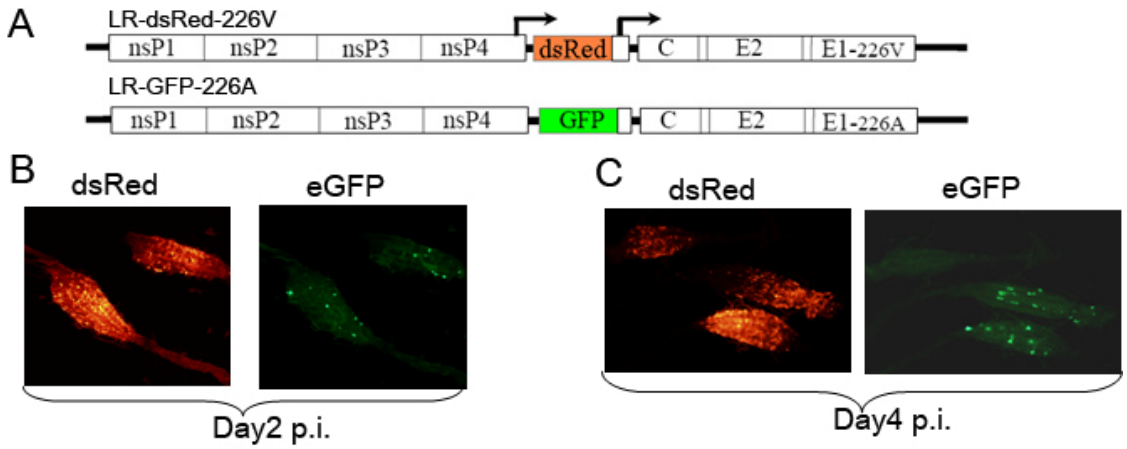


Figure 4.4 Competition of the LR-dsRed-226V and LR-GFP-226A in orally infected *Ae. albopictus.*

Blood meal contained 5.95 $Log_{10}TCID_{50}$/ml of 1:1 mixture of LR-dsRed-226V and LR-GFP-226A.
A - genome organization of viruses LR-dsRed-226V and LR-GFP-226A
B and C - dsRed and eGFP fluorescence in the midguts of *Ae. albopictus* at 2 and 4 dpi.

however, to address potential concerns that dsRed expression might influence the ability of CHIKV to infect and disseminate in *Ae. albopictus*, the reciprocal combination of fluorescent proteins was expressed in the backbone of CHIKV with E1-226V and E1-226A residues (Figure 4.4).

Days p.i.	Midgut infection rates. Infected / Total (%)		p value [a]
	LR-dsRed-226V	LR-GFP-226A	
2	5/5 (100%)	4/5(80%)	0.500
4	8/8 (100%)	6/8 (75%)	1.00
7	5/5 (100%)	5/5(100%)	0.499
14	8/8 (100%)	7/8 (87,5%)	0.499

Table 4.4 Infection rates of the LR-GFP-226V and LR-dsRed-226A in orally infected *Ae. albopictus.*

a - p value was calculated using Fisher's exact test.

Reciprocal substitution of the fluorescent proteins in CHIKV with E1-226V and E1-226A backbone did not affect the outcome of the competition for colonization of midgut cells of orally infected *Ae. albopictus*. Although under these given conditions both LR-dsRed-226V and LR-GFP-226A were able to simultaneously infect almost 100% of midguts from analyzed mosquitoes (Table 4.4), the dsRed fluorescence (which corresponds to LR-dsRed-226V virus) was markedly more abundant within individual midguts as compared to eGFP fluorescence (which corresponds to LR-GFP-226A virus) (Figure 4.4).

The dissemination rates of the LR-dsRed-226V in orally infected *Ae. albopictus*

Days p.i.	Salivary gland infection rates: SG Infected / Total (%)					
	Oral infection			Intrathoracic infection		
	LR-dsRed-226V	LR-GFP-226A	p value [a]	LR-dsRed-226V	LR-GFP-226A	p value [a]
2	ND	ND	ND	10/10 (100)	10/10 (100)	1
4	ND	ND	ND	10/10 (100)	10/10 (100)	1
7	4/10 (40)	0/10 (0)	0.043	10/10 (100)	10/10(100)	1
14	5/10 (50)	0/10 (0)	0.016	10/10 (100)	10/10 (100)	1

Table 4.5 Comparison of the salivary gland infection rates in the orally and intrathoracically infected *Ae. albopictus*.

Blood meal contained 5.95 $Log_{10}TCID_{50}$/ml of 1:1 mixture of LR-dsRed-226V and LR-GFP-226A. Inoculum for intrathoracic infection contained 1.95 $Log_{10}TCID_{50}$/mosquito of 1:1 mixture of LR-dsRed-226V and LR-GFP-226A viruses.
a - p value was calculated using Fisher's exact test.
ND– data is not determined

were 40% and 50% on days 7 and 14, respectively, which is significantly higher compared to dissemination rates of LR-GFP-226A (Table 4.5). However, the dissemination rates of LR-dsRed-226V and LR-GFP-226A in intrathoracically infected *Ae. albopictus* were indistinguishable. Both viruses were present in 100% of salivary glands as early as 2 dpi and no marked differences in the number of cells expressing GFP or dsRed were observed. These results are almost identical to data obtained in experiments using LR-GFP-226V and LR-dsRed-226A viruses, indicating that GFP and dsRed proteins do not differentially affect CHIKV viral infectivity and dissemination properties in *Ae. albopictus*. These data also additionally support our previous observations suggesting that the increase in dissemination rates observed for CHIKV with E1-226V mutations resulted from more efficient midgut infection, but not from more efficient virus infection and propagation in *Ae. albopictus* secondary organs such as salivary glands.

To further investigate the role of increased midgut infectivity associated with the E1-A226V mutation on CHIKV replication in *Ae. albopictus* bodies and virus transmission by this vector, competition experiments between LR-ApaI-226V and LR-226A were conducted similarly to those described in Chapter 3 (Figure 4.5). In orally infected mosquitoes the RNA of the LR-ApaI-226V was markedly more abundant as compared to RNA derived from LR-226A. However, in intrathoracically infected *Ae. albopictus* both viruses were present in similar quantities, and LR-226A RNA was found to be slightly more abundant in both replicas of 10 mosquitoes per replica.

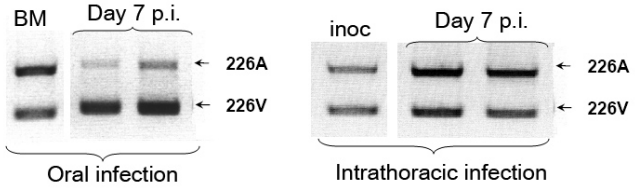


Figure 4.5 Competition between LR-ApaI-226V and LR-226A in bodies of *Ae. albopictus* mosquitoes.

At 7 dpi, viral RNA was extracted from two pools of 10 mosquitoes orally or intrathoracically infected with 1:1 mix of LR-ApaI-226V and LR-226A and processed as described in Chapter 3.
BM - ratio of the RNAs derived from LR-ApaI-226V and LR-226A in blood meal.
Inoc - initial ratio of LR-ApaI-226V and LR-226A in the inoculum used for intrathoracic infection.

The effect of intrathoracic route of infection on the *Ae. albopictus* transition of CHIKV to the vertebrate host was also analyzed (Figure 4.6). In Chapter 3 I showed that

in mosquitoes infected orally with LR-ApaI-226V and LR-226A viruses, only RNA derived from LR-ApaI-226V virus was present in six individual suckling mice (6/6 for LR-ApaI-226V versus 0/6 for LR-226A, p=0.001 by Fisher`s exact test), however, when mosquitoes were infected intrathoracically with a 1:1 mixture of these viruses and on day 7 p.i allowed to feed on suckling mice, no significant differences were observed in the distribution of viral RNAs. The RNA derived from LR-226A was the only RNA species in one out of six mice and present in an amount equivalent to LR-ApaI-226V in four out of six mice (5/6 for LR-ApaI-226V versus 5/6 for LR-226A, p=0.77 by Fisher`s exact test). These data indicate that the evolutionary success of the CHIKV strains bearing the E1-A226V mutation in the conditions when *Ae. albopictus* serves as the main CHIKV vector is most likely attributed to an increase in CHIKV infectivity for the midgut cells. This mutation allows more efficient dissemination in the mosquito body and leads to more efficient virus transmission to the next vertebrate host.

DISCUSSION

Infection of *Ae. albopictus* mosquitoes via the intrathoracic route does not result in significant differences in the kinetics of virus growth, virus dissemination rates, effectiveness of replication in mosquito bodies and virus transmission to suckling mice

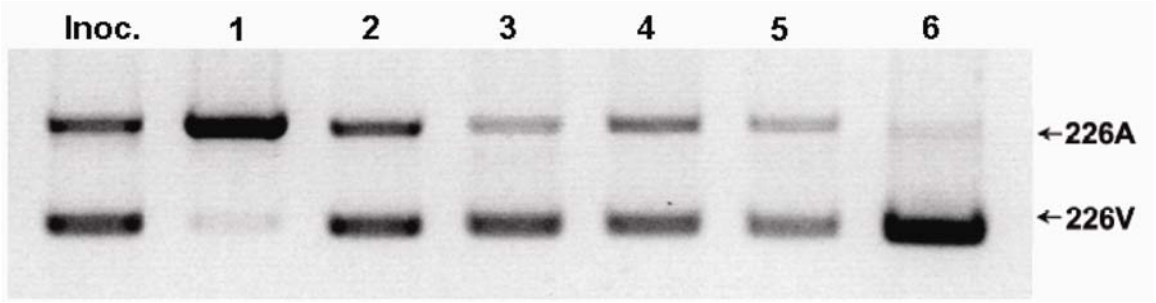


Figure 4.6 Effect of routes of infection on CHIKV transmission by *Ae. albopictus* mosquitoes.

Ae. albopictus mosquitoes were intrathoracically infected with a 1:1 mixture of 10 pfu (5 pfu per virus) of LR-Apa-226V and LR-226A viruses. At 7 dpi, the mosquitoes were presented with a 2-3 day old suckling mouse (Swiss Webster).
Mice were returned to their cage and sacrificed on day 3 post exposure. Blood from each individual mouse (≈50 µl) was collected and immediately mixed with 450 µl of TRIzol reagent for RNA extraction.
Inoc - initial ratio of LR-ApaI-226V and LR-226A in inoculum. 1-6 ratio of LR-ApaI-226V and LR-226A RNA in six individual mice.

between CHIKV with either E1-226V of E1-226A residues. However, in orally infected mosquitoes the E1-A226V mutation was associated with significant increases in all of these parameters. Since the main difference between the oral and intrathoracic routes of infection is that when injected intrathoracically, CHIKV does not need to infect and replicate in midgut cells, and can directly infect secondary organs, we concluded that midgut infection and replication are the major determinants of the transmissibility of epidemic CHIKV strains with E1-A226V mutation.

A similar observation was reported by Smith et al. (2008) in the study of infection and replication of epizootic and enzootic strains of VEEV in the most important epizootic VEEV vector – *Ae. taeniorhynchus* mosquitoes. Earlier it was shown that the PE2 gene of the epizootic VEEV strains contains the main determinants of increased VEEV infectivity to *Ae. taeniorhynchus* (Brault et al., 2002). The epizootic VEEV strain 3908 bound to the midguts of *Ae. taeniorhynchus* significantly more efficiently as compared to the enzootic 68U201 strain, and infected a significantly higher number of individual cells within a single midgut (Smith et al., 2008). Interestingly, the differences in the infection rates of the GFP-expressing replicon particles constructed based on the 3908 and 68u201 strains of VEEV were not statistically significant when the particles were administered intrathoracically ($p=0.106$). Although more eGFP expressing cells were detected in mosquitoes infected with replicons based on the epizootic 3908 strain, when fully infectious VEEV were injected intrathoracically the strain 68U201 replicated to only slightly lower titers as compared to the 3908 strain. Based on these observations, the authors concluded that the midgut infection is the primary limitation to transmission of the enzootic strains of VEEV by *Ae. taeniorhynchus* (Smith et al., 2008).

Earlier Weaver et al. (1984) showed that although the enzootic VEEV vector *Cx. taeniopus* is extremely susceptible to infection with VEEV subtype IE, virus dissemination and transmission is dependent on titer in the blood meal. Less than 0.7 Log_{10}pfu/ml was sufficient to infect almost 100% of mosquitoes, however, the mean dissemination rate was significantly lower as compared to an experiment where 5.2 Log_{10}pfu/ml of the same virus was presented to *Cx. taeniopus* (Weaver et al., 1984). These authors concluded that a mesenteron (midgut) escape barrier (MEB) exists in *Cx. taeniopus* which modulates the efficiency of viral dissemination from midguts into haemocoel and believed that permeability of this barrier is blood-meal-titer related.

Similar conclusions of an existence of a MEB were presented by Kramer et al. (1981) who were studying dissemination of the WEEV in *Cx. tarsalis* mosquitoes. The authors showed that the number of infected mosquitoes transmitting WEEV gradually increases with increase in the blood meal virus titers used for mosquito infection (Kramer et al., 1981).

It has been suggested that the permeability of the MEB in particular mosquito species may vary depending on the particular virus strain used for infection. Studies of oral infectivity of *Ae. aegypti* with yellow fever virus (YFV) vaccine strain 17D, showed that the virus is able only to infect midgut epithelia, but did not progress to a disseminated infection. However, Asibi (the wild type strain of YFV) disseminates in ~ 80% of mosquitoes (McElroy et al., 2006). Similarly, epizootic VEEV subtype I-AB can infect ~ 20% of *Cx. taeniopus,* but fails to disseminate in any of the infected mosquitoes (Weaver et al., 1984). Interestingly, in both cases intrathoracic inoculation of these viruses into mosquito vectors led to 100% infection, and, at least in case of 17D, it also led to a 100% virus dissemination rate (McElroy K. personal communication (Weaver et al., 1984). Unfortunately, the midgut infection rate of YFV strain 17D and VEEV subtype I-AB are significantly lower than that of YFV Asibi and VEEV subtype IE in *Ae. aegypti* and *Cx. taeniopus* respectively, making it problematic to quantitatively characterize effects of MEB for each of these viruses.

Based on these previous reports, we could not exclude the possibility that the CHIKV E1-A226V mutation may modulate the ability of CHIKV to penetrate the MEB. Although the effect of E1-A226V on CHIKV permeability was not directly studied, evidence suggests that penetration of the midgut is comparable between CHIKV with either E1-226 A or V residues. It has been shown that salivary gland infection rates of the 05.115 and 06.21 strains of CHIKV for *Ae. albopictus* are blood meal-dose-dependent. To achieve a 50% dissemination rate with strain 05.115 the required blood meal titer is at least ~1.5 Log_{10}pfu/ml higher than for strain 06.21 (Vazeille et al., 2007). Interestingly, comparison of the OID_{50} values of the LR-GFP-226V and LR-GFP-226A virus pair, and/or the 37997-GFP226V and 37997-GFP226A pair demonstrates a very similar (~1.5-2.0 $Log_{10}TCID_{50}$/ml) difference in the blood meal titer required for infection (Figure 3.5, Figure 3.6). This suggests that regardless of the amino acid residue at the E1-226 position of CHIKV, an increase in infection rates correlates with a proportional increase in

dissemination efficiency from the midguts into the heamocoel. If the MEB permeability was significantly different between CHIKV with the E1-226A and V residues, then one would predict that differences in infection and dissemination rates would not be identical for these two viruses, especially considering that replication of both viruses is indistinguishable in *Ae. albopictus* if the midgut is bypassed by intrathoracic inoculation (Figure 4.2 Figure 4.5).

CONCLUSIONS

The results of this study clearly indicate that adaptation of CHIKV to transmission by *Ae. albopictus* requires the E1-A226V mutation, which acts primarily by increasing CHIKV midgut infectivity for this mosquito species. This increase in midgut infectivity/replication is the most important phenotypic effect of this mutation, which later determines other phenotypic manifestations such as increased dissemination and transmission of the virus by *Ae. albopictus*. These findings justify our primary interest in CHIKV infectivity of midgut cells of *Ae. albopictus* which will be the main focus of the following chapters.

CHAPTER 5: THE E1-A226V MUTATION MODULATES CHIKV CHOLESTEROL DEPENDENCE OF AND DECREASES THE PH THRESHOLD FOR VIRUS FUSION

ABSTRACT

As previously described, we demonstrated that the E1-A226V mutation is responsible for adaptation of chikungunya virus (CHIKV) to *Ae. albopictus* mosquitoes by increasing virus infectivity for midgut cells (Chapter 3; Tsetsarkin et al., 2007). However the molecular mechanism explaining the positive effect of E1-A226V on the CHIKV infectivity for midgut cells of *Ae. albopictus* remains largely unknown. In experiments described in this chapter I performed a detailed mutagenesis analysis of position E1-226. I found that CHIKV can tolerate a significant variability at this position. Analysis of viruses with specific mutations at E1-226 in *Ae. albopictus* mosquitoes revealed that these mutations can be classified into three groups: highly infectious (V, I, M and L); intermediately infectious (H and P); and poorly infectious (A, S, T, G, F and D). Analysis of three-dimentional models of the E1 protein of CHIKV constructed based on the E1 structure of Semliki Forest virus (SFV) suggests that an increase in interactions between methyl and/or methylene groups of the aliphatic residues at E1-226 with methylene groups at position E1-86P located in the fusion loop might be responsible for the observed phenotypic effects of the E1-226 mutation. Using cholesterol depleted C6/36 cells we demonstrated that the E1-A226V mutation is associated with an increase in cholesterol-dependency of CHIKV. Analysis of viruses with specific mutations at E1-226, and other CHIKV genome regions which modulate cholesterol dependency of the virus, indicates that there is no clear mechanistic correlation between dependency for cholesterol and increased infectivity to *Ae. albopictus* mosquitoes. I also showed that the E1-A226V mutation increases the pH dependency of the CHIKV fusion reaction; however, subsequent genetic analysis failed to support an association between CHIKV dependency for pH and mosquito infectivity phenotypes. Based on these data, I concluded that the E1-A226V mutation probably acts at the different steps of the CHIKV life cycle, affecting multiple functions of the virus.

INTRODUCTION

I previously demonstrated that the E1-A226V mutation is responsible for adaptation of CHIKV to *Ae. albopictus* mosquitoes by increasing viral infectivity for midgut cells (Chapter 3; Tsetsarkin et al., 2007). This increase leads to more efficient dissemination of the virus into salivary glands and more efficient virus transmission to vertebrate host (Chapter 3; Chapter 4). These observations provided a plausible explanation for the epidemiologic success of the CHIKV bearing this mutation during the 2005-2007 CHIKV outbreaks that were maintained by *Ae. albopictus* mosquitoes. The investigation of the underlying molecular mechanism of action of E1-A226V mutation would require a cell culture model that mimics the effect of this mutation *in vitro*. Unfortunately, there are no available cell culture systems derived from the midgut epithelial cells for any mosquito species. Also, the E1-A226V-associated increase in infectivity cannot be reproduced using *Ae. albopictus*-derived C6/36 cells, nor mammalian BHK-21 or Vero cells. This makes the detailed investigation of the molecular mechanism responsible for the observed effects of the E1-A226V mutation problematic. In these circumstances the alternative approach for studying such mechanisms could be the characterization of particular parameters of the infection process for CHIKV which differ between CHIKV with E1-226A and CHIKV with E1-226V residues, and then using a panel of viruses with different mutations to investigate correlation between this parameter and mosquito infectivity phenotype.

The envelope of CHIKV (like all other alphaviruses) consists of a lipid bilayer in which 240 copies of E2-E1 heterodimers are embedded (Strauss and Strauss, 1994). The E1 protein lies below E2 and mediates fusion of the viral and cellular membranes during viral entry (Strauss and Strauss, 1994). E2 is believed to mediate virus interactions with cells surface receptor(s), followed by receptor-mediated endocytosis. The fusion of alphaviruses is triggered by the acidic pH of endosomes and consists of several well-characterized stages (for review (Kielian, 2006), Chapter 1.1.7).

Most of the previously characterized determinants of vector specificity are located in the E2 protein of alphaviruses, and it was suggested that adaptation of alphaviruses to particular mosquito vector occurs through acquisition of the specific mutations in E2 protein, which allows virus interaction with specific receptor presented in this mosquito

(Chapter 1.2.5). However, the mutation responsible for adaptation of CHIKV to *Ae. albopictus* occurrs in the E1 protein; therefore the receptor-mediated explanation of its effect on viral fitness in this mosquito seems implausible, suggesting that this mutation acts at steps in virus entry following receptor-mediated endocytosis.

It has been shown that mutations at the same position of the E1 protein (E1-226) of SFV can modulate the cholesterol requirements for SFV entry into and exit from cells (Vashishtha et al., 1998). The *in vitro* experiments using wild-type (wt) SFV, which has proline at E1-226, showed that the presence of cholesterol in liposomes is required for: 1) fusion of the virus with liposomes (White and Helenius, 1980), 2) binding of the E1-ectodomain to liposome membrane, and 3) E1 acid-dependent conformational changes such as epitope exposure, homotrimerization, and acquisition of trypsin resistance (Kielian and Helenius, 1985; Kielian and Jungerwirth, 1990; Klimjack et al., 1994). *In vivo* studies using cholesterol-depleted mosquito C6/36 cells showed that cholesterol is also required for efficient wt SFV and SINV entry into and exit from cells (Lu et al., 1999; Marquardt et al., 1993; Phalen and Kielian, 1991; Vashishtha et al., 1998). Subsequent studies using different sterol analogs showed that a 3β-hydroxil group is the most critical portion of the sterol molecule that is required for SFV fusion (Kielian and Helenius, 1984; Phalen and Kielian, 1991).

Subsequent *in vivo* studies showed that a single amino acid substitution E1-P226S in SFV and triple substitution at E1-A226S, K227G, N228M was sufficient to significantly increase virus production in, virus infectivity to and virus exit from a cholesterol-depleted C6/36 cells (Lu et al., 1999; Vashishtha et al., 1998). *In vitro* studies showed that this mutation is responsible for a significant increase in SFV fusion with cholesterol-free liposomes. Also, using soluble E1 protein, it was shown that E1-P226S makes conformational changes in the protein strikingly less cholesterol-dependent than that of wt virus. A later study using lipid-mixing assays showed that *in vitro* fusion of wt and E1-P226S mutant viruses with cholesterol-containing liposomes have comparable kinetics, activation energies, and sphingolipid dependence. Additionally the pH thresholds of fusion of wt and E1-P226S mutant were indistinguishable (Chatterjee et al., 2000). The mutagenesis analysis of the position E1-226 of SFV and SINV showed that overall conformation of the E1 region around the E1-226 (rather than some specific chemical group at this particular position) determines virus cholesterol dependence

(Kielian et al., 2000). Together, these findings indicate that the E1-P226S mutation acts at a so-called "lipid sensing" step which occurs earlier and differs from the stable insertion of the E1 fusion peptide into the host cell target membrane, E1 epitope exposure and E1 homotrimerization. It was hypothesized that the presence or absence of cholesterol in target membranes is detected by the E1 protein, which influences the kinetics of E1 conformational changes during fusion (Kielian et al., 2000). However, the detailed mechanism explaining the effect of this mutation at E1-226 on cholesterol dependence of alphaviruses remains unknown.

Interestingly, in the later study using VEEV enveloped pseudo-typed murine leukemia virus, it was shown that the entry of VEEV into cells is resistant to cholesterol depletion (Kolokoltsov et al., 2006), suggesting that cholesterol dependence is not absolute and probably depends on particular virus/host species relationships.

The role of the E1-P226S mutation, which confers a cholesterol-independent phenotype of SFV in cell culture, was also investigated in *Ae. albopictus* mosquitoes (Ahn et al., 1999) which as with all insects, do not possess the enzymatic pathways required for cholesterol production. Instead, they obtain required sterols from dietary sources (Canavoso et al., 2001; Clayton, 1964). The E1-P226S mutation confers more efficient SFV replication in intrathoracicaly injected *Ae. albopictus*, particularly at low multiplicities of infection (Ahn et al., 1999). Also, this study showed that propagation of SFV containing the E1-P226S mutation in mosquitoes does not result in virus reversion to a wt residue. Considering that previously I showed that the E1-A226V mutation confers the adaptation of CHIKV to midgut cells of *Ae. albopictus,* I hypothesized that E1-A226V mutation mediates this effect by modulating cholesterol dependence of CHIKV. In this chapter I investigated possible correlation between cholesterol dependence of CHIKV and particular CHIKV infectivity phenotypes in *Ae. albopictus* mosquitoes.

MATERIALS AND METHODS

Viruses and plasmids

The viruses and plasmids encoding full length infectious clones of the LR2006 OPY1 strain pCHIK-LR ic (GenBank accession number EU224268), GFP-expressing full

length clone pCHIK-LR 5'GFP (GenBank accession number EU224269), pLR-ApaI-226V and LR-226A have been previously described (Chapter 2; Chapter 3; Tsetsarkin et al., 2006; Vanlandingham et al., 2005b). The plasmid p37997-226A (pCHIK-37997ic, GenBank accession number EU224270) and p37997-226V have been described (Chapter 3; Vanlandingham et al., 2005b). Plasmids encoding a full length clone of SINV Toto1101 has been previously described (Rice et al., 1987)**.**

Two point mutations at E1-226 and E1-227 were introduced into pToto-1101 to produce a cholesterol-independent SINV mutant, designated Toto-1101SG. The plasmid was constructed by simultaneous cloning of 2 DNA fragments into pToto-1101. Fragment 1 was generated using a fusion PCR method. Initially, two PCR reactions were run on pToto-1101. The first reaction (PCR1) was run using Sin-F and Toto-SG-R primers set; for second reaction (PCR2) Toto-SG-F and Rep5-R2 primers were used (Table 5.1; Table 2.1). 0.5 µl of each amplicons generated by PCR1 and PCR 2 were used as templates for PCR3 using primers Sin-F and Rep5-R2. The resultant amplicon was digested by *BssH*II and *Stu*I restrictases. Fragment 2 (979 nt) was generated by digestion of pToto-1101 with *Stu*I and *Xho*I restrictases. Fragments 1 and 2 were cloned by *BssH*II and *Xho*I into pToto-1101. The resultant plasmid was designated as pToto-1101-SG and the PCR-generated region was completely sequenced.

A series of point mutations at E1-226 (S, T, G, I, P, F, M, H, L) was introduced into pCHIKV-LR i.c. using the strategy similar to those described for pLR-GFP-226A. Briefly, plasmids were constructed by simultaneous cloning of 2 DNA fragments into pCHIKV-LR i.c. The first fragment was generated by fusion PCR method using 41855-Xho-F, 41855-StR1 and one pair of primers encoding desired amino acid substitution (Table 5.1; Table 2.1). This fragment was digested with *Xho*I and *Kpn*I restrictases. Fragment 2 (961 nt) was generated by digestion of pCHIKV-LR i.c. with *Kpn*I and *Pac*I restrictases. Fragments 1 and 2 were cloned by *Xho*I and *Pac*I into pCHIKV-LR i.c. The resultant plasmids were designated as pLR-226X, where X is a particular amino acid residue at position E1-226. PCR-generated regions of pLR-226X were completely sequenced using primers 41855-XhoF and 41855-StF1 (Table 2.1).

Virus	Primer name	Sequence
LR-226G	181-F	5’CTGCAGAGACCGGCTGGGGGTACGGTACACGTG
	181-R	5’CACGTGTACCGTACCCCCAGCCGGTCTCTGCAG
LR-226I	182-F	5’CTGCAGAGACCGGCTATAGGTACGGTACACGTG
	182-R	5’CACGTGTACCGTACCTATAGCCGGTCTCTGCAG
LR-226S	184-F	5’GTACTGCAGAGACCGGCTTCGGGTACGGTACACGTG
	184-R	5’CACGTGTACCGTACCCGAAGCCGGTCTCTGCAGTAC
LR-226T	185-F	5’GTACTGCAGAGACCGGCTACGGGTACGGTACACGTG
	185-R	5’CACGTGTACCGTACCCGTAGCCGGTCTCTGCAGTAC
LR-226P	198-F	5’GTACTGCAGAGACCGGCTCCGGGTACGGTACACGTG
	198-R	5’CACGTGTACCGTACCCGGAGCCGGTCTCTGCAGTAC
LR-226F	199-F	5’GTACTGCAGAGACCGGCTTTCGGTACGGTACACGTG
	199-R	5’CACGTGTACCGTACCGAAAGCCGGTCTCTGCAGTAC
LR-226M	200-F	5’GTACTGCAGAGACCGGCTATGGGTACGGTACACGTG
	200-R	5’CACGTGTACCGTACCCATAGCCGGTCTCTGCAGTAC
LR-226L	207-F	5’GTACTGCAGAGACCGGCTCTGGGTACGGTACACGTG
	207-R	5’CACGTGTACCGTACCCAGAGCCGGTCTCTGCAGTAC
LR-226H	208-F	5’GTACTGCAGAGACCGGCTCACGGTACGGTACACGTG
	208-R	5’CACGTGTACCGTACCGTGAGCCGGTCTCTGCAGTAC
LR-226D	209-F	5’GTACTGCAGAGACCGGCTGACGGTACGGTACACGTG
	209-R	5’CACGTGTACCGTACCGTCAGCCGGTCTCTGCAGTAC
LR-226K	230-F	5’GTACTGCAGAGACCGGCTAAGGGTACGGTACACGTG
	230-R	5’CACGTGTACCGTACCCTTAGCCGGTCTCTGCAGTAC
LR-226N	231-F	5’GTACTGCAGAGACCGGCTAACGGTACGGTACACGTG
	231-R	5’CACGTGTACCGTACCGTTAGCCGGTCTCTGCAGTAC
SIN-226SG	Sin-F	5’GCATTACTACCATCGCCATC
	Toto-SG-F	5’-TCAAGCCTTCCTCCGGGAACGTGCATGTCC
	Toto-SG-R	5’-GGACATGCACGTTCCCGGAGGAAGGCTTGAGTAG

Table 5.1 Specific primers implemented for construction of plasmids used in this study.

To introduce S, T, G, I, P, F, M, H, L residues individually at E1-226 in the background of eGFP-expressing CHIKV (pLR-GFP-226V), the DNA fragment of 4709 nt was generated by digestion of LR-GFP-226V with *Age*I and *Xho*I. This fragment was cloned into each of pLR-226X plasmids by *Age*I and *Xho*I sites. The resultant plasmids were designated pLR-GFP-226X where X is a specific desired amino acid residue at position E1-226. Presence of the eGFP gene in the plasmid was confirmed by restriction digestion analysis. Presence of correct substitutions leading to the desired amino acid mutations at E1-226 was confirmed by sequencing of the plasmids using primer 41855-stF1 (Table 2.1). Additionally D, K, N residues were directly introduced into pLR-GFP-226V using the strategy similar to those described for pLR-GFP-226A.

A plasmid encoding the full-length i.c. of LR2006 OPY1 strain containing mutations previously identified in the cholesterol-independent plaque purified clone of LR-ApaI-226V (Clone#1) was generated by cloning of two cDNA fragments into pCHIKV-LR 3**'**GFP. Fragment 1 was amplified based on RNA isolated form Clone#1 virus using 41855-XhoF and 41855-stR1 primers. This fragment was digested with *Xho*I and *Kpn*I restrictases. Fragment 2 (961 nt) was generated by digestion of pLR-GFP-226V with *Kpn*I and *Pac*I restrictases. Fragments 1 and 2 were simultaneously ligated and cloned by *Xho*I and *Pac*I sites into pCHIKV-LR 3'GFP. This cloning strategy allowed easy screening of recombinant plasmids, which in contrast to the vector plasmid do not contain eGFP gene. The resultant plasmid was named pLR-Cl#1, and the PCR-generated region was completely sequenced using primers 41855-XhoF, 41855-stF1 and 41855-stR1 (Table 2.1) to confirm introduction of G10189T (E1-A66S), G10201A (E1-D70N) and A10660C (E1-R223R) mutations. To introduce a single point mutation, C10660A, into pLR-Cl#1, the two DNA fragments of 1082 nt. and 1249 nt. were generated by digestion of pLR-Cl#1 with *Xho*I and *EcoR*V and digestion of pLR-GFP-226V with *EcoR*V and *Pac*I restrictases, respectively. Both DNA fragments were cloned simultaneously into pCHIKV-LR 3**'**GFP by *Xho*I and *Pac*I sites. The resultant plasmid was named pLR-Cl#1-10660A, and sequenced from 41855-Xho-F and 41855-stR1 primers. eGFP expressing version of this plasmid was generated using the same strategy as was described for pLR-GFP-226X constructs. The resultant clone was designated as pLR-GFP-Cl#1-10660A and sequenced from 41855-XhoF.

A plasmid encoding full length i.c. of CHIKV containing two mutations G10659T (E1-Q222H) and G10970A (E1-G326D), which were previously identified in the cholesterol-independent plaque purified clone#10 (Cl#10) of LR-ApaI-226V virus was generated by cloning of two cDNA fragments into pCHIKV-LR 3'GFP. Fragment 1 was amplified based on RNA isolated form Cl#10 virus using 41855-XhoF and 41855-HindR primers. This fragment was digested with *Xho*I and *Hind*III restrictases. Fragment 2 (2138 nt) was generated by digestion of pCHIKV-LR 3'GFP with *Hind*III and *BamH*I restrictases. Fragments 1 and 2 were simultaneously ligated and cloned into pCHIKV-LR 3'GFP by *Xho*I and *Hind*III sites. This cloning strategy allowed easy screening of recombinant plasmids, which in contrast to vector plasmid do not contain eGFP gene. The resultant plasmid was named pLR-Cl#10, and the PCR-generated region was completely sequenced using primers 41855-XhoF, 41855-stF1 and 41855-Hind-R (Table 2.1).

To introduce the individual mutation G10659T (E1-Q222H) into pCHIKV-LR i.c. the 41855-cDNA fragment was amplified from Cl#10 virus RNA using 41855-XhoF and 41855-stR1, and cloned into pCHIKV-LR 3'GFP using a strategy identical to those used for construction of LR-Cl#1. The resultant clone was designated as pLR-Q222H and sequenced from 41855-XhoF and 41855-stR1.

To introduce a second mutation which was previously discovered in Cl#10 virus (E1-G326D) into pCHIKV-LR i.c., two DNA fragments were cloned into pCHIKV-LR 3'GFP. Fragment 1 of 2883 nt. containing this E1-G326D mutation, was generated by digestion of pLR-Cl#10 with *Bgl*I and *Kpn*I restrictases. Fragment 2 (1798 nt) was generated by digestion of pCHIKV-LR i.c. with *Kpn*I and *Bgl*I restrictases. Fragments 1 and 2 were simultaneously ligated and cloned into pCHIKV-LR i.c. by *Bgl*I sites. *Bgl*I restrictase recognizes 2 sites in the pCHIKV-LR i.c., but after endonuclease digestion creates different sticky 3' end overhangs. This allowed simultaneous cloning of two DNA fragments by *Bgl*I sites. The resultant clone was designated as pLR-G326D and sequenced from 41855-Hind-R and 41855-stR1 primers. eGFP-expressing versions of the pLR-Cl#10, pLR-Q222H and pLR-G326D plasmids were generated using the same strategy as it was described for pLR-GFP-226X constructs. The resultant clones were designated as pLR-GFP-Cl#10, pLR-GFP-Q222H and pLR-GFP-G326D and sequenced from 41855-StR1.

In vitro growth of CHIKV in standard and cholesterol-depleted C6/36 cells

To investigate if the mutations in E1 protein influenced cholesterol dependence of the CHIKV, cholesterol-depleted C6/36 cells were prepared by five passages in L-15 or MEM alpha medium containing 10% FBS treated with 2% CAB-O-Sil (Acros Organics) for 12 h at room temperature as previously described (Weinstein, 1979). (CAB-O-Sil is a hydrated colloidal silica that adsorbs lipoproteins/cholesterol and allowes esay removal of cholesterol from bilogycal solutions. CHIKV growth curves were determined by infecting cholesterol-depleted and normal C6/36 cells at multiplicities of infection (MOI) of 0.1 and 1.0, respectively, by rocking for 1 h at 25°C. The cells were washed three times with medium and 5.5 ml of fresh L-15 or MEM alpha medium supplied with 10 % of standard or CAB-O-Sil treated FBS was added to the flask. At the indicated times post-infection, 0.5 ml of medium was removed and stored at -80ºC until titrated. The volume of medium was then restored by adding 0.5 ml of appropriate medium. Viral titers from tissue culture supernatant were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml as previously described (Higgs et al., 1997).

C6/36 cells infectivity assay

Primary infections of cholesterol-depleted C6/36 cells with CHIKV containing specific mutations in the E1 glycoprotein were quantified using an infectious center assay. Confluent monolayers of sterol-depleted C6/36 cells in 96 well plates were infected with 10 µl of serial ten-fold dilutions of eGFP expressing CHIK viruses, for 1 h at room temperature. The virus dilutions were made in MEM alpha medium containing 10% FBS treated with 2% CAB-O-Sil. Following infection, 100 µl of MEM alpha medium containing 10% cholesterol-depleted FBS and 0.8% of carboxy methyl cellulose (which prevents spread of viruses beyond the initial focus of infection) were added to each well. Plates were incubated for 20 h at 30ºC. Experiments were performed in triplicates, and eGFP expressing foci were counted and results were normalized to 10^6 infectious centers/ml on control cells.

Adaptation of LR-ApaI-226V to cholesterol-depleted C6/36 cells

LR-ApaI-226V virus was passed four times in cholesterol-depleted C6/36 cells maintained in L-15 medium. At first passage, a confluent monolayer of cholesterol-depleted C6/36 in 25cm^2 tissue culture flask was infected with LR-ApaI-226V at an MOI of 0.1. At 3 dpi, 1 ml of tissue culture supernatant was used to infect new cholesterol-

depleted C6/36 in 25cm^2 tissue culture flasks. After the fourth passage in cholesterol-depleted C6/36 cells, tissue culture supernatant was harvested at 2 dpi and stored at -80ºC until needed. To obtain the plaque-purified clones, supernatant of the passage four was titrated by plaque assay on Vero cells as previously described (Lemm et al., 1990). At 2 dpi ten individual plaques below the agarose were pierced with a 200 µl tip and used for infection of standard C6/36 cells in L-15 medium in one well of 12 well plate. At 2 dpi culture supernatants were harvested and stored at -80ºC until needed.

Sequencing of the cholesterol-independent clones of CHIKV

Viral RNA from ten plaque-purified, cholesterol-independent clones of CHIKV was extracted using a IAamp Viral RNA Purification kit (Qiagen, Valencia, CA) following standard manufacturer's protocol. RNA was reversed transcribed from random hexamer primers using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was amplified from 41855-stF1 and 41855-Xmn-R primers set and from 41855-Xho-F and 41855-stR1 primers (Table 2.1) using Taq DNA polymerase (New England Biolabs, Ipswich, MA), and sequenced from 41855-Xho-F,41855-stF1 and 41855-Xmn-R primers using ABI PRISM model 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Virus competition assay in cells treated with NH_4Cl

BHK-21 or C6/36 cells in 12 well plates were maintained in L-15 medium supplied with 10% FBS. Thirty min prior to infection, cell culture media was replaced with 0.5 ml of the L-15 media containing various amounts of NH_4Cl (the endosomal acidification inhibitor) as indicated (see results). After a 30 min incubation at room temperature, 50µl of a 1:1 mixture of LR-ApaI-226V and LR-226A virus (10^7pfu/ml) was added to each well in 12 well plates and infection was allowed to continue for 1h at room temperature. Cells were washed once with 0.8 ml of L-15 media containing NH_4Cl, then 0.8 ml of fresh L-15 medium containing various amounts of NH_4Cl was added to each well and cells were incubated for 48h at 37ºC for BHK-21 or 28ºC for C6/36 cells. Cell culture supernatants were harvested and used for RNA extraction, or titration on Vero cells. Virus RNA was processed as was discussed previously (Chapter 3).

Cell-cell fusion assay

For comparative studies of the pH threshold of the CHIKV fusion reaction, confluent monolayers of C6/36 cells were infected (MOI of 0.5-1.0) with eGFP

expressing CHIKV containing specific mutations in the E1 glycoprotein. Cells were incubated for 24h in 0.5 ml of L-15 medium supplemented with 10% FBS to allow cell surface expression of viral glycoproteins. Cells were washed once with 0.5 ml of L-15 and the cell-to-cell fusion reaction was triggered by incubating the cell monolayers for 2 min at 30 ºC in 1 ml of pre-warmed (30 ºC) L-15 medium whose pH was previously adjusted to desired values. Fusion reactions were stopped by replacement of the fusion medium with 0.5 ml of standard L-15 media (pH 7.4). Cells were incubated for 3h at 28 ºC to allow polykaryons to develop. Cells were fixed with 3.5% formaldehyde and stained with 1 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Fusion indexes (percent of fusion) was calculated as (1-c/n)x100%, where c is the number of eGFP expressing cells, n is the number of nuclei (n≥70). Experiments were performed in duplicate for each virus.

Oral infection of mosquitoes

Ae. albopictus (Galveston strain) were infected in an Arthropod Containment Level 3 (ACL-3) insectary as described previously (Chapter 2; Chapter 3; McElroy et al., 2006; Vanlandingham et al., 2005b). To estimate the OID_{50} values, serial ten-fold dilutions of viruses (derived directly after electroporation of *in vitro* transcribed RNA into BHK-21 cells) were made in L-15 medium followed by mixing the samples with defibrinated sheep blood. Mosquitoes were dissected at 7 dpi and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one focus of eGFP expressing cells was present in the midgut. The experiments were performed once or twice for each virus. OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).

For stability assays, 3-5 mosquitoes infected with the highest dilution of the selected viruses were triturated in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) at 7 dpi, and total mosquito and viral RNA was extracted according to the manufacturer's instructions. Viral cDNA was amplified using 41855-Xho-F and 41855-stR1 primers (Table 2.1), and the region of interest was directly sequenced using an ABI PRISM model 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Three-dimentional modeling of the CHIKV E1 protein

Three-dimentional models of CHIKV E1 protein with different mutations at E1-226 were constructed based on an experimentally resolved atomic structure of the monomeric form of the SFV E1 protein [PDB ID: 2alaA (Roussel et al., 2006)] using a Swiss-Model program available at (http://swissmodel.expasy.org//SWISS-MODEL.html). The calculated 3-D models were analyzed using PyMol molecular viewer.

RESULTS

Effect of mutation at E1-226 on CHIKV infectivity to *Ae. albopictus* and cholesterol dependence

To determine if a requirement for cholesterol in the cell membrane is important for CHIKV, cholesterol-depleted C6/36 cells were produced by five passages of the cells in L-15 medium containing 10% FBS treated with 2% CAB-O-Sil for 12 h at room temperature as previously described (Weinstein, 1979). To test the quality of the selected cells, growth kinetics of SINV strain Toto1101 and cholesterol-independent virus Toto1101-SG, which contained two mutations (E1-A226S and E1-K227G) were compared in cholesterol-depleted C6/36 cells (Figure 5.1). As was shown previously, SINV Toto1101 production was significantly inhibited in cholesterol-depleted C6/36 cells (Lu et al., 1999). Although, Toto1101SG virus produced a lower titer in BHK-21 cells after RNA electroporation (Table 5.2) and developed smaller size plaques on Vero cells (data not shown), in the cholesterol-depleted C6/36 cells this virus grew to 3 $Log_{10}TCID_{50}$/ml higher titer than Toto1101, which correlates with the cholesterol-independent phenotype

Virus	Specific infectivity	Virus titer	
		24h	48h
Toto1101	3.5×10^5	8.52	8.52
Toto1101SG	2.5×10^5	6.95	6.52

Table 5.2 Specific infectivity and virus titers after electroporation.

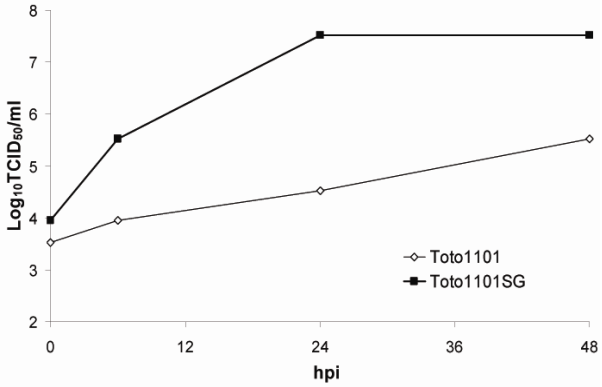


Figure 5.1 Growth kinetics of Toto1101 and Toto1101SG in cholesterol-depleted C6/36 cells.

Cholesterol depleted cells in L-15 medium were infected at an MOI of 0.1.

of this virus. This shows that results obtained using my C6/36 cells perfectly reproduce previously published data.

To determine if a requirement for cholesterol in the cell membrane is important for CHIKV, I analyzed the cholesterol dependence of LR-ApaI-226V, LR-226A, 37997-226V and 37997-226A viruses (Figure 5.2). Growth curves of E1-226A and E1-226V viruses in the background of the Indian Ocean (LR) and West African (37997) strains of CHIKV were almost indistinguishable when grown in C6/36 cells maintained in L-15 supplied with standard 10% FBS. However, when the cells were depleted of cholesterol, LR- 226A and 37997-226A viruses replicated significantly more rapidly than LR-226V and 37997-226V viruses, reaching 3 $Log_{10}TCID_{50}$/ml higher titer at 1, 2 and 3 dpi. These data indicate that the E1-A226V mutation in CHIKV is responsible for acquisition of strong cholesterol-dependence in the cell membrane. Interestingly, alanine at E1-226 was previously shown to confer a cholesterol-independent phenotype of SFV (Kielian et al., 2000). Based on this result I hypothesize that increase in CHIKV infectivity for midgut cells of *Ae. albopictus* correlates with the acquisition of the cholesterol-dependent phenotype, whereas low dependence on cholesterol for growth in C6/36 cells will be associated with low midgut infectivity.

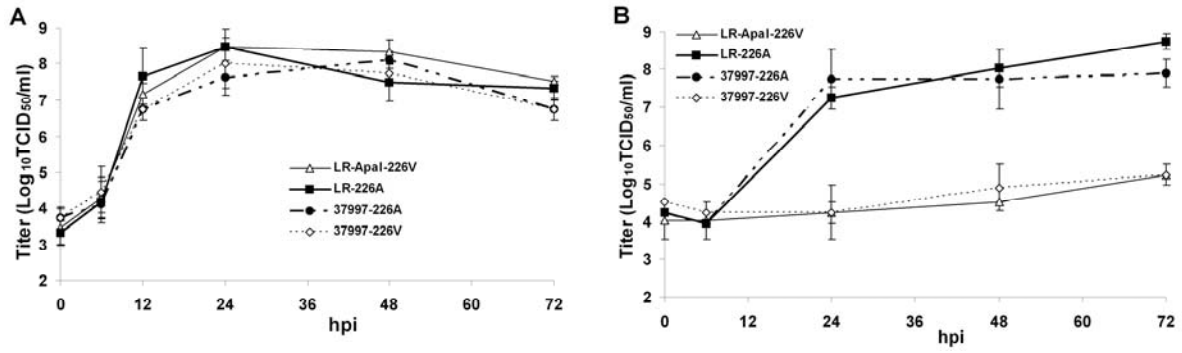


Figure 5.2 Effect of E1-A226V mutation on *in vitro* growth of CHIKV in standard (A) and cholesterol depleted (B) C6/36 cells.

Confluent monolayers of standard (A) and cholesterol depleted (B) C6/36 cells n L-15 medium were infected with LR-ApaI-226V, LR-226A, 37997-226A and 37997-226V viruses at an MOI of 1.0(A) and an MOI of 0.1 (B). Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments.
hpi – hours post-infection.

Template used for *in vitro* transcription.	E1-226 [a]	Time [b]	Specific infectivity (pfu/µg of RNA)	Virus titer[c]	
				24 h	48 h
pLR-ApaI-226V	V	48	9.1×10^5	6.95	7.52
pLR-226A	A	48	8.3×10^5	6.52	7.52
pLR-226S	S	48	7.0×10^5	7.95	7.95
pLR-226T	T	48	6.5×10^5	7.95	7.95
pLR-226G	G	48	10.0×10^5	6.52	7.52
pLR-226I	I	72	5.0×10^5	6.52	6.52
pLR-226P	P	48	9.5×10^5	7.52	6.52
pLR-226F	F	72	9.8×10^5	6.95	6.52
pLR-226M	M	72	9.9×10^5	7.52	7.52
pLR-226H	H	72	10.0×10^5	6.95	6.95
pLR-226L	L	96	10.0×10^5	6.95	6.95

Table 5.3 Specific infectivity and virus titers after electroporation.

a – amino acids at position of E1-226
b – time (h) required for visible plaques to develop
c – Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml

To better understand the amino acid requirements at position E1-226 for increased CHIKV midgut infectivity, and to understand how it correlates with cholesterol dependence of CHIKV, I constructed a number of CHIKV containing a single amino acid substitutions at E1-226 and tested these viruses in *Ae. albopictus* and in cholesterol depleted C6/36 cells. Initially, mutations encoding S, T, G, and I residues at E1-226 were introduced into the backbone of non-eGFP expressing pCHIKV-LR i.c. and specific infectivity values, virus titers after electroporation and plaque sizes were compared to pLR-ApaI-226V and pLR-226A constructs (Table 5.3). It was previously shown that for SFV, E1-226S ensured a cholesterol independent phenotype, whereas SFV which has E1-226T, was cholesterol dependent (Kielian et al., 2000). Analysis of G and I residues at E1-226 would also be very informative because they represent a control of the effects of amino acid size at E1-226 on CHIKV phenotypes in *Ae. albopictus* and in cholesterol depleted C6/36 cells.

Specific infectivity values were comparable between all these constructs, indicating that introduction of S, T, G and I at E1-226 is not lethal for CHIKV replication in BHK-21 cells (Table 5.3). LR-226S and LR-226T viruses replicated to titers higher than those of LR-ApaI-226V and LR-226A. Titers of LR-226G were comparable, however LR-226I grew slower (Table 5.3). Also the LR-226S, LR-226T and LR-226G

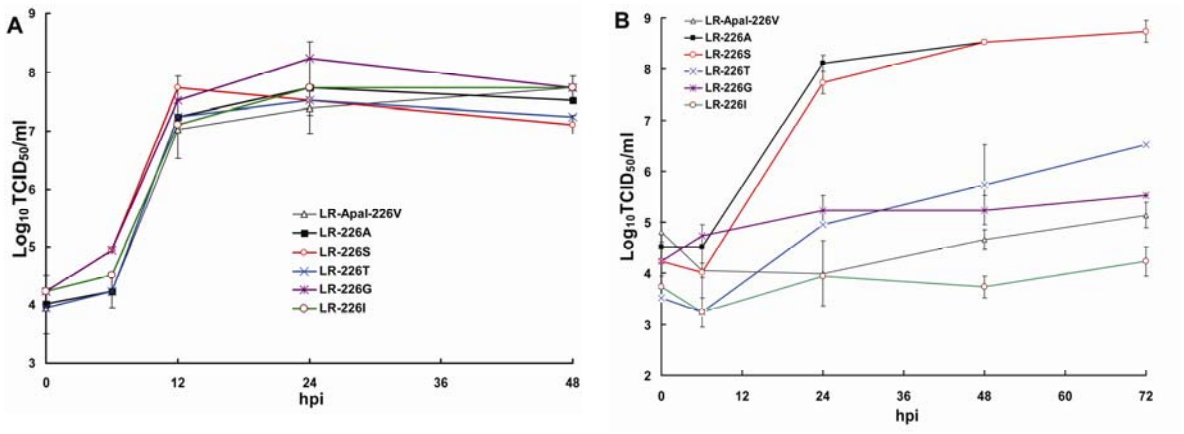


Figure 5.3 Effect of S, T, G and I residues at E1-226 on growth of CHIKV in standard (A) and cholesterol depleted (B) C6/36 cells.

Standard cells were infected at an MOI of 1.0. Cholesterol depleted cells in MEM-alpha medium were infected at an MOI of 0.1. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments.

developed plaques on Vero cells on day 2 post electroporation (p.e.) which were comparable in size with LR-ApaI-226V and LR-226A, however, LR-226I developed visible plaques only on day 3 p.e. This indicates that introduction of isoleucine at E1-226 attenuates CHIKV in vertebrate cell culture.

Template used for *in vitro* transcription.	E1-226 [a]	Time [b]	Specific infectivity	Virus titer[c]	
				24h	48h
pLR-GFP-226V	V	48	$4.8x10^5$	6.52	6.52
pLR-GFP-226A	A	48	$3.3x10^5$	6.95	6.95
pLR-GFP-226S	S	48	$7.0x10^5$	7.95	7.95
pLR-GFP-226T	T	48	$6.5x10^5$	7.95	7.95
pLR-GFP-226G	G	48	$10.0x10^5$	6.52	7.52
pLR-GFP-226I	I	72	$5.0x10^5$	6.52	6.95
pLR-GFP-226P	P	48	$7.2x10^5$	6.95	6.95
pLR-GFP-226F	F	72	$5.0x10^5$	ND	6.95
pLR-GFP-226M	M	72	$5.0x10^5$	ND	6.72
pLR-GFP-226H	H	72	$5.0x10^5$	5.95	6.95
pLR-GFP-226L	L	96	$3.0x10^5$	5.95	6.52
pLR-GFP-226D	D	96	$5.0x10^5$	6.52	6.52
pLR-GFP-226K	K	No	$5.0x10^2$	ND	ND
pLR-GFP-226N	N	No	$2.0x10^3$	ND	ND

Table 5.4. Specific infectivity and virus titers after electroporation.

a – amino acids at position of E1-226.
b – time (h) required for visible plaques to develop.
c – Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml
ND- titer is not determined

Growth kinetics of LR-226S, LR-226T, LR-226G and LR-226I were compared in standard and cholesterol-depleted C6/36 cells, and compared with growth of LR-ApaI-226V and LR-226A viruses (Figure 5.3). All viruses grew very efficiently in standard C6/36 cells (Figure 5.3A) and BHK-21 cells (data not shown). However, in cholesterol-depleted cells only LR-226S demonstrated growth kinetic comparable with LR-226A. Growth of all other viruses was significantly attenuated, reaching 3-4 $Log_{10}TCID_{50}$/ml lower titers on 24, 48, and 72 hpi as compared with LR-226A and LR-226S (Figure 5.3 B).

To further investigate the effects of S, T, G and I residues at E1-226 on the CHIKV requirement for cholesterol, the mutations encoding these amino acids were introduced into the backbone of eGFP-expressing i.c. of CHIKV (Table 5.4), and then specific infectivity of these viruses were determined in standard and cholesterol-depleted C6/36 cells (Figure 5.4). The infectivity of LR-GFP-226A and LR-GFP-226S for cholesterol-depleted C6/36 was 2-2.5 Log_{10}IC/ml higher than the infectivity of CHIKV with V, G, I residues. This is in agreement with the growth kinetics of these viruses in cholesterol-depleted C6/36 cells. This indicates that cell infectivity of E1-226V, G- and I- containing virus is much more dependent on the presence of cholesterol, than cell infectivity of 226A and 226S viruses. Interestingly, the cell infectivity of LR-GFP-226T virus was significantly lower ($p<0.01$ Student`s t-test) than LR-GFP-226A and LR-GFP-226S viruses, but was significantly higher than that of LR-GFP-226V, LR-GFP-226G and LR-GFP-226I viruses, indicating CHIKV dependence for cholesterol can be expressed as a spectrum of different phenotypes.

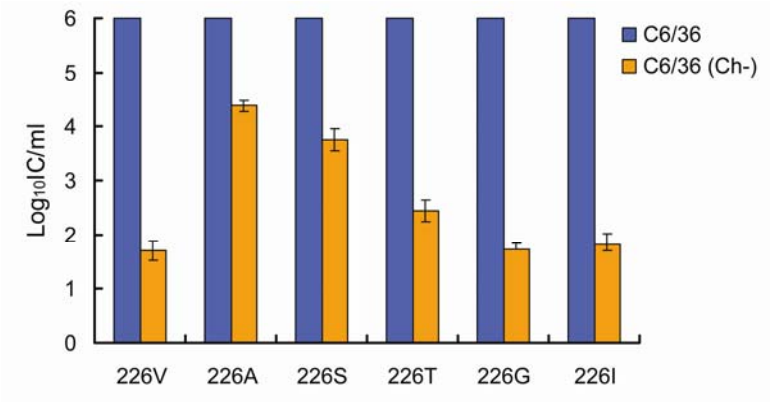


Figure 5.4 Effect of S, T, G and I residues at E1-226 on CHIKV infectivity for cholesterol depleted C6/36 cells.

Standard (blue bars) and cholesterol depleted (yellow bars) C6/36 cells were infected with serial dilutions of eGFP expressing CHIKV containing indicated residues at E1-226. Results are normalized for 10^6 viral infections of standard C6/36 cells. Data indicate an average of three experiments ± standard deviation.
IC-infectious center.

To investigate the relationship between CHIKV dependence for cholesterol and CHIKV infectivity for midgut cells of *Ae. albopictus* mosquitoes, serial dilutions of LR-GFP-226S, LR-GFP-226T, LR-GFP-226G and LR-GFP-226I viruses were orally presented to *Ae. albopictus* mosquitoes. CHIKV infectivity was analyzed by the expression of eGFP in the midgut cells at 7 dpi (Figure 5.5, Figure 3.5; Table 5.5). OID_{50} values of LR-GFP-226S, LR-GFP-226T and LR-GFP-226G in *Ae. albopictus* were between 5.35 and 5.94 $Log_{10}TCID_{50}$/ml, which were not significantly different from the OID_{50} value of LR-GFP-226A virus (5.45 $Log_{10}TCID_{50}$/ml). This indicates that cholesterol dependence of CHIKV does not directly correlate with an increase in midgut infectivity. LR-GFP-226T and LR-GFP-226G both were significantly more cholesterol-dependant as compared to LR-GFP-226A and LR-GFP-226S; however, all four viruses demonstrated almost identical infectivity for *Ae. albopictus* mosquitoes.

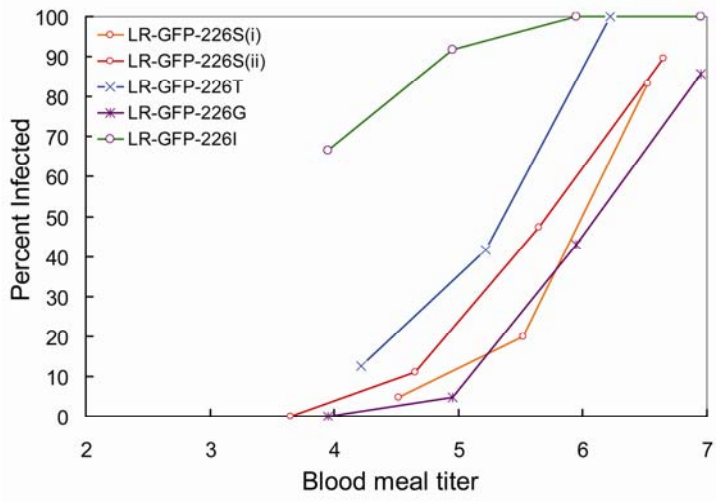


Figure 5.5 Effect of S, T, G and I residues at E1-226 on CHIKV infectivity for *Ae. albopictus.*

Serial ten-fold dilutions of eGFP expressing CHIKV containing S, T, G or I residues at E1-226 were made to produce blood meals. The experiments were performed once for E1-226T, G and I viruses or twice forLR-GFP-226S virus (i and ii).

Infectivity of LR-GFP-226I was almost identical to infectivity of epidemic CHIKV containing E1-226V residue ($p<0.1$). Both valine and isoleucine have additional methyl and ethylene groups in their side chains as compared to alanine or glycine, which are probably required for an increase in CHIKV infectivity for midgut cells of *Ae. albopictus.* The presence of a hydroxyl group in the side chains of serine and threonine apparently has a negative effect on CHIKV infectivity to *Ae. albopictus*.

Previously I showed that the E1-226I residue attenuates CHIKV replication in vertebrate cell lines (Table5.3; Table5.4), which could be associated with accumulation of revertant and pseudo-revertant mutations in the virus genome. To determine if the E1-

226I residue undergoes reversion prior to or during mosquito infection, the region containing the E1-226I residue was sequenced in viral RNA extracted from four infected mosquitoes at 7 dpi (Table 5.6). Results showed that all analyzed mosquitoes were infected with viruses containing E1-226I, indicating that the E1-226I residue is stable in these conditions.

Backbone	E1 226[a]	Exp[b]	N m[c]	OID_{50}[d]	C_{95}[d]	p-value
LR-GFP-226A	A	Com	194	5.45	NG	-
LR-GFP-226V	V	Com	261	3.52	NG	p<0.01
LR-GFP-226S	S	1	70	5.94	5.68-6.24	p>0.1
		2	131	5.68	5.45-5.92	p>0.1
LR-GFP-226T	T	1	72	5.35	5.09-5.61	p>0.1
LR-GFP-226G	G	1	107	5.93	5.67-6.19	p>0.1
LR-GFP-226I	I	1	98	3.54	1.94-4.02	p<0.01
LR-GFP-226P	P	1	98	4.78	4.47-5.10	p<0.05
		2	137	4.86	4.43-5.11	p<0.05
LR-GFP-226F	F	1	112	5.62	5.32-5.99	p>0.1
LR-GFP-226M	M	1	117	3.30	2.49-3.68	p<0.01
LR-GFP-226H	H	1	119	4.69	4.44-4.91	p<0.05
		2	99	4.68	4.40-4.93	p<0.05
LR-GFP-226L	L	1	105	3.85	3.57-4.16	p<0.01
LR-GFP-226D	D	1	48	5.72	5.37-6.08	p>0.1

Table 5.5 Effect of mutations at E1-226 on CHIKV-GFP virus infectivity for *Ae. albopictus* midgut cells.

Exp – experiment number
N m – number of mosquitoes used to estimate OID_{50} value
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
p-value – comparison of statistical significance of difference in OID_{50} values for CHIKV with indicated residues at E1-226 and OID_{50} value of LR-GFP-226A.
Comb – combined summary of two independent experiments.
NG – data is not given

To further investigate the amino acid requirement at position E1-226 for increased CHIKV midgut infectivity and cholesterol dependence, we constructed CHIKV containing the following residues at E1-226 (P, F, M, H, L). All constructs had

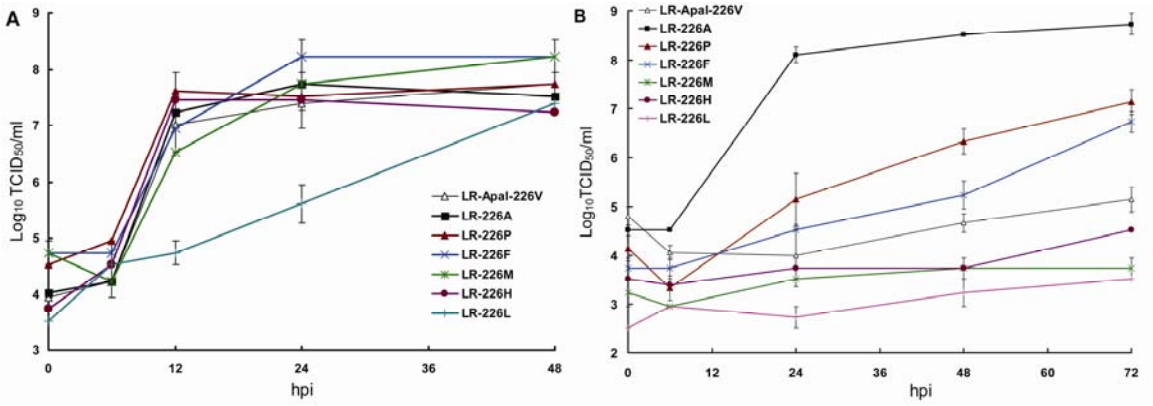


Figure 5.6 Effect of P, F, M, H and L residues at E1-226 on growth of CHIKV in standard (A) and cholesterol depleted (B) C6/36 cells.

Standard cells were infected at an MOI of 1.0. Cholesterol depleted cells in MEM-alpha medium were infected at an MOI of 0.1. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments. hpi – hours post-infection.

comparable specific infectivity values of *in vitro* transcribed RNAs and produced moderate to high virus titers in BHK-21 cells (Table 5.3), indicating that these residues are not lethal for CHIKV, at least in BHK-21. However, constructs with F, M and H residues developed plaques only at 72 hpe in Vero cells, and LR-226L developed plaques at 96 hpe, indicating an attenuation pattern of these viruses.

Growth kinetics of all these viruses (except LR-226L) were comparable in standard C6/36 cells (Figure 5.6 A) and BHK-21 cells (data not shown). However, growth of these viruses was significantly inhibited at 24, 48 and 72 hpi as compared to LR-226A or LR226S viruses. Interestingly, the titer of LR-226P virus was not significantly different from that of LR-ApaI-226V at 24 hpi (p=0.14 student`s t-test), but became

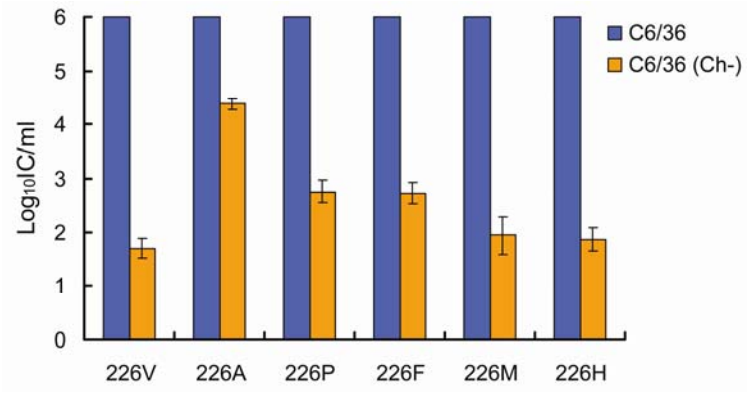


Figure 5.7 Effect of P, F, M and H residues at E1-226 on CHIKV infectivity for cholesterol depleted C6/36 cells.

Standard (blue bars) and cholesterol depleted (yellow bars) C6/36 cells were infected with serial dilutions of eGFP expressing CHIKV containing indicated residues at E1-226. Results are normalized for 10^6 viral infections of standard C6/36 cells. Data indicate an average of three experiments ± standard deviation.
IC-infectious center.

significantly different at 48 and 72 hpi. The P, F, M, H residues were also introduced into e-GFP expressing i.c. of CHIKV (Table 5.4) and specific infectivity of resultant viruses for cholesterol-depleted cell was compared with LR-GFP-226V and LR-GFP-226A viruses (Figure 5.7). Infectivities of all viruses were significantly lower as compared to the infectivity of LR-GFP-226A, however, infectivities of LR-GFP-226P and LR-GFP-226F were significantly higher than that of LR-GFP-226V virus, which agrees with growth kinetics results. These data additionally confirm my earlier observation that CHIKV dependence on cholesterol can be expressed as a spectrum of different phenotypes.

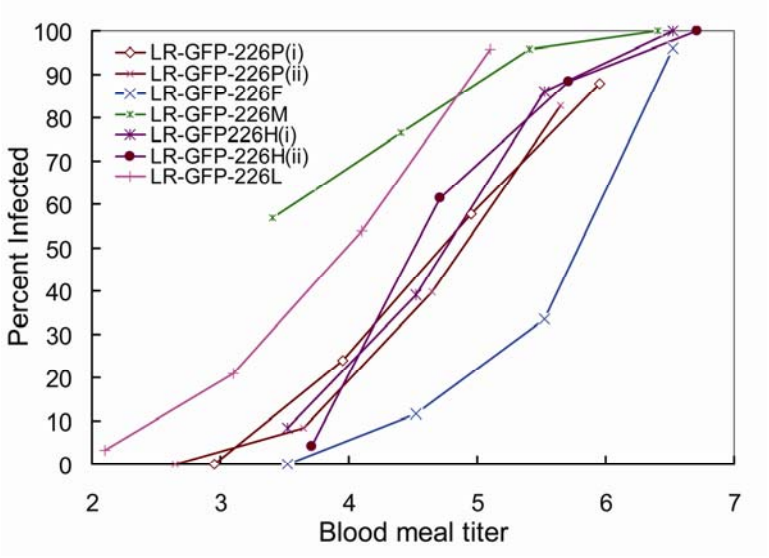


Figure 5.8 Effect of P, F, M, H and L residues at E1-226 on CHIKV infectivity for *Ae. albopictus.*

The experiments were performed once for E1-226 F, M and L viruses or twice for LR-GFP-226P and LR-GFP-226H virus (i and ii)

Also, infectivity of eGFP expressing viruses containing P, F, M, H, L residues at E1-226 were quantitated in *Ae. albopictus* mosquitoes (Figure 5.8; Table5.5). The OID_{50} of LR-GFP-226F for *Ae. albopictus* was similar to the OID_{50} value of LR-GFP-226A ($p>0.1$), indicating that phenylalanine at E1-226 does not lead to increased CHIKV infectivity. Since LR-GFP-226F was found to be much more cholesterol-dependent as compared to LR-GFP-226A, this experiment additionally supported my earlier observation that dependence for cholesterol does not correlate with increased *Ae. albopictus* midgut infectivity. OID_{50} values of LR-GFP-226M and LR-GFP-226L were indistinguishable as compared with the high infectivity viruses LR-GFP-226V and LR-GFP-226I, suggesting that V, I, M and L all share similar structural properties, which are important for CHIKV infectivity for *Ae. albopictus*. Interestingly, LR-GFP-226P and LR-GFP-226H demonstrated an intermediate infectivity phenotype, which was statistically

different from highly infectious viruses such as LR-GFP-226 (V or I) and/or low infectivity viruses such as LR-GFP-226 (A or S).

The stability of LR-GFP-226 F, M, H, L was also analyzed (Table 5.6). All analyzed mosquitoes infected with CHIKV containing F, M and H residues were found to replicate RNA encoding the F, M, and H amino acids at E1-226, respectively, indicating that these viruses are stable in these conditions. Two of four mosquitoes infected with virus derived from the pLR-GFP-226L construct contained RNA encoding only the E1-226L residue, however, sequence results from the remaining two mosquitoes indicated that two RNA species (encoding E1-226L and E1-226P) were simultaneously present in these mosquitoes. These data indicate that LR-GFP-226L is unstable and evolves quickly to substitute the attenuating E1-226L residue.

Virus	Time	N m	E1-226
LR-GFP-226I	72	4	1-I; 2-I; 3-I; 4-I
LR-GFP-226F	72	3	1-F; 2-F; 3-F
LR-GFP-226M	72	3	1-M; 2-M; 3-M
LR-GFP-226H	72	3	1-H; 2-H; 3-H
LR-GFP-226L	96	4	1-L; 2-L/P; 3-L/P, 4-L

Table 5.6 Stability of selected mutations at E1-226 in *Ae. albopictus* mosquitoes.

Time - time (h) required for visible plaques to develop.
N m - number of individual mosquitoes analyzed
E1-226 – residue at E1-226 in viral RNA isolated from individual mosquito
P/L means that virus with leucine and proline are simultaneously present in this mosquito.

To investigate possible intramolecular interactions associated with large aliphatic amino acids at E1-226 with the other regions of E1 and E2 proteins, the highly attenuated E1-226L mutant was harvested on day 2 post-electroporation, and then was additionally propagated in BHK-21 cells. The resultant virus was used for plaque assay on Vero cells and 10 individual clones (five large (3-4 mm) and five small (1.5-2 mm) plaques) were selected at 2 dpi. Clones were propagated once in C6/36 cells, and the 9500-11000 nt genome region was sequenced. All 10 plaque-purified virus contained the same single amino acid substitution E1-L226P, indicating that the appearance of direct reversion at E1-226 is more favorable than accumulation of a resuscitating mutations in any other region of the CHIKV genome. Considering that the introduction of at least 6 different amino acids (A, V, G, S, T, P) was shown to result in viable, non-attenuated CHIKVs, I concluded that specific abrogation of the E1-L226P

mutation by using any other leucine-encoding codons other than CTG, will not be successful to select rescue mutations at regions other than E1-226. Interestingly, four out of five small-plaque-derived clones had an additional silent mutation G10650A (E1-L219L) which is located in the close proximity to E1-226 position. The role of this mutation remains unknown.

The intermediate midgut infectivity of LR-GFP-226H virus was surprising, because all other residues, which increase CHIKV infectivity, generally belong to a class of aliphatic non-polar amino acids (V, I, M, L and P). To investigate if the electric charge of amino acids at E1-226 is associated with increased infectivity for *Ae. albopictus,* three more viruses containing K, D, and N residues at E1-226 in the backbone of eGFP-expressing CHIKV were constructed. The specific infectivity of LR-GFP-226D RNA was in the range of 10^5-10^6 pfu/µg of RNA; however, plaques developed only on day 4 post RNA transfection and were very small in size (Table 5.4). This indicates that aspartic acid at E1-226, although it attenuates CHIKV, it is not lethal to the virus for growth in BHK-21 and Vero cells. The OID_{50} value of this virus in *Ae. albopictus* was determined to be 5.72 $Log_{10}TCID_{50}$/ml, which is not significantly different as compared to LR-GFP-226A ($p>0.1$), indicating that E1-226D does not increase CHIKV infectivity (Table 5.5).

The specific infectivity of LR-GFP-226K and LR-GFP-226N were $5.0x10^2$ and $2.0x10^3$ pfu/µg RNA, respectively (Table 5.4), which is 2-3 Log_{10}pfu/µgRNA lower than the specific infectivity of all other constructs. This indicates that viruses are not viable, and the appearence of the plaques in the lower dilutions is most likely attributed to reversions or pseudo-revertion mutations in the virus genome. Only 40% of BHK-21 cells transfected with LR-GFP-226N expressed eGFP at 24 hpe (which corresponds to usual transfection efficiency of 30 to 90%). All of these infected cells died at 48 hpe; however, the intact monolayer of non-GFP expressing cells survived in the same tissue culture flask. Surprisingly, 100% of BHK-21 cells transfected with LR-GFP-226K expressed eGFP at 24 hpe., and all of the cells died at 2 dpe. This indicates that the LR-GFP-226K virus was released from RNA-transfected BHK-21 cells and infected intact cells. To explain the very low specific infectivity value of LR-GFP-226K, I hypothesized that LR-GFP-226K virus can infect and replicate in BHK-21 cells, but for some reason fails to infect/replicate in Vero cells, which were used for the specific infectivity studies.

To test this hypothesis I titrated LR-GFP-226K virus, which was harvested at 48 hpe., by plaque assay on both BHK-21 and Vero cells. The titration on BHK-21 cells yielded about 2-3 log higher titer than that from Vero cells ($1.2x10^7$ versus $4x10^4$ pfu/ml), indicating that LR-GFP-226K is much more infectious to BHK-21 cells as compared to Vero cells. The plaques, which appeared in Vero cells titrations in very low dilutions, were probably attributed to compensatory mutations in the LR-GFP-226K genome. Since the E1-226N mutation was lethal for CHIKV in BHK-21cells and the E1-226K mutation prevents CHIKV infectivity in Vero cells (which were used for virus titration in all previous OID_{50} experiments), LR-GFP-226K and LR-GFP-226K viruses were not tested in *Ae. albopictus.*

Mutations in other CHIKV genome regions which control cholesterol dependency of CHIKV

The mutagenesis analysis of the position E-226 in CHIKV showed that all of the residues that confer an increased infectivity phenotype in *Ae. albopictus* also were responsible for increased dependence for cholesterol in the target membrane. To investigate if increased CHIKV dependence for cholesterol is always associated with increased infectivity in *Ae. albopictus*, the LR-ApaI-226V virus, which has the E1-226V residue and has been shown to be highly cholesterol-dependent, was used to select mutations (at positions other than E1-226) which can control cholesterol-dependence of CHIKV. LR-ApaI-226V was passed four times in cholesterol depleted C6/36 cells. After the first passage the virus titer was 5.52 $Log_{10}TCID_{50}$/ml at 3 dpi., but at 2 dpi. of passage four the titer was 7.95 $Log_{10}TCID_{50}$/ml, indicating of successful adaptation to cholesterol depleted cells. The passage four virus

Clone #	Mutation (nt)	Mutation (aa)
1	10189 G→T 10201 G→A 10660 A→C	E1-A66S E1-D70N E1-R223R
2	10760 T→C	E1-V226A
3	10760 T→C 10337 A→C	E1-V226A E1-K114T
4	10760 T→C	E1-V226A
5	10189 G→T 10201 G→A 10660 A→C	E1-A66S E1-D70N E1-R223R
6	10760 T→C	E1-V226A
7	10189 G→T 10201 G→A 10660 A→C 10760 T→C	E1-A66S E1-D70N E1-R223R E1-V226A
8	10760 T→C	E1-V226A
9	10760 T→C	E1-V226A
10	10659 G→T 10970 G→A	E1-Q222H E1-G326D

Table 5.7 Summary of adaptation experiment.

was used for plaque assay on Vero cells and 10 individual clones were selected at 2 dpi. Clones were propagated once in C6/36 cells, and the 9500-11000 nt. genome region was sequenced (Table 5.7).

The majority (7 out of 10) of sequenced clones contained the E1-V226A mutation, which has been shown to decrease cholesterol dependence of CHIKV. This mutation was the only substitution found in 5 clones (#2, 4, 6, 8, 9), and in clones #3 and #7 it appeared in context with other mutations. The second most common variant (3 out of 10) contained three nucleotide substitutions (10189 G→T; 10201 G→A; 10660 A→C); two coding (E1-A66S and E1-D70N) and one synonymous (E1-R223R). In two clones (#1 and #5) these three changes were the only found mutations, but in clone #7, these mutations were accompanied by an E1-V226A substitution. The least common variant (1 out of 10) contained two nucleotide substitutions (10659 G→T and 10970 G→A) leading to two amino acid changes (E1-Q222H and E1-G326D). To confirm that selected plaque-purified clones were indeed adapted to grow in cholesterol-depleted C6/36 cells, the growth kinetics of the clones #1, #2 and #10 were compared in cholesterol-depleted C6/36 cells (Figure 5.9). All viruses grew significantly faster than the original virus. Interestingly, growth kinetics of these viruses correlate with the frequency of isolation of the particular mutations after adaptation of LR-ApaI-226V to cholesterol-depleted C6/36 cells. Thus clone #2 virus, which has a single E1-V226A mutation (isolated in 70% of sequences), grew faster than clone #1 virus which has triple mutation (isolated in 30% of sequences), whereas clone #1 grew faster than clone #10 (its mutations were present in 10% of sequences).

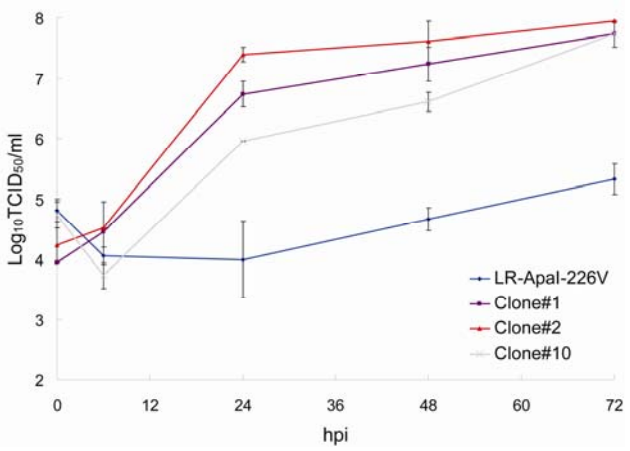


Figure 5.9 Growth of plaque-purified clones #1, #2 and #10 in cholesterol-depleted C6/36 cells.

Cholesterol depleted cells in MEM-alpha medium were infected at an MOI of 0.1. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments.

To confirm that mutations [10189 G→T(E1-A66S); 10201 G→A(E1-D70N); 10660 A→C (E1-R223R)] in clone#1 and (10659 G→T and 10970 G→A) in clone#10

viruses control CHIKV cholesterol dependence, these mutations were introduced into the pCHIKV-LR i.c. and resultant viruses were tested in both BHK-21 cells, and in standard and cholesterol-depleted C6/36 cells (Table 5.8; Figure 5.10 A, B). Growth kinetics of the i.c. derived virus LR-Cl#1 in cholesterol-depleted C6/36 cells was indistinguishable compared to cholesterol-adapted virus (Clone#1). This indicates that 10189 G→T; 10201 G→A and 10660 A→C mutations indeed modulate cholesterol dependence of CHIKV. The third of these mutations [10660 A→C (E1-R223R)] does not lead to an amino acid substitution, however, it is located at the close proximity to position E1-226. This mutation was present in all three individual clones (#1, #5 and #7), which have two other coding mutations (10189 G→T; 10201 G→A), suggesting that 10660 A→C might be important for modulating cholesterol dependency of CHIKV. To test this possibility, the 10660 C→A mutation from the pLR-ApaI-226V was introduced into pLR-Cl#1, and resultant virus (pLR-Cl#1-10660A) was analyzed in standard and cholesterol-depleted C6/36 cells (Figure 5.10 A, B). This virus replicated as efficiently as LR-Cl#1 or Clone#1 viruses in cholesterol-depleted C6/36 cells indicating that the silent mutation 10660A→C is not implicated in cholesterol sensitivity of CHIKV.

Template used for *in vitro* transcription.	Mutation in E1 as compared to LR-ApaI-226V virus	Specific infectivity (pfu/µg of RNA)	Virus titer[a]	
			24h	48h
pLR-ApaI-226V	NO	9.1×10^5	6.95	7.52
pLR-GFP-226V	NO	4.8×10^5	6.52	6.52
pLR-Cl#1	E1-A66S E1-D70N	7.0×10^5	6.95	6.95
pLR-Cl#1-10660A	E1-A66S E1-D70N	10.0×10^5	7.52	7.95
pLR-GFP-Cl#1-10660A	E1-A66S E1-D70N	9.5×10^5	7.52	6.52
pLR-Cl#10	E1-Q222H E1-G326D	5.0×10^5	6.52	7.23
pLR-GFP-Cl#10	E1-Q222H E1-G326D	10.0×10^5	7.52	6.95
pLR-Q222H	E1-Q222H	10.0×10^5	7.95	7.52
pLR-GFP-Q222H	E1-Q222H	6.0×10^5	7.52	7.52
pLR-G326D	E1-G326D	8.0×10^5	6.52	7.52
pLR-GFP- G326D	E1-G326D	10.0×10^5	7.52	7.95

Table 5.8 Specific infectivity and virus titers after electroporation.

a – Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml

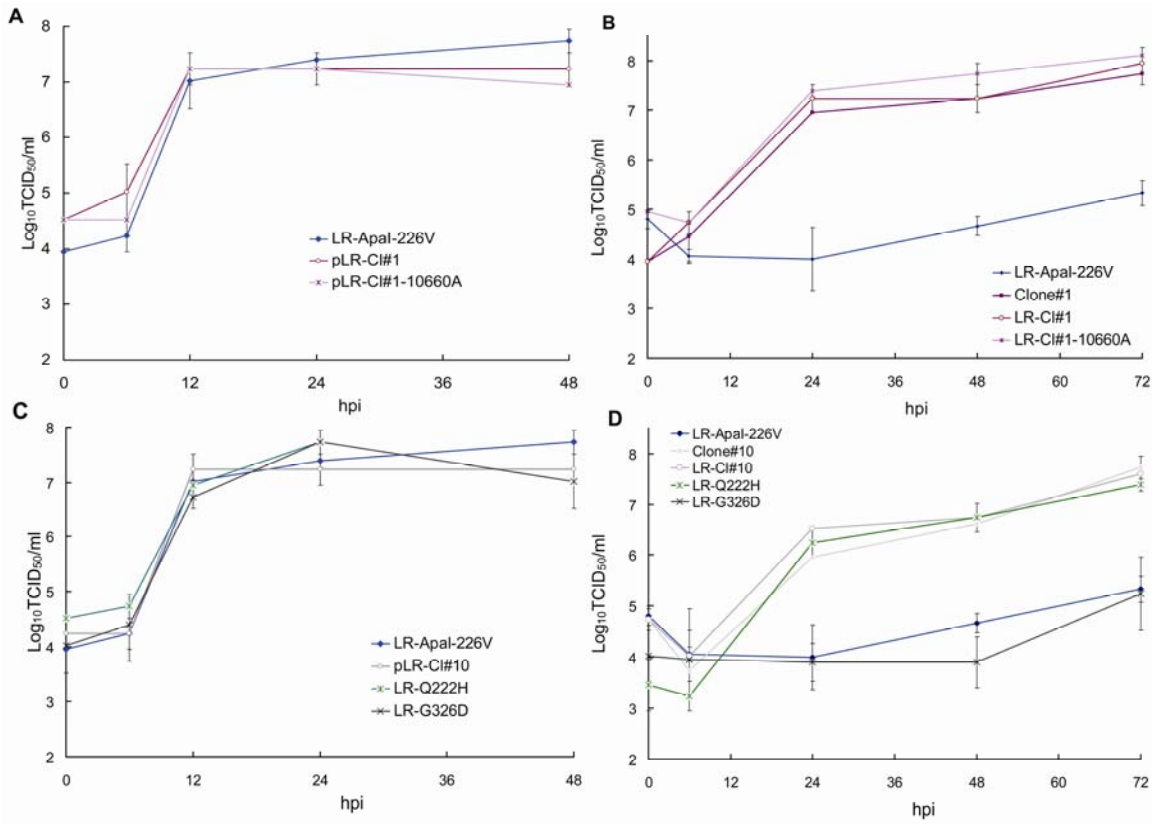


Figure 5.10 Growth kinetics of CHIKV with mutations in E1 protein identified in Clone#1 virus (A and B) and in Clone#10 virus (C and D) in standard (A and C) and cholesterol depleted (B and D) C6/36 cells.

Standard cells were infected at an MOI of 1.0. Cholesterol depleted cells were infected at an MOI of 0.1. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments.
hpi – hours post-infection

Growth kinetics of LR-Cl#10 virus showed that 10659 G→T (E1-Q222H) and 10970 G→A (E1-G326D) mutations identified in Clone#10 virus also control cholesterol-dependence of CHIKV. LR-Cl#10, and the Clone#10 viruses replicated identically in both standard and cholesterol-depleted C6/36 cells (Table 5.8; Figure 5.10 C, D). To determine individual effects of substitutions E1-Q222H and E1-G326D on cholesterol dependency of CHIKV, these changes were individually introduced into the pCHIKV-LR i.c. and the resultant viruses were tested as described above (Table 5.8;). Introduction of the individual E1-Q222H mutation into the backbone of the pCHIKV-LR i.c. resulted in virus which grew as efficiently as LR-Cl#10 and Clone#10 viruses in both standard and cholesterol-depleted C6/36 cells (Figure 5.10 C, D). The E1-G326D substitution had no effect on growth of CHIKV (LR- G326D virus) in standard C6/36

cells, but the growth of this virus was significantly inhibited ($p>0.1$ at 24, 48 and 72 hpi) in cholesterol-depleted C6/36 cells as compared with LR-Cl#10 and Clone#10 virus. This result indicates that only E1-Q222H (but not E1-G326D) is important for determining the cholesterol-independent phenotype of Clone#10 virus.

The eGFP expressing versions of LR-Cl#1-10660A (LR-GFP-Cl#1-10660A), LR-Cl#10 (LR-GFP-Cl#10), LR-Q222H (LR-GFP-Q222H) and LR-G326D (LR-GFP-G326D) were constructed and specific infectivities of these viruses were determined in standard and cholesterol-depleted C6/36 cells (Figure 5.11). The specific infectivities of LR-Cl#1-10660A, LR-GFP-Cl#10 and LR-GFP-Q222H viruses were significantly higher ($p<0.01$ Student`s t test) in cholesterol-depleted C6/36 cells as compared with LR-GFP-226V virus, which genetically corresponds to LR-ApaI-226V virus, which was used for the adaptation experiment. Specific infectivity of LR-GFP-G326D in cholesterol-depleted C6/36 cells was almost identical to that of LR-GFP-226V virus ($p=0.84$ Student`s t test). These data are in agreement with growth kinetics data (Figure 5.10), and demonstrate that the modulation of cholesterol sensitivity by mutation E1-A66S and E1-D70N from Clone#1 and E1-Q222H from clone#10 virus occurs (at least in part) at the level of cell`s entry.

To correlate the roles of the mutations E1-A66S and E1-D70N from Clone #1 virus and E1-Q222H and E1-G326D from Clone #10 virus in modulating cholesterol-

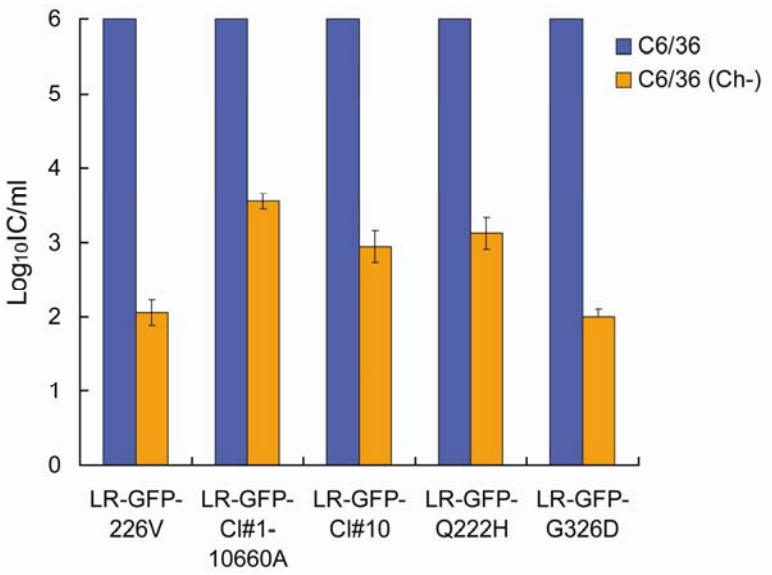


Figure 5.11 Effect of mutations in E1 protein identified in Clone#1 virus and in Clone#10 virus on CHIKV infectivity for cholesterol depleted C6/36 cells.

Standard (blue bars) and cholesterol depleted (yellow bars) C6/36 cells were infected with serial dilutions of eGFP expressing CHIKV containing mutations at at positions other than E1-226. Results are normalized for 10^6 viral infections of standard C6/36 cells. Data indicate an average of three experiments ± standard deviation.
IC-infectious center

dependency of CHIKV with the role of these mutations in CHIKV infectivity to midgut of *Ae. albopictus* mosquitoes, the OID_{50} values of LR-GFP-Cl#1-10660A, LR-GFP-Cl#10, LR-GFP-Q222H and LR-GFP-G326D viruses were determined as described above (Figure 5.12; Table 5.9). Infectivity of LR-GFP-Cl#1-10660A virus was almost identical to infectivity of LR-GFP-226V, indicating that E1-A66S and E1-D70N mutations do not affect mosquito infectivity of CHIKV. These data also indicate that cholesterol-dependence of CHIKV does not directly correlate with increased mosquito

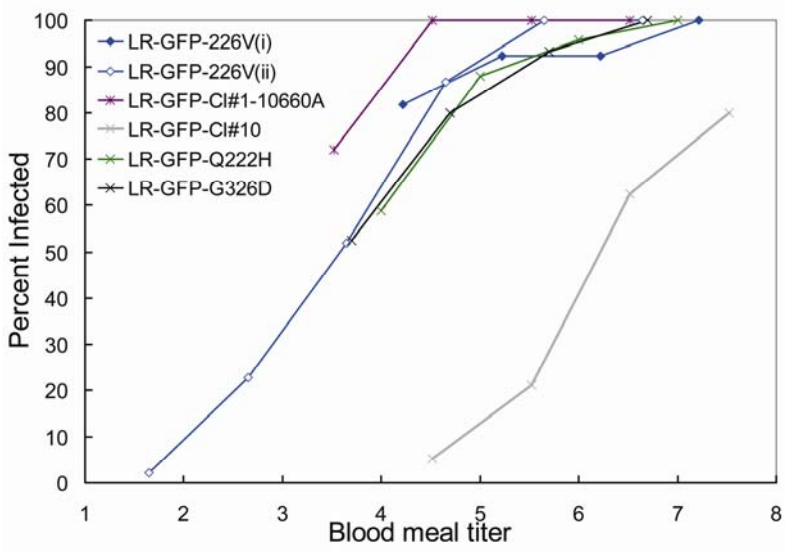


Figure 5.12 Effect of mutations: E1-A66S and E1-D70N from Clone #1 virus and E1-Q222H and E1-G326D from Clone #10 virus on CHIKV infectivity for *Ae. albopictus.*

Backbone	Mutation in E1 as compared to LR-ApaI-226V virus	Exp	N m	OID_{50}	C_{95}	p value
LR-GFP-226V	NO	Comb	261	3.52	NG	
LR-GFP-Cl#1-10660A	E1-A66S E1-D70N	1	80	3.41	3.32-3.50	p>0.1
LR-GFP-Cl#10	E1-Q222H E1-G326D	1	72	6.31	5.93-6.80	p<0.01*
LR-GFP-Q226H	E1-Q222H	1	85	3.68	1.82-4.25	p>0.1
LR-GFP-G226D	E1-G326D	1	81	3.65	2.43-4.13	p>0.1

Table 5.9 Effect of mutations: E1-A66S and E1-D70N from Clone #1 virus and E1-Q222H and E1-G326D from Clone #10 virus on CHIKV infectivity for *Ae. albopictus.*

Exp – experiment number
N m – number of mosquitoes used to estimate OID_{50} value
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
Comb – combined summary of two independent experiments.
p-value – comparison of statistical significance of difference in OID_{50} values for CHIKV with indicated mutations in E1 protein and OID_{50} value of LR-GFP-226V.

infectivity. Interestingly, OID_{50} value of LR-GFP-Cl#10 was significantly higher (low infectivity) than the OID_{50} of LR-GFP-226V ($p<0.01$), however, OID_{50} values of LR-GFP-Q222H (3.68) and LR-GFP-G326D (3.65) viruses (which express mutations isolated in Clone#10 virus individually) were indistinguishable from the OID_{50} of LR-GFP-226V (3.52). Since the E1-Q222H mutation was shown to be the only mutation from Clone #10 responsible for modulating cholesterol-dependency of CHIKV, it additionally confirms that cholesterol-dependency of CHIKV does not correlate with CHIKV infectivity for midgut cells of *Ae. albopictus* mosquitoes. The significant increase in CHIKV OID_{50} values due to combined expression of E1-Q222H and E1-G326D (LR-GFP-Cl#10), is unexpected and required additional investigation, which is beyond the scope of this dissertation project.

E1-A226V mutation is responsible for decrease in fusion pH threshold

It was assumed that fusion of CHIKV takes place in the acidic conditions of the endosomes. The pH dependence of the fusion reaction can be assessed by measuring sensitivity of virus entry to inhibition by a variety of agents that act to raise the pH of the endosome above the threshold required for fusion (Kielian et al., 1986). In this study we analyzed the sensitivity of CHIKV with either E1-226A or E1-226V residues to inhibition with the known lysotropic agent

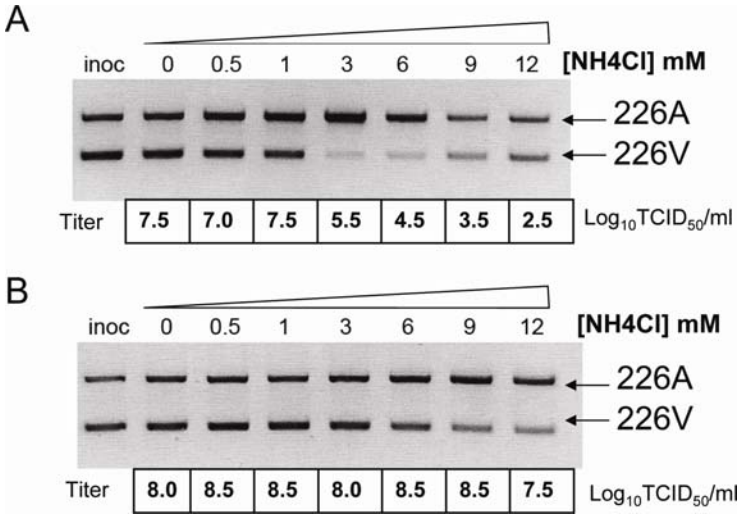


Figure 5.13 Effect of NH_4Cl on competition between LR-ApaI-226V and LR-226A viruses for growth in C6/36 (A) and BHK-21 cells.

Thirty min prior to infection cells were preincubated with L-15 media containing various amount of NH_4Cl. Then cells were infected with 1:1 mixture of LR-ApaI-226V and LR-226A viruses (10^7pfu/ml) and incubated for 48h in L-15 media containing various amount of NH_4Cl. Cell culture supernatants were harvested and used for RNA extraction or titration on Vero cells. Virus RNA was processed as was discussed previously in Chapter 3.

ammonium chloride (NH_4Cl). This compound has been used previously to demonstrate the effect of mutations in both SFV and SINV on their fusion thresholds (Glomb-Reinmund and Kielian, 1998a; Glomb-Reinmund and Kielian, 1998b). The LR-ApaI-226V and LR-226A viruses were mixed in a 1:1 ratio and used to infect C6/36 or BHK-21 cells pretreated with different concentrations of NH_4Cl. At 48 hpi., viral RNA from the cell culture supernatant was extracted and processed as described earlier (Chapter 3: Materials and Methods). LR-ApaI-226V was markedly more sensitive to inhibition with NH_4Cl than was LR-226A virus. Thus in C6/36 cells at the presence of 3, 6, and 9 mM of NH_4Cl the majority of CHIKV RNA in the supernatant was derived from LR-226A virus (Figure 5.13 A). Similarly, in BHK-21 cells, LR-226A virus outcompeted LR-ApaI-226V at NH_4Cl concentrations of 9 and 12 mM (Figure 5.13 B). These data suggest that CHIKV with the E1-A226V mutation requires a low pH to trigger the fusion reaction. To confirm these findings we compared the pH thresholds of the cell-cell fusion of the cells infected with LR-GFP-226A and LR-GFP-226V viruses. Infected C6/36 cells were treated for 2 min with L-15 medium adjusted to the indicated pH and cell-cell fusion efficiency was calculated as (1-c/n)x100%, where c is a number of eGFP expressing cells, n is number of nuclei (n≥70). In two independent experiments

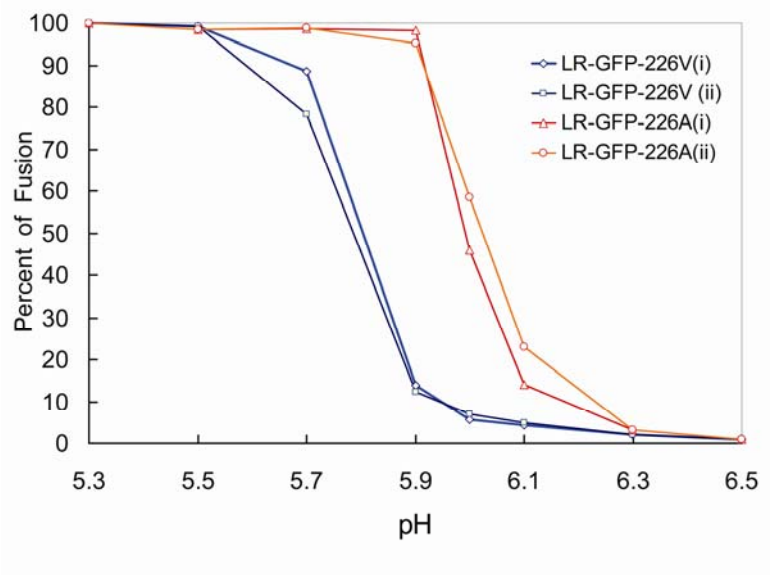


Figure 5.14 Effect of E1-A226V mutation on CHIKV induced cell-cell fusion.

C6/36 cells infected with LR-GFP-226A or LR-GFP-226V viruses were incubated for 2 min with L-15 medium which pH was previously adjusted to desired values. The reaction was abrogated by replacement of the fusion medium with 0.5 ml of standard L-15 media. Percent of fusion was calculated as (1-c/n)x100%, where c is a number of eGFP expressing cells, n is number of nuclei (n≥70). Two independent experiments were performed for each virus (i and ii). Each individual experiment was performed in duplicates and results are expressed as an average of duplicates.

the LR-GFP-226V virus triggered cell-cell fusion reaction at ~0.2 lower pH as compared to LR-GFP-226A virus (Figure 5.14).

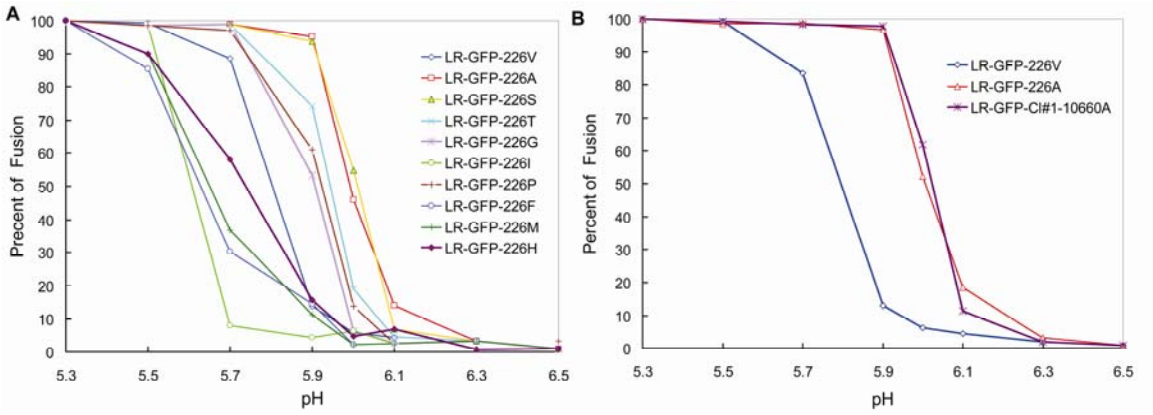


Figure 5.15 Effect of mutations at E1-226 (A) and E1-A66S and E1-D70N mutations (B) on CHIKV induced cell-cell fusion.

C6/36 cells were infected with eGFP expressing viruses and processed as described in legend for Figure 5.14. Experiments were performed in duplicates for each virus, and results are expressed as an average of duplicates.

To investigate if CHIKV mosquito infectivity phenotypes correlated with different thresholds for their fusion reactions, the cell-cell fusion assay was conducted for eGFP-expressing viruses containing S, T, G, I, P, F, M and H residues at E1-226 (Figure 5.15A). No apparent correlation was found between these two parameters. Thus, mosquito infectivity of LR-GFP-226F virus was found to be similar to the infectivity of LR-GFP-226A virus (Table 5.15A), however, LR-GFP-226F virus triggered the cell-cell fusion reaction at a pH even lower than pH of fusion of highly infectious virus LR-GFP-226V (pH~5.6 vs pH~5.8 respectively). On the other hand, mosquito infectivity of LR-GFP-Cl#1-10660A virus was identical to that of LR-GFP-226V, however, LR-GFP-Cl#1-10660A fused C6/36 cells at pH markedly higher than LR-GFP-226V (Table 5.15B).

DISCUSSION

In the present study I performed a detailed mutagenesis analysis of position E1-226. I found CHIKV can tolerate a significant variability at position E1-226. Depending on specific amino acids at this position, CHIKV exhibited various arrays of phenotypes in

cell culture conditions. These phenotypes may be broadly categorized as follows (see Table 5.3 and Table 5.3):

1) – viruses that have S, T, G, P were almost indistinguishable from parental virus containing V or A;

2) – introduction of I, F, M and H residues resulted in viruses that were slightly attenuated;

3) – introduction of L and D residues resulted in significant attenuation of CHIKV;

4) – introduction of N completely inactivated CHIKV;

5) – cell type-specific attenuation of CHIKV containing K at E1-226.

The last virus (LR-GFP-226K) is particularly interesting because it shows that position E1-226 can differentially modulate infectivity of CHIKV to relatively closely related cell culture systems such as BHK-21 and Vero. This suggests that the mechanism by which alphaviruses enter the cell can vary even between these two systems. This phenomenon requires further investigation.

Analysis of viruses with specific mutations at E1-226 in *Ae. albopictus* mosquitoes revealed that effects of these mutations can be classified into three groups as follows:

1) – amino acids I, M and L resulted in viruses that were highly infectious to midgut cells of *Ae. albopictus*. OID_{50} values of these viruses were not significantly different from CHIKV with valine at E1-226;

2) – expression of amino acids S, T, G, F and D at E1-226 resulted in viruses with low infectivity to *Ae. albopictus*. OID_{50} values of these viruses were statistically indistinguishable from pre-epidemic form of CHIKV with E1-226A residue.

3) – an intermediate infectivity phenotype was attributed to P and H residues. OID_{50} values of these viruses were statistically higher than that of highly infectious viruses (V, I, M, L), but in the same time lower than that of low infectivity viruses (Table 5.5; Figure 5.5; Figure 5.8).

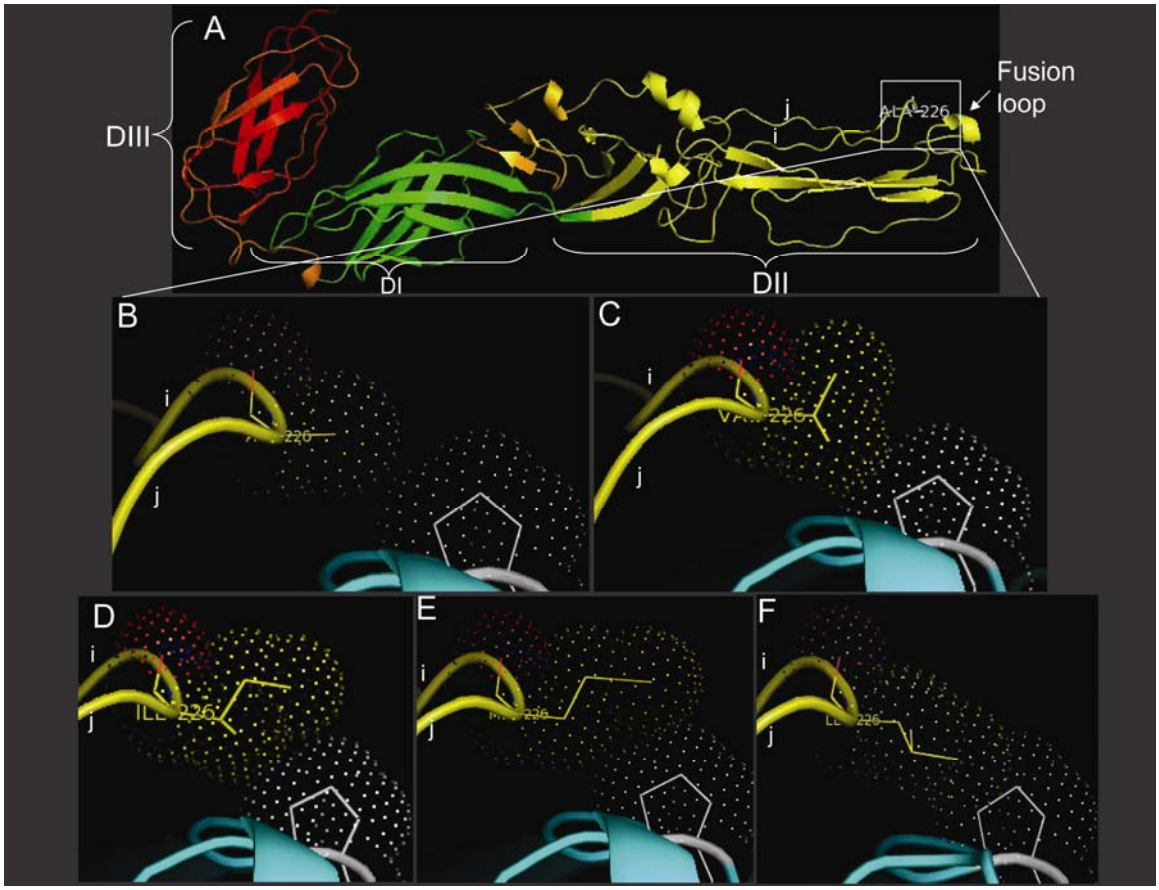


Figure 5.16 Predicted structures of CHIKV E1 protein with different mutations at E1-226 constructed based on experimentally solved atomic structure of monomer form of E1 protein of SFV (PDB ID: 2alaA).

A –full view of predicted structure of E1 molecule of CHIKV with E1-226A residue. Domain I is in green; Domain II is in yellow; Domain III is in red.
B to F – interactions of the tip of ij loop with different residues at E1-226 (yellow) with E1-86P residue in fusion loop (white).
B – E1-226A; C – E1-226V; D – E1-226I; E – E1-226M; F – E1-226L

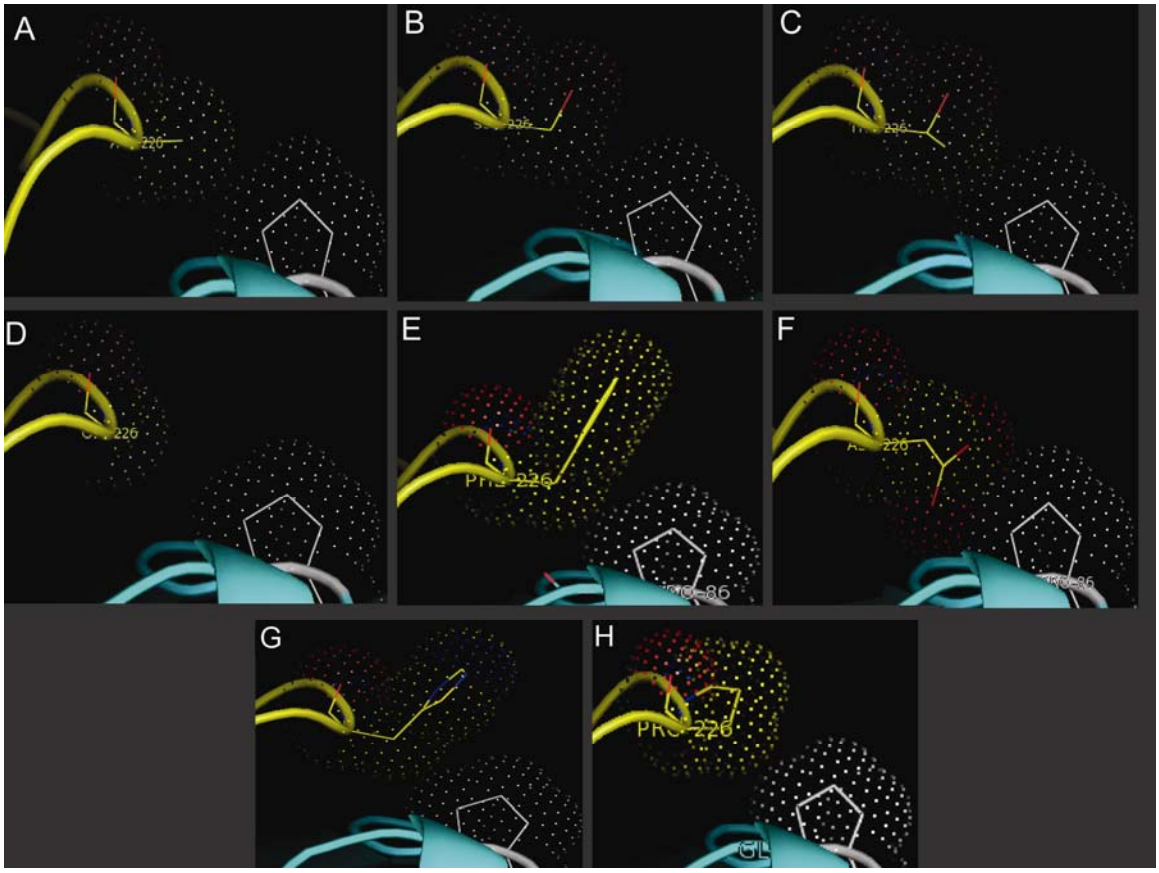


Figure 5.17 Interactions of the tip of ij loop with different residues at E1-226 (yellow) with E1-86P residue in fusion loop (white).

A to F – residues at E1-226 associated with low CHIKV infectivity to *Ae. albopictus*.
A – E1-226A; B – E1-226S; C – E1-226T; D – E1-226G; E – E1-226F; F – E1-226D

G and H – residues at E1-226 associated with intermediate CHIKV infectivity to *Ae. albopictus.* G – E1-226H; H – E1-226P;

The common features shared by residues at E1-226 that ensure a high infectivity phenotype of CHIKV in *Ae. albopictus* (V, I, L and M) is that they belong to class of non-polar aliphatic amino acids. Interestingly, all residues that do not fall into this category (A, S, T, G, F, D, P, H) were associated with significantly lower infectivity to *Ae. albopictus.* To better understand the role of non-polar aliphatic amino acids I constructed 3-D models of CHIKV E1 protein with these mutations based on experimentally resolved monomeric forms of the SFV E1 protein (Roussel et al., 2006) (Figure 5.16; 5.17). It appears that a general requirement for an increase in infectivity for *Ae. albopictus* is associated with the presence of at least two more aliphatic carbon atoms connected to the β-carbon of alanine. Also these models suggest that an increase in

interactions between methyl (CH_3-) and/or methylene (-CH_2-) groups of the aliphatic residues at E1-226 with methylene groups of proline at E1-86 (and possibly glycine at E1-90G) that are located in the fusion loop, might be responsible for the phenotypic effect of the E1-226 mutation (Figure 5.16). Thus aliphatic amino acids at E1-226 form a close contact with E1-86P, however, for all other amino acids these contacts are disrupted/weakened because of special factors (G, A, P, F, H), or because of the presence of the hydroxyl or carboxyl groups in side chain of S, T and D (Figure 5.17).

The current model of the alphavirus membrane fusion process postulates that upon exposure to low pH and E2-E1 heterodimer dissociation, the fusion loop and/or adjacent ij loop (with position E1-226) acts as sensor of lipid composition of target membranes, which then regulates the stable insertion of the fusion loop into membrane of endosomes (Chatterjee et al., 2000; Gibbons et al., 2004a; Kielian et al., 2000). Therefore, the mutations (V, I, M, L) that modulate interactions between the fusion and ij loops may act by affecting this lipid sensor step. It also possible that mutations at the tip of the ij loop (E1-226) act independently of the fusion loop by changing over conformation of E1 protein during this lipid sensing step.

To investigate the effect of mutations at E1-226 on the CHIKV requirement for cholesterol, I analyzed growth kinetics and infectivity of CHIKV with different mutations at E1-226 in cholesterol-depleted C6/36 cells. The pre-epidemic CHIKV with alanine at E1-226 infected and grew much more efficiently in C6/36 cells without cholesterol as compared to the mutated CHIKV (V, I, M, and L at E1-226) with a high infectivity phenotype in *Ae. albopictus* (Figures 5.3; 5.4; 5.6; 5.7). This discovery suggests that CHIKV dependence for cholesterol is connected to infectivity to *Ae. albopictus.* Interestingly, the recent study showed that the E1 protein of alphaviruses can directly bind to cholesterol, which associates with membrane fusion properties of the viruses (Umashankar et al., 2008). This suggests that direct binding to cholesterol can be responsible for an increase in CHIKV infectivity for *Ae. albopictus*. However, analysis of viruses that showed intermediate and decreased infectivity to *Ae. albopictus,* does not support the conclusion that CHIKV dependence for cholesterol is mechanistically correlated with infectivity to *Ae. albopictus.* These viruses demonstrated broad variety of cholesterol-dependence phenotypes that do not correlate with mosquito infectivity profile. The simplest explanation for these discrepancies is that dependence for

cholesterol and infectivity to *Ae. albopictus* are two independent phenotypic effects of the E1-226 mutations. Alternatively, dependence for cholesterol might be controlled at several steps of the infectious process. Cholesterol-independent viruses (such as LR-226A and LR-226S) do not rely on cholesterol at each of these steps, however, cholesterol-dependent viruses (such as LR-226V and LR-226G) may require cholesterol at the different (not overlapping) steps, and the CHIKV-cholesterol interaction at steps which are connected to *Ae. albopictus* infectivity, is not necessary (does not occur) for viruses which showed decreased infectivity for *Ae. albopictus.*

All of the residues at E1-226 that are associated with high infectivity for *Ae. albopictus* also demonstrated high dependence to cholesterol. To address the question if cholesterol-dependence is absolutely required for high mosquito infectivity, we characterized the mutations in CHIKV genome regions other than E1-226, which can make CHIKV less cholesterol-dependent (Table 5.7, Table 5.8; Figure5.9; Figure5.10). We showed that introduction of E1-A66S and E1-D70N mutations into the i.c. of CHIKV with valine at E1-226 significantly releases cholesterol-dependence of CHIKV; however, these mutations had no effect on CHIKV infectivity to *Ae. albopictus* mosquitoes. Similarly, introduction of a single E1-Q222H mutation makes CHIKV significantly less cholesterol-dependent; however, it does not affect mosquito infectivity of CHIKV to *Ae. albopictus*. These data indicate that cholesterol dependence can be modulated independently of the phenotypes of infectivity to *Ae. albopictus* mosquito. Altogether these observations and previously discussed data on the roles of individual mutations at E1-226, indicate that cholesterol dependence of CHIKV cannot serve as an indicator of infectious properties of CHIKV in *Ae. albopictus* mosquito.

Interestingly, all substitutions that control cholesterol-dependency of CHIKV occurred in the E1 region that has never previously been implicated in modulating cholesterol dependency of alphaviruses. Previously it was shown that beside the position E1-226, the mutations in “the hinge” region of the E1 protein (positions: E1-44 and E1-178) could also control cholesterol dependency of SFV. Interestingly, E1-44 and E1-178 mutations also control SFV dependency for sphingolipids, and it was proposed that these mutations in hinge region control flexibility of DI and DII domains, which are believed to be important for the biological function of the molecule. (Chatterjee et al., 2002). The effector mechanism of mutations from clone #1 (E1-A66S and E1-D70N) and Clone#10

(Q222H) on CHIKV cholesterol dependency are not known. The mutations E1-A66S and E1-D70N are located in Domain II in the bc loop which is involved in interactions with Domain I of the adjacent E1 protein and E2 protein (Roussel et al., 2006). Therefore, it is possible that these mutations generally affect the stability of the CHIKV particle. The Q222H is located in the middle of the ij loop and probably acts by controlling the flexibility and the topology of the tip of this loop, which interacts with the fusion loop and target membrane.

Three out of ten (30%) of plaque-purified viruses selected for growth in cholesterol-depleted C6/36 cells had a silent mutation (10660 A→C (E1-R223R), which was always isolated with two other mutations (E1-A66S and E1-D70N). The high isolation frequencies of the 10660 A→C mutation (no other silent mutations were detected in any other region of all ten sequenced clones) and close proximity to position E1-226, led to the hypothesis that this mutation may play a role in modulating cholesterol dependence of CHIKV. However, the growth of CHIKV that had only two coding mutations; E1-A66S and E1-D70N in cholesterol-depleted C6/36 cells, was indistinguishable from the triple mutant [E1-A66S, E1-D70N and E1-R223R (10660 A→C)]. This suggests that 10660 A→C mutation most likely does not play a role in modulating cholesterol-dependency of CHIKV; however, more detailed experiments addressing a precise role of each of these three mutations, are required to completely discard this possibility. It is also important to mention that four out of five (80%) CHIKV clones derived from small-plaque viruses generated after propagation of LR-226L virus in BHK-21 cells have, in addition to the E1-L226P mutation, another silent mutation E1-L219L (10650 G→A) that is also located in close proximity to the E1-226 position. The phenomenon of the appearance of silent mutations at the region around E1-226 begs for further investigation, but it is already tempting to suggest that codon usage for particular residues around position E1-226 may influence the folding of the nascent E1 polypeptide during translation, which later can modulate its biological properties. This phenomenon has been described for several genes including the EgFABP1 protein from *E.coli* (Cortazzo et al., 2002) and MDR1 from humans (Fung and Gottesman, 2009; Kimchi-Sarfaty et al., 2007).

Competition experiments between LR-ApaI-226V and LR-226A in the presence of inhibitor of endosomal acidification (NH_4Cl), and cell-cell fusion experiments

demonstrated that the E1-A226V mutation is responsible for a decrease in the pH required for triggering of a fusion reaction (Figures 5.13 and 5.14). The observed difference in pH dependence between LR-GFP-226V and LR-GFP-226A viruses was relatively small (~0.2); however, I believe that it still can have significant impact on the location of where the fusion reaction occurs within endosomal compartments. Thus, lower pH suggests that fusion of E1-226V viruses would occur not in early endosomes (pH 6.5-6.0), but rather in late endosomes (pH<6.0) which have a quite different lipid composition (Marsh and Helenius, 2006). Interestingly, substitutions E1-226I and E1-226M that were found to significantly increase CHIKV infectivity to *Ae. albopictus,* also led to a decrease in pH of fusion reaction. However, the further analyses of the effects of different mutations at E1-226 on pH of fusion reaction failed to support the association between pH-dependence of the fusion reaction and CHIKV phenotypes in *Ae. albopictus* (Figure5.15 A and B). This indicates that a decrease in the pH of fusion reaction due to E1-A226V mutation is probably not directly responsible for the observed increase in CHIKV infectivity to *Ae. albopictus* mosquitoes.

There was also no clear correlation between pH and cholesterol dependence of CHIKV. Thus, although it seems that viruses that have lower cholesterol dependency for cell entry have a tendency to trigger fusion at higher pH, there are two exceptions that contradict this rule. The E1-226F mutation was responsible for intermediate dependency for cholesterol, but required very low pH to trigger fusion. However, CHIKV with E1-226G was highly cholesterol dependent, but triggered fusion at markedly higher pH than the other highly cholesterol dependent virus. Interestingly, the E1-P226S mutation in SFV which is also responsible for modulation of cholesterol dependence, was shown to have no effect on SFV pH dependence for cell entry (Chatterjee et al., 2000).

CONCLUSIONS

In this study I demonstrated that the mutation E1-A226V is associated with multiple effects on CHIKV physiology, such as cholesterol and pH dependency and mosquito infectivity. The detailed analyses of CHIKV with different mutations at E1-226 and other genome regions revealed that there is no clear correlation between these

parameters. This indicates that E1-A226V mutation probably acts at different steps of CHIKV life cycle affecting multiple functions of the virus.

CHAPTER 6: EPISTATIC ROLES OF E2 GLYCOPROTEIN MUTATIONS IN ADAPTATION OF CHIKUNGUNYA VIRUS TO *AE. ALBOPICTUS* AND *AE. AEGYPTI* MOSQUITOES

ABSTRACT

Between 2005 and 2007 Chikungunya virus (CHIKV) caused its largest outbreak/epidemic in documented history, affecting parts of Africa, the Indian Ocean islands, India, and Europe. An unusual feature of this ongoing epidemic is the involvement of *Ae. albopictus* as a principal vector. Previously we have demonstrated that a single mutation, E1-A226V, significantly changed the ability of the virus to infect and be transmitted by this vector when expressed in the background of well characterized CHIKV strains LR2006 OPY1 and 37997. However, in the current study I demonstrate that introduction of the E1-A226V mutation into the background of an infectious clone (i. c.) derived from the Ag41855 strain (isolated in Uganda in 1982) does not significantly increase infectivity for *Ae. albopictus*. In order to elucidate the genetic determinants that affect CHIKV sensitivity to the E1-A226V mutation in *Ae. albopictus*, the genomes of the LR2006 OPY1 and Ag41855 strains were used for construction of chimeric viruses and viruses with a specific combination of point mutations at selected positions. Based upon the midgut infection rates of the derived viruses in *Ae. albopictus* and *Ae. aegypti* mosquitoes, a critical role of the mutations at positions E2-60 and E2-211 on vector infection was revealed. The E2-G60D mutation was an important determinant of CHIKV infectivity for both *Ae. albopictus* and *Ae. aegypti,* but only moderately modulated the effect of the E1-A226V mutation in *Ae. albopictus.* However, the effect of the E2-I211T mutation with respect to mosquito infections was much more specific, strongly modifying the effect of the E1-A226V mutation in *Ae. albopictus*. In contrast, CHIKV infectivity for *Ae. aegypti* was not influenced by the E2-I211T mutation.

The occurrence of the E2-60G and E2-211I residues among CHIKV isolates was analyzed, revealing a high prevalence of E2-211I among strains belonging to the Eastern/Central/South African (ECSA) clade. This suggests that the E2-211I might be important for adaptation of CHIKV to some particular conditions prevalent in areas

occupied by ECSA strains. The E2-60G was found in 4 out of 39 strains with no apparent close phylogenetic relationships among them.

I showed that the E2-211I residue significantly attenuated growth of CHIKV in BHK-21 cells after standard infection; however, no differences in the kinetics of virus production were observed for CHIKV with either the E2-211I or the E2-211T residues in BHK-21 cells electroporated with *in vitro* transcribed RNA. Also I showed that the mutations at E2-211 do not affect the pH threshold of fusion reaction in BHK-21 and C6/36 cells.

The effect of mutations at E2-211 on CHIKV binding to midgut epithelial cells or brush border membrane fraction of *Ae. albopictus* was also analyzed, revealing no difference between binding capacity of viruses with E2-211I or E2-211T residues. However, using virus overload protein binding assay (VOPBA) I demonstrated that mutations at E2-211 can significantly alter the ability of CHIKV to interact with specific proteins associated with the brush border membrane fraction of *Ae. albopictus.* The significance of these findings is discussed. These newly described determinants of CHIKV mosquito infectivity for *Ae. albopictus* and *Ae. aegypti* are of particular importance for studies aimed at the investigation of the detailed mechanisms of CHIKV adaptations to its vector species.

The effect of the E1-A226V mutation on infectivity of VEEV, ONNV and Mayaro virus (MAYV) to *Ae. albopictus* was analyzed. For these viruses the E1-A226V mutation was not associated with a significant increase in viral infectivity, indicating that possible adaptation of alphaviruses other than CHIKV to *Ae. albopictus* mosquitoes would probably require mutation(s) in the genome region other than at position E1-226.

Substantial parts of this chapter have been accepted for publication into the PLoS ONE journal.

INTRODUCTION

The recent massive epidemics of Chikungunya virus (CHIKV) in Africa, the Indian Ocean islands, India, and the small outbreak in Europe have elevated this arthropod-borne virus (arbovirus) to the status of a major global health problem (Simon et al., 2008). CHIKV, a member of the *Alphavirus* genus in the family *Togaviridae,* is

transmitted to humans by *Aedes* (*Stegomyia*) spp. mosquitoes, primarily *Ae. aegypti*. However, transmission by a previously unrecognized vector species, *Ae. albopictus,* has been a critical contributor facilitating recent epidemics (Bonilauri et al., 2008; de Lamballerie et al., 2008; Delatte et al., 2008; Enserink, 2008; Pages et al., 2009; Reiter et al., 2006; Schuffenecker et al., 2006).

Phylogenetic analysis of CHIKV strains obtained during outbreaks circulating in the *Ae. albopictus*-human transmission cycles have identified the independent acquisition of a common mutation, namely E1-A226V, in strains isolated from different geographic regions (de Lamballerie et al., 2008; Schuffenecker et al., 2006), suggesting that this mutation is associated with specific genetic adaptation to *Ae. albopictus* mosquitoes. Recently we demonstrated that the E1-A226V mutation significantly increases the ability of CHIKV to infect and be transmitted by a laboratory-adapted colony of *Ae. albopictus* mosquitoes when expressed in the background of the well-characterized La Reunion LR2006 OPY1 and West-African 37997 CHIKV strains (Tsetsarkin et al., 2007). Furthermore, CHIKV isolates from Reunion Island possessing valine at position E1-226 disseminate significantly more efficiently to the salivary glands of *Ae. albopictus* mosquitoes collected from La Reunion Island and Mayotte, as compared with CHIKV isolates bearing alanine at this position (Vazeille et al., 2007). Taken together, these findings provide compelling evidence that the E1-A226V mutation is a major genetic determinant of adaptation of CHIKV to a new vector species, *Ae. albopictus,* and provide a plausible explanation for how this mutant CHIKV caused an epidemic in a region lacking the more typical urban vector, *Ae. aegypti*.

Ae. albopictus mosquitoes are native to Southeast Asia, but have recently spread globally due to the advent of modern transportation, with the current geographic range including Europe, Africa, the Middle East, North and South America and the Caribbean (Benedict et al., 2007; Gratz, 2004). As a consequence of this recent range expansion, the pathogens transmitted by this species may be introduced or reemerge in new areas. This scenario was exemplified in August and September of 2007, when the CHIKV–*Ae. albopictus* transmission cycle was established for the first time in Europe, with an estimated 254 human cases in Italy (Bonilauri et al., 2008; Delatte et al., 2008; Enserink, 2007; Rezza et al., 2007). Besides CHIKV and Dengue virus, *Ae. albopictus* has been demonstrated to be susceptible to infection by several clinically important arboviruses

including; Eastern equine encephalitis virus (Mitchell et al., 1992; Scott et al., 1990), and Venezuelan equine encephalitis virus (VEEV) (Beaman and Turell, 1991; Fernandez et al., 2003), yellow fever virus (Mitchell et al., 1987), West Nile virus (Sardelis et al., 2001; Sardelis et al., 2002; Turell et al., 2001), Japanese encephalitis virus (Weng et al., 1997), and Rift Valley fever virus (Moutailler et al., 2008), among others. Understanding the mechanism(s) responsible for adaptation of arboviruses to a new vector may enhance our ability to predict spatial and temporal epidemic risks, and to direct vector control efforts towards specific arthropods, and so will enhance our ability to reduce the incidence of these diseases.

Previous investigations of the effects of the E1-A226V mutation on CHIKV infection of midguts, dissemination into salivary glands, and transmission to a vertebrate host by *Ae. albopictus* suggested that the epidemiologic success of CHIKV with the E1-A226V mutation was most likely due to enhanced midgut infectivity (Tsetsarkin et al., 2007; Vazeille et al., 2007). The ability of CHIKV with A or V residues in position E1-226 to disseminate to the salivary glands and be transmitted to suckling mice by orally infected and intrathoracically injected *Ae. albopictus* was also compared. When intrathoracically injected into the mosquito hemocoel, CHIKV does not need to infect midgut cells and can directly infect secondary organs including the salivary glands. Since in intrathoracically infected *Ae. albopictus* the E1-A226V mutation did not enhance dissemination/transmission rates (Chapter 4), it was thus concluded that the effect of this mutation occurs before virus is released from the midgut into the hemocoel. These data, together with previous findings (Tsetsarkin et al., 2007; Vazeille et al., 2007), support the hypothesis that increased *Ae. albopictus* midgut infectivity resulting from the A226V mutation plays a primary role in enhanced viral transmission.

Little is known about the molecular mechanisms responsible for the selective advantage associated with the E1-A226V mutation. Earlier we demonstrated that the E1-A226V mutation was responsible for modulation of the CHIKV cholesterol dependence for entry and replication in C6/36 cells depleted of cholesterol. This mutation is also responsible for modulation of pH dependence of the CHIKV fusion reaction. This suggests that specific lipid composition in the particular endosomal compartments of *Ae. albopictus* midgut cells might provide an advantage for entry of CHIKV with the E1-A226V mutation. However, detailed investigation of the effects of different amino acids

at E1-226 and mutations in the other CHIKV genome regions, which can also modulate CHIKV cholesterol and pH dependence, showed that there is no correlation between cholesterol and/or pH dependence and increased infectivity to *Ae. albopictus* mosquitoes (Chapter 5).

The majority of the previously described determinants of vector specificity of different alphaviruses are located within the E2 glycoprotein, circumstantially indicating that the process of alphavirus adaptation to new mosquito species occurs via adaptation to a specific cell surface receptor expressed in this mosquito (Brault et al., 2004b; Brault et al., 2002; Myles et al., 2003; Pierro et al., 2007; Pierro et al., 2008). Here, I characterize mutations in the E2 protein that differentially affect *Ae. albopictus* CHIKV midgut infectivity based on the presence of E1-226A or E1-226V residues. Based on my data I conclude that a mutation at position E2-60 influences CHIKV infectivity for *Ae. albopictus*, regardless of the mutations in position E1-226, and also modulates CHIKV infectivity for *Ae. aegypti*. Furthermore, substitutions at E2-211 are crucial for CHIKV sensitivity to the E1-226V mutation in *Ae. albopictus* mosquitoes, but have no effect on CHIKV infectivity for *Ae. aegypti*, and are widely dispersed among CHIKV isolates. These findings provide greater insight into the complexity of the molecular mechanisms involved in adaptation of CHIKV to a new vector.

MATERIALS AND METHODS

Viruses and plasmids

The plasmids encoding full-length infectious clones (i.c.), derived from CHIKV LR2006 OPY1 strain CHIK-LR i.c. (GenBank accession number EU224268) and GFP-expressing full-length clone LR-GFP-226V (CHIK-LR 5'GFP, accession number EU224269) were previously described (Chapter 2; Tsetsarkin et al., 2006; Tsetsarkin et al., 2007).

Construction of full-length i.c. of CHIKV strain Ag41855 (pAg41855 i.c.)

The plasmid encoding full-length and full-length eGFP expressing i.c. of the Ag41855 strain of CHIKV was generated using methodology similar to those described previously for CHIKV strains LR2006 OPY1 and 37997 (Tsetsarkin et al., 2006; Vanlandingham et

al., 2005b). The Ag41855 strain of CHIKV was obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch, Galveston, TX. This strain was isolated in 1982 in Uganda from a human and was passed three times in suckling mice and twice in Vero cells before being used for i.c. construction.

Name	Sequence
41855-Asc-R	5'- AGATGGCGCGCCTGATTAGTGTTTAGGTAC
GFP-Pme-R	5'-CCTTCGTTTAAACTACTTGTACAGCTCGTCCA
Chik-LR-Pme-F	CCTTCGTTTAAACCATGGCCACCTTTGCAAG
41855-226V-F	ACTGCAGAGACCGGCTGTGGGTACGGTACATGT
41855-226V-R	ACATGTACCGTACCCACAGCCGGTCTCTGCAGT
E2-60D-F	5'-TCGGAATAAAGACGGATGACAGCCACGATTGGACCAAG
E2-60D -R	5'-CTTGGTCCAATCGTGGCTGTCATCCGTCTTTATTCCGA
E2-162A-F	5'-ACGTGCAGAGCACCGCCGCAACTACCGAGGAGATAGAG
E2-162A-R	5'-CTCTATCTCCTCGGTAGTTGCGGCGGTGCTCTGCACGT
E2-211T-F	5'-GCTCAAATGAAGGACTAACAACTACAGACAAAGTGA
E2-211T-R	5'-TCACTTTGTCTGTAGTTGTTAGTCCTTCATTTGAGC
41855-StF2	5'-GTAGCACTAGAACGCATCAG
37997-E2-211I-F	5'-CTCAAACGAGGGACTGATAACCACAGACAAAGTGA
37997-E2-211I-R	5'-TCACTTTGTCTGTGGTTATCAGTCCCTCGTTTGAG
Chik-F1	5'-CTAAATAGGTACGCACTACAGC
Chik-R1	5'-CGGCGTCGCAAAAGCAGTAG-3
VEE-164-F	5'-TGCTGCAGAGACCCAAAGTAGGAGCGATCCATGTG
VEE-164-R	5'-CACATGGATCGCTCCTACTTTGGGTCTCTGCAGCA
VEE-250-F	5'-TGCTGCAGAGACCCGCAGTAGGAGCGATCCATGTGC
VEE-250-R	5'-GCACATGGATCGCTCCTACTGCGGGTCTCTGCAGCA
VEE-Cla-F	5'-AACAGTTCAGCCAGTGCACA
VEE-Nde-R	5'- TGACAATCACCTTTGCACGT
VEE-St-F1	5'-GCCTGCTCAAGTGCGTGTG
May-226V-F	5'-CTGGCAAGACCAGCAGTCGGCAACATCCACGTCC
May-226V-R	5'-GGACGTGGATGTTGCCGACTGCTGGTCTTGCCAG
May-EcorV-R	5'-TTATAGGCGCATCAGTCAGT
May-Xho-F	5'-GGTACTATGAACTGTTGCA
Onn-226V-F	5'-ACTGCAAAGACCAGCGGTAGGAGCAATACACGT
Onn-226V-R	5'-ACGTGTATTGCTCCTACCGCTGGTCTTTGCAGT
Onn-R5	5'-ATCCACTCTTCATGGTAATC
Sg-Bgl-R	5'-TTAGTGTCTGCTAAACGATAC

Table 6.1 Specific primers implemented for construction of plasmids used in this study.

The viral RNA was extracted using the Viral RNA Purification kit (Qiagen, Valencia, CA), and used as a template for cDNA production as was described previously (Chapter 2). cDNA was completely sequenced using primers depicted in Table 2.1. The 5' end of Ag41855 cDNA was amplified and sequenced using FirstChoice® RLM-RACE kit (Ambion, Austin, Texas). 3' terminal part of the genome was amplified using the 3' RACE method (Frohman, 1993) as described previously (Chapter 2).

The modified version of pSinRep5 plasmid (Invitrogen, Carlsbad, CA), which has an additional *Cla*I site introduced at 9330 nt in front of the Sp6 promoter (Tsetsarkin K. unpublished data), was used as a backbone for the clone construction. At the first step, two cDNA fragments (1a and 1b) of Ag41855 strain, which correspond to the 5' end (1-1131 nt.) and 3' end (11220- 11811) of the viral genome, were amplified using Chik-Sp6-F2 and 41855-Ngo-R (Fragment 1a) and Chik-F3 and Chik-pA-R (Fragment 1a) primers sets. Fragment 1a was digested with *Cla*I restrictase and phosphorylated using T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA). Fragment 1b was digested using *Xmn*I and *Not*I. Fragments 1a and 1b were cloned simultaneously using *Cla*I and *Not*I sites into pSinRep5-ClaI. The resultant plasmid was named **p41855-1** and sequenced from Chik-ns-F5 and SinRep5-Sacv-F primers.

At the second step, two PCR fragments (2a and 2b) were generated. Fragment 2a was amplified using the Chik-ns-F5 and Chik-nsR4 primer set. This amplicon was digested using *NgoM*IV restrictase and phosphorylated using PNK. Fragment 2b was amplified using 41855-Xho-F and 41855-Hind-R primers, digested using *Xho*I restrictase and phosphorylated with PNK. Fragments 2a and 2b were cloned simultaneously by *NgoM*IV and *Xho*I sites into **p41855-1**. The resultant plasmid was named **p41855-2** and the PCR generated region of the plasmid was sequenced using primers indicated in Table 2.1.

In the third step, the 3661-8269 nt genome region of Ag41855 strain was cloned into pBluescript II SK(+) (Stratagene, La Jolla, CA). Two cDNA fragments (3a and 3b) were generated: fragment 3a was amplified using Chik-nsF3 and 41855-Eag-R primers; fragment 3b was amplified using the 41855-nsF1 and 41855-Mlu-R primers set. Fragment 3a was digested with *Sac*II and *Eag*I restrictases; Fragment 3b was phosphorylated using PNK and digested with *Eag*I restrictase. Fragments 3a and 3b were

cloned simultaneously using *Sac*II and *EcoR*V sites into pBluescript II SK(+). The resultant plasmid was named **p41855-3** and PCR generated region of the plasmid was sequenced using primers indicated in Table 2.1.

In the final step, two overlapping cDNA fragments (4a, 4b) were generated: fragment 4a (3477 nt.) was produced by digestion of **p41855-3** with *Sac*II and *Bgl*I restrictases; fragment 4b was amplified using primers 41855-BglI-F and 41855-Xho-R primers set, and the amplicon was digested with *Bgl*I and *Xho*I restrictases. Fragments 4a and 4b were cloned simultaneously by *Sac*II and *Xho*I into **p41855-2.** The resultant plasmid was named **pAg41855 i.c.** and the PCR generated region of the plasmid was sequenced using primers indicated in Table 2.1.

Construction of full-length eGFP expressing i.c. of the Ag41855 strain of CHIKV

The eGFP-expressing version of the full-length i.c. for the Ag41855 strain of CHIKV (p41855-GFP-226A) was constructed in a two steps cloning strategy similarly to those described previously for pCHIKV-LR 5'GFP (Chapter 2).

In the first step, three DNA fragments (1a, 1b and 1c) were cloned simultaneously by *Sac*II and *Pac*I sites into the **pAg41855 i.c**. Fragment 1a of 3027 nt was generated by digestion of **pAg41855 i.c.** with *Sac*II and *Bsr*GI. Fragment 2b was PCR-amplified from **pAg41855 i.c.** using the Chik 41855-nsF3 (Table 2.1) and 41855-Asc-R (Table 6.1) primers set, and digested with *BsrG*I and *Asc*I restrictases. Fragment 2c of 731 nt. was generated by digestion of pCHIK-37997 i.c. (GenBank accession number EU224270) with *Asc*I and *Pac*I restrictases. The resultant plasmid was named **pAg41855-GFP-1** and the PCR-generated region of the plasmid was sequenced using primers indicated in Table 2.1. In the second step, 3 cDNA fragments (2a, 2b and 2c) were generated. Fragment 2a of 729 nt. was produced by digestion of LR-GFP-226V (pCHIKV-LR 5'GFP) with *Asc*I and *Pme*I restrictases. Fragment 2b was amplified by PCR from **pAg41855 i.c.** using the Chik-LR-Pme-F and 41855-MluR primers set (Table 2.4 and 2.1 respectively) and digested with *Pme*I and *Bgl*I restrictases. Fragment 2c of 3844 nt. was generated by digestion of **pAg41855 i.c .**with *Bgl*I and *Pac*I restrictases. Fragments 2a, 2b and 2c were cloned simultaneously by *Sac*II and *Pac*I sites into the **pAg41855-GFP-1.** The resultant plasmid was named **p41855-GFP-226A** and the PCR generated region of the plasmid was sequenced using primers indicated in Table 2.1.

Construction of p41855-GFP-226V and p41855-226V plasmids

The p41855-GFP-226V construct was produced using a strategy similar to those previously described for the LR-GFP-226A constructs (Chapter 5). Briefly, plasmids were constructed by simultaneous cloning of 2 DNA fragments into **p41855-GFP-226A**. The first fragment was generated by fusion PCR method using (41855-Xho-F and 41855-226V-R) and (41855-226V-F and 41855-StR1) primers sets (Table 2.1; Table 6.1). This fragment was digested with *Xho*I and *Kpn*I restrictases. Fragment 2 (961 nt.) was generated by digestion of **pAg41855 i.c.** with *Kpn*I and *Pac*I restrictases. Fragments 1 and 2 were cloned by *Xho*I and *Pac*I into **pAg41855 i.c**. The resultant plasmid was designated as p41855-GFP-226V and the PCR generated region was completely sequenced using primers 41855-XhoF and 41855-StF1 (Table 2.1).

A non-eGFP-expressing version of p41855-GFP-226V was constructed by cloning of a 2336 nt. fragment generated by digestion of p41855-GFP-226V with *Xho*I and *Not*I restrictases into *Xho*I and *Not*I sites of **pAg41855 i.c.** The resultant construct was designated as p41855-226V and was sequenced from the 41855-St-F1 primer (Table 2.1).

Construction of p41855/LR-GFP-226V, p41855/LR-GFP-226A and pLR/41855-GFP-226V plasmids

To generate the plasmid encoding chimeric viruses containing the E2 protein of LR2006 OPY1 strain in the backbone of the Ag41855 strain, the fragment of 8021-9225 nt. (which corresponds to 152-553 aa. in the structural polypeptide) was introduced into p41855-GFP-226V and p41855-GFP-226A (Figure 6.1). The plasmid was constructed by cloning of 2 cDNA fragments (A and B): fragment A of 1204 nt. was generated by digestion of pCHIKV-LR i.c. with *Bgl*I and *Sac*II restrictases; fragment B of 3477 nt. was generated by digestion of p41855-GFP-226A (used for p41855/LR-GFP-226A) or p41855-GFP-226V (used for p41855/LR-GFP-226V) with *Bgl*I and *Sac*II. The fragments were cloned simultaneously into p41855-GFP-226A by *Bgl*I sites. The pLR/41855-GFP-226V plasmid encoding the reverse chimeric virus containing the E2 protein of Ag41855 strain in the backbone of LR-GFP-226V (LR2006 OPY1 strain) was generated using the same strategy: fragment A of 1204 nt. was generated by digestion of **pAg41855 i.c.** with *Bgl*I and *Sac*II; fragment B of 3477 nt. was generated by digestion of pLR-GFP-226V with *Bgl*I and *Sac*II and fragments A and B were cloned into pLR-GFP-226V by *Bgl*I

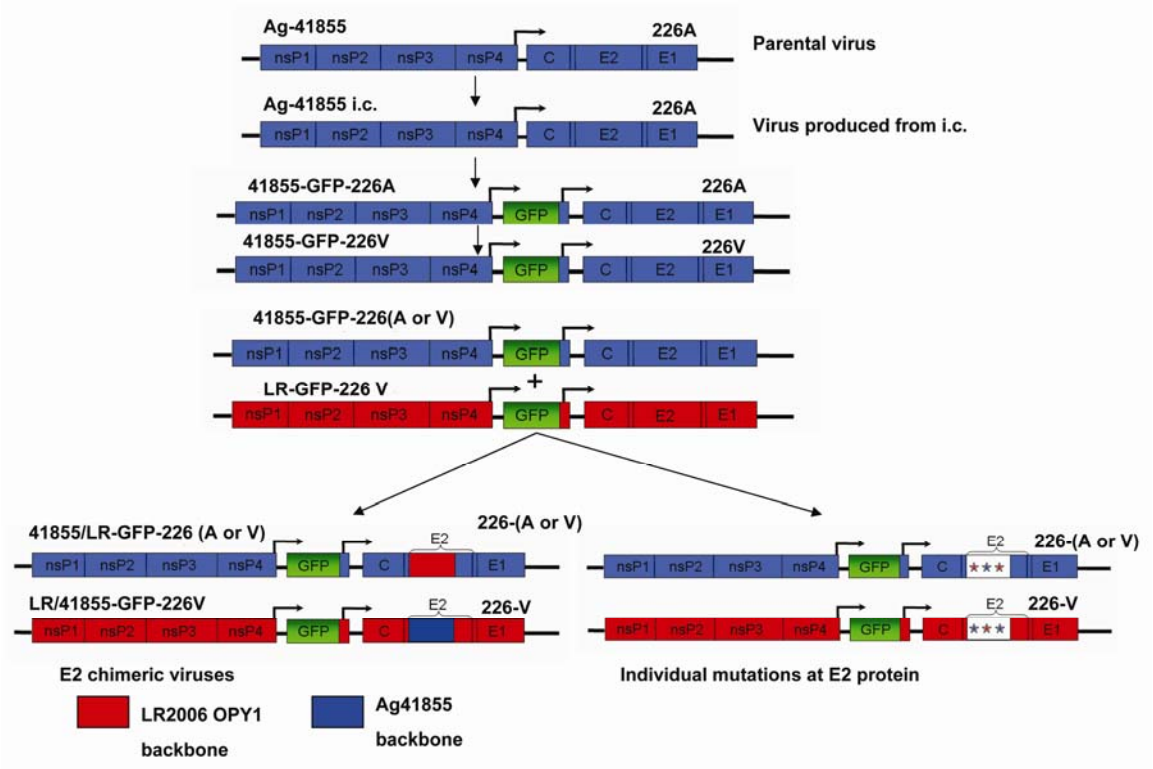


Figure 6.1 Schematic representation of the viruses used in mosquito infectivity studies.

sites. The presence of the desired inserts in these constructs was confirmed by restriction digestion analysis and direct sequencing from the 41855-BglI-F primer (Table 2.1)

Introduction of single mutations E2-G60D, E2-V162A and E2-I211T into the p41855-GFP-226V plasmid

The single mutations E2-G60D, E2-V162A and E2-I211T from the LR2006 OPY1 CHIKV strain were introduced into the backbone of eGFP-expressing i.c. p41855-GFP-226V using a similar strategy. These plasmids were constructed by simultaneous ligation and cloning of 2 cDNA fragments (A and B) into *Bgl*I sites of p41855-GFP-226V. Fragment A, which is different for each mutation to be introduced, was generated by a fusion PCR technique using the **pAg41855 i.c** plasmid as a template and primers sets (41855-BglI-F and E2-X-R) and (E2-X-F and 41855-Xho-R) for the first PCR (Table 6.1 and 2.1), followed by a second PCR from 41855-BglI-F and 41855-Xho-R primers using amplicons generated in the first PCR as templates [where X is either 60D, 162A or 211T (Table 6.1)]. The resultant amplicons were digested with *Bgl*I and *Sac*II restrictases. Fragment B of 3477 nt. was common for all constructs. It was generated by digestion of

p41855-GFP-226V with *Bgl*I and *Sac*II restrictases. Fragments A and B were cloned simultaneously into *Bgl*I of the p41855-GFP-226V plasmid. The resultant plasmids were named p41855-GFP-226V-(E2-60D), p41855-GFP-226V-(E2-162A) and p41855-GFP-226V-(E2-211T) and PCR generated region was completely sequenced using primers 41855-BglI-F and 41855-Xho-R (Table 2.1).

Construction of the plasmids which contain combination of two mutations (E2-G60D and E2-V162A), (E2-G60D and E2-I211T) and (E2-V162A and E2-I211T) in the backbone of p41855-GFP-226V

The double mutations (E2-G60D and E2-V162A), (E2-G60D and E2-I211T) and (E2-V162A and E2-I211T) from the LR2006 OPY1 CHIKV strain were introduced into the backbone of eGFP-expressing i.c. p41855-GFP-226V using a strategy similar to those described for individual mutants E2-G60D, E2-V162A and E2-I211T. These plasmids were constructed by simultaneous ligation and cloning of 2 cDNA fragments (A and B) into *Bgl*I sites of p41855-GFP-226V. Fragment B of 3477 nt. was common for all constructs. It was generated by digestion of p41855-GFP-226V with *Bgl*I and *Sac*II restrictases. Fragment A was unique for each of the plasmids and was produced using a fusion PCR technique. To generate Fragment A which encodes double mutant (E2-G60D and E2-V162A), the fusion PCR was run using p41855-GFP-226V-(E2-162A) as a template and (41855-BglI-F and E2-60D-R) and (E2-60D-F and 41855-Xho-R) primers sets. To generate Fragment A which encodes double mutant (E2-G60D and E2-I211T) the fusion PCR was run using plasmid p41855-GFP-226V-(E2-211T) as a template and (41855-BglI-F and E2-60D-R) and (E2-60D-F and 41855-Xho-R) primers sets. To generate fragment A which encodes double mutant (E2-V162A and E2-I211T) the fusion PCR was run using plasmid p41855-GFP-226V-(E2-211T) as a template and (41855-BglI-F and E2-162A-R) and (E2-162A-F and 41855-Xho-R) primers sets. Individual fragments A were digested with *Bgl*I and *Sac*II restrictases. The individual fragments A and B were cloned simultaneously into the *Bgl*I site of the p41855-GFP-226V plasmid. The resultant plasmids were named p41855-GFP-226V-(E2-60D/162A), p41855-GFP-226V-(E2-60D/211T) and p41855-GFP-226V-(E2-162A/211T) and the PCR generated region was completely sequenced using primers 41855-BglI-F and 41855-Xho-R (Table 2.1).

Construction of the plasmids which contain single (E2-G60D, E2-V162A and E2-I211T) or double (E2-G60D and E2-V162A), (E2-G60D and E2-I211T) and (E2-V162A and E2-I211T) mutations in the backbone of p41855-GFP-226A

To introduce the mutations at positions E2-60, E2-162 and E2-211 from the LR2006 OPY1 strain into the backbone of eGFP-expressing i.c. p41855-GFP-226A the plasmids encoding appropriate combination of E2 mutations in the backbone of p41855-GFP-226V were digested with *Age*I and *Xho*I restrictases and the fragment of 4709 nt. was cloned into *Age*I and *Xho*I sites of **pAg41855 i.c.** The presence of the desired inserts in the resultant constructs was confirmed by restriction digestion analysis and direct sequencing from the 41855-St-R1primer (Table 2.1).

Construction of the plasmids which contain combinations of single (E2-D60G and E2-T211I) or double (E2-D60G and E2-T211I) and (E2-A162V and E2-T211I) mutations in the backbone of pLR-GFP-226V

The plasmids encoding the mutations in the E2 protein from the Ag41855 strain in the backbone of pLR-GFP-226V were generated using a strategy similar to those described for constructs encoding E2 mutations of the LR2006 OPY strain in the backbone of p41855-GFP-226V. To introduce E2-D60G and E2-T211I mutations individually, the plasmids p41855-GFP-226V-(E2-162A/211T) and p41855-GFP-226V-(E2-60D/162A) were digested with *Bgl*I and *Sac*II restrictases and resultant cDNA fragments of 1204 nt. were cloned simultaneously with a 3477 nt. cDNA fragment derived from digestion of pCHIKV-LR i.c. with *Bgl*I and *Sac*II restrictases, into pLR-GFP-226V plasmid by *Bgl*I sites. To introduce double mutations (E2-D60G and E2-T211I) and (E2-A162V and E2-T211I), the plasmids p41855-GFP-226V-E2-162A and p41855-GFP-226V-E2-60D were digested with *Bgl*I and *Sac*II restrictases and the resultant cDNA fragments of 1204 nt. were cloned simultaneously with a fragment of 3477 nt. into the pLR-GFP-226V plasmid by *Bgl*I sites. The resultant plasmids designated as pLR-GFP-226V-(E2-60G), pLR-GFP-226V-(E2-211I), pLR-GFP-226V-(E2-60G/211I) and pLR-GFP-226V-(E2-162V/211I) were sequenced from primer 41855-StF2 (Table 2.1).

Introduction of individual mutations E2-G60D and E2-I211T and double mutation (E2-G60D and E2-I211T) into p41855-226V

To produce CHIKV with individual mutations E2-G60D and E2-I211T and double mutation (E2-G60D and E2-I211T) in the backbone of non-eGFP expressing i.c. of Ag p41855-226V, the plasmids p41855-GFP-226V-(E2-60D), p41855-GFP-226V-

(E2-211T) and p41855-GFP-226V-(E2-60D/211T) were digested with *Bgl*I restrictase. The resultant fragments of 4681 nt. were cloned individually into p41855-226V by *Bgl*I sites. The resultant fragments were named p41855-226V-(E2-60D), p41855-226V-(E2-211T) and p41855-226V-(E2-G60D/E2-I211T) and sequenced from the 41855-St-F2 primer (Table 2.1)

Introduction of individual mutation E2-T211I into pCHIKV-LR i.c. and p37997-226A

To introduce the E2-T211I mutation into the pCHIKV-LR i.c., the p41855-GFP-(60D/162A) plasmid was digested with *Bgl*I and *Sac*II restrictases and a fragment of 1204 nt. was cloned simultaneously with a fragment of 3477 nt. which was generated by digestion of pCHIKV-LR i.c., into pCHIKV-LR i.c. by *Bgl*I sites. The resultant plasmid was named LR-226V-(E2-211I) and sequenced from primer 41855-StF2 (Table 2.1)

The E2-T211I mutation was also introduced into p37997-226A. The plasmid was produced by simultaneous ligation of two cDNA fragments (A and B). Fragment A was generated using a fusion PCR technique using p37997-226A plasmid as a template and (Chik-F1 and 37997-E2-211I-R) and (37997-E2-211I-F and Chik-R1) primers sets (Table 6.1). The resultant amplicon was digested with *NgoM*IV and *Pst*I restrictases. Fragment B of 2696 nt. was generated by digestion of p37997-226A plasmid with *Pst*I and *Not*I restrictases. The fragments A and B were cloned by *NgoM*IV and *Not*I sites into p37997-226A. The resultant plasmid was designated as p37997-226A-(E2-211I), and entire RCR generated region of this plasmid was sequenced.

Introduction of the E1-A226V mutation into: PM1-738/GFP i.c. (eGFP expressing i.c. of VEEV), pMayaro-GFP (eGFP expressing ic of Mayaro virus) and pONNV-GFP (eGFP expressing i.c. of ONNV)

A single E1-A226V mutation was introduced into an eGFP expressing i.c. of VEEV subtype ID designated as plasmid PM1-738/GFP i.c. This i.c. was developed based on the ZPC-738 strain of VEEV subtype ID (GenBank accession no. AF100566) (Anishchenko et al., 2004). A cDNA fragment encoding the E1 protein region containing the E1-A226V mutation was generated using a fusion PCR technique as described in Chapter 3. PM1-738/GFP i.c. was used as a template for fusion PCR reactions using (VEE-Cla-F and VEE-164-R) and (VEE-164-F and VEE-Nde-R) primers sets (Table 6.1) followed by PCR from VEE-Cla-F and VEE-Nde-R primers. The amplicon was digested with *Cla*I and *Nde*I restrictases and cloned by *Cla*I and *Nde*I sites into PM1-738/GFP i.c. The resultant plasmid designated as pVEE-GFP-226V was sequenced from VEE-Cla-F,

VEE-Nde-R and VEE-St-F1 primers (Table 6.1). To introduce a double mutant E1-K225A and E1-A226V, the same strategy was used as for PM1-738/GFP-226V, but instead of primers VEE-164-F and VEE-164-R, we used primers VEE-250-F and VEE-250-R (Table 6.1). The resultant plasmid was named PM1-738/GFP-225A226V.

The eGFP expressing i.c. of MAYV (pMayaro-GFP) was developed by Tsetsarkin K. and Forrester N (unpublished data) based on IQT 4235 strain of MAYV, isolated in 1997 in Loreto, Peru. To introduce the E1-A226V mutation into pMayaro-GFP, two cDNA fragments (A and B) were generated. Fragment A was produced by a fusion PCR technique using pMayaro-GFP as a template and two primers sets (May-Xho-F and May-226V-R) and (May-226V-F and May-EcorV-R), followed by PCR from May-Xho-F and May-EcorV primers. The amplicon was digested with *Xho*I and *EcoR*V restrictases. Fragment B of 1619 nt. was generated by digestion of pMayaro-GFP with *EcoR*V and *Bgl*I restrictases. Fragments A and B were cloned simultaneously by *Xho*I and *Bgl*I sites of pMayaro-GFP. The resultant plasmid was named pMayaro-GFP-226V and the PCR-generated region was completely sequenced.

The eGFP expressing i.c. of ONNV virus (pONN-GFP) was developed by Tsetsarkin K. (unpublished data) based on the SG650 strain of ONNV. Strain SG650 (GenBank accession no. AF079456) was isolated from human serum in Uganda in 1996 and has been passed three times in Vero cells and once in *An. gambiae* mosquitoes before being used for i.c. construction. To introduce the E1-A226V mutation into pONN-GFP, two cDNA fragments (A and B) were generated. Fragment A was produced by fusion PCR using pONN-GFP as a template and two primers sets (Onn-F5 and Onn-226V-F) and (Onn-226V-R and Sg-Bgl-R), followed by PCR from Onn-F5 and Sg-Bgl-R primers. The amplicon was digested with *BamH*I and *Sca*I restrictases. Fragment B of 1033 nt. was generated by digestion of pONN-GFP with *Sca*I and *Not*I restrictases. Fragments A and B were cloned simultaneously using *BamH*I and *Not*I sites into pONN-GFP. The resultant plasmid was named pONN-GFP-226V and the PCR-generated region was completely sequenced.

All plasmids were purified by either centrifugation in cesium chloride gradients or by using QIAGEN Plasmid Mini Kits (QIAGEN, Valencia, CA) following the manufacturer's instructions. All plasmids derived from CHIKV, ONNV and VEEV i.c. were linearized with *Not*I. pMayaro-GFP and pMayaro-GFP-226V were linearized with

*Pac*I. All plasmids were *in vitro* transcribed from the minimal SP6 promoter using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) following the manufacturer's instructions. The yield and integrity of synthesized RNA were analyzed by agarose gel electrophoresis in the presence of 0.25 μg/ml of ethidium bromide. RNA (~10 μg) was electroporated into $1x10^7$ BHK-21 (baby hamster kidney) cells as previously described and cells were then transferred to 75 cm^2 tissue culture flasks with 15 ml of Leibovitz L-15 (L-15) medium. Supernatants were collected at 24 and 48 h post-electroporation and stored at -80ºC. Electroporation efficiency was estimated using an infectious centers assay as previously described (Chapter 2; Tsetsarkin et al., 2006).

Cells and mosquitoes

BHK-21 and C6/36 cells were maintained at 37 ºC and 28 ºC respectively in L-15 medium supplemented with 10% FBS. *Ae. aegypti* (white-eyed Higgs variant of the Rexville D strain) and *Ae. albopictus* (Galveston strain) were reared at 27ºC and 80% relative humidity under a 16h light: 8h dark photoperiod, as previously described (Vanlandingham et al., 2005b). Adults were kept in paper cartons supplied with 10% sucrose on cotton balls. To promote egg production, females were fed on anaesthetized hamsters once per week. All animal manipulations were performed in accordance with National Institutes of Health and UTMB humane laboratory animal use approved protocols. Viral titers from tissue culture supernatants were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml as previously described (Chapter 2; Higgs et al., 1997).

Oral infection of mosquitoes

Most of the studies of oral infectivity of CHIKV in *Ae. aegypti* and *Ae. albopictus* mosquitoes were performed using eGFP-expressing viruses. *Ae. aegypti* and *Ae. albopictus* were infected in an Arthropod Containment Level 3 insectary as described previously (McElroy et al., 2006; Vanlandingham et al., 2006). To estimate the Oral Infectious Dose 50% values (OID_{50}), frozen stocks of viruses were thawed at 37 ºC and four to five 10-fold serial dilutions of virus were made in L-15 medium followed by mixing the samples with an equal volume of defibrinated sheep blood. Each viral dilution was presented to 50 4-5-day post-eclosion *Ae. aegypti* or *Ae. albopictus* female

mosquitoes (starved for 24 h) using a Hemotek membrane feeding system (Discovery Workshops, Accrington, Lancashire, United Kingdom) fitted with a murine skin membrane. Mosquitoes were permitted to feed for 1 h, after which engorged mosquitoes [stage >3b (Pilitt and Jones, 1972)] were returned to the cages for maintenance.

At 7 days post-infection (dpi) mosquitoes were dissected and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one focus of eGFP-expressing cells was present in the midgut. To compare oral infectivity of non-eGFP-expressing viruses 16 to 24 mosquitoes from each viral dilution were collected at 7 dpi , individually triturated in 1ml of L-15 media and titrated as described (Vanlandingham et al., 2005b). A mosquito was considered infected if it contained more than 0.94 $Log_{10}TCID_{50}$ infection units (limit of detection). The experiments were performed once or twice for each virus. OID_{50} values and confidence intervals were calculated using PriProbit program (version 1.63). The SAS equivalent method was used to calculate the fiducial limits (confidence intervals), assuming normal function distribution and an "all or nothing" response parameter. The difference between two OID_{50} values was considered statistically significant if 95% fiducial limits did not overlap.

Phylogenetic analyses

The entire E2-6K-E1 genome regions (2772 nt.) of the selected CHIKV strains were kindly provided by Sara Volk or obtained from GenBank. Sequences were aligned with ClustalW method in Lasergene v7.0; gap introductions in the nucleotide alignment were refined using amino acid alignments to preserve codon homology. The phylogeny was produced using neighbor-joining and maximum parsimony methods available in the PAUP* v4.0b 10 package (Sinauer Associates, Inc., Sunderland, MA). Bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates to assess reliability of the grouping.

Purification of the CHIKV using stepped sucrose gradient

Sub-confluent monolayers (75-80%) of BHK-21 cells in two T-175 flasks were infected with different CHIKVs with mutations in E2 proteins at an MOI of 0.2-0.5. At 2 dpi. cell culture supernatant was clarified by centrifugation at 2,000 g for 10 min at 4 ºC.

Supernatant was placed on top of a 20% sucrose cushion made in 1x TNE buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA pH 7.5) and centrifugated at 26,000 rpm (rotor: Beckman SW28) for 4 h. at +4 ºC. The pellet was resuspended in 2ml of TNE buffer and layered on top of a 20%-60% stepped sucrose gradient in 19x89 mm Beckman centrifuge tubes. Tubes were centrifuged at 35,000 rpm (rotor: Beckman SW41) for 1 h. at 4 ºC. The virus band ~ 1ml was aspirated using a glass pipette, dissolved in 3ml of 1x TNE buffer and placed in Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Billerica, MA). Virus was concentrated by centrifugation at 3,200 g for 40 min at 4 ºC. The liquid phase was adjusted to 1 ml by 1x TNE and 200 µl aliquots of virus were stored at -80 until needed. To determine the quality of the purified virus, 15 µl of virus was mixed with 5 µl of 4xSDS loading buffer with β-MeEtOH (200mM Tris-HCl (pH 6.8), 20% β-MeEtOH 4% SDS, ~0.01% Bromphenol blue, 40%Glycerol), boiled for 5 min and separated in a10% SDS PAGE. The gel was stained with coomassie blue R250 (0.25% coomassie blue R250, 45% methanol, 10% glacial acetic acid). Protein concentration of purified virus stock was determined using the BCA protein assay kit (Pierce, Rockford, IL.) according to the manufacturer`s instructions.

Purification of brush border membrane (BBM) from midguts of *Ae. albopictus* and *Ae. aegypti* mosquitoes

BBM was isolated according to (Mourya et al., 1998) with minor modifications. From 300 to 800 4-5 days old *Ae. albopictus* or *Ae. aegypti* female mosquitoes were dissected under a dissecting microscope at 4 ºC in ice cold PBS. The midguts were transferred into 2ml of Buffer A (17mM Tris-HCl, 5mM EGTA, 300mM Mannitol, 1mM PMSF and 1xComplete Protease Inhibitor Cocktail (CPIC) (Roche Applied Science, Indianapolis, IN) pH 7.4). Midguts were homogenized with 50 strokes of a glass Dounce homogenizer. The total volume was adjusted to 4.5 ml with buffer A and solution was centrifugated at 800g for 5 min at 4 ºC. The supernatant was transferred into a new tube and 0.5 ml of 120mM $MgCl_2$ was added to the mix. The supernatant was incubated on ice for 20 min followed by centrifugation at 3,200g for 10 min at 4 ºC. The supernatant was transferred to a new tube, and the pellet was resuspended in 6 ml of buffer B (17mM Tris-HCl (pH 7.4), 5mM EGTA, 300mM Mannitol, 12 mM $MgCl_2$, 1mM PMSF, and 1x CPIC), followed by centrifugation at 3,200g for 10 min 4 ºC. Supernatants from first and

second centrifugations were combined and centrifugated at 30,000g for 1h at 4 ºC. The pellet was resuspended in buffer A in proportions of (membranes from 2 midguts)/1 µl of buffer A and stored at -80ºC until needed. Protein concentration of brush border membranes was determined using the BCA protein assay kit (Pierce, Rockford, IL.).

Membrane proteins from C6/36 cells were purified using a similar methodology. Cells were grown at 28 ºC in L-15 medium supplemented with 10% FBS. Confluent monolayers were washed with PBS, scraped from the cell culture flask and centrifugated at 800g for 5 min Cells were washed once with PBS, and once with buffer A. Cells were resuspended in 3 ml of buffer B and disrupted with 30 strokes of a glass Dounce homogenizer. Homogenate was centrifugated at 3,200g for 10 min at 4 ºC. The supernatant was transferred into new tube and 8 ml of buffer B was added. Supernatant was kept on ice for 20 min followed by centrifugation at 30,000g for 1 h at 4 ºC. The pellet was resuspended in ice cold buffer A and protein concentration was determined using the BCA protein assay kit.

Virus overload protein binding assay (VOPBA)

VOPBA was performed as described previously (Ludwig et al., 1996) with minor modifications. 15 µl (1µg/µl) of membrane proteins were mixed with 5 µl of 4x loading buffer without β-MeEtOH (200mM Tris-HCl (pH 6.8), 2% SDS, ~0.01% Bromphenol blue, 40%Glycerol), incubated on ice for 5 min and loaded onto 8 or 10% SDS-PAGE gel. Proteins were separated by SDS-PAGE, followed by transfer to nitrocellulose membrane. Membranes were incubated overnight in 5 ml of renaturalization buffer (1% BSA (Fisher Scientific), 5% low fat milk (Bio-Rad Laboratories, Hercules, CA) in PBS) at 4 ºC. Membranes were washed twice with 1% low fat milk in PBS, followed by incubation with 200 µg of purified CHIKV in 4 ml of 1% low fat milk in PBS for 1 h. at room temperature (RT). Membranes were washed three times in washing buffer (1% low fat milk, 0.05% Tween 20 in PBS), followed by incubation with anti-CHIKV hyperimmune mouse ascitic fluid (Powers et al., 2000) diluted 1/500 in washing buffer for 1 h at RT. Membranes were washed three times in washing buffer and incubated with secondary sheep anti-mouse immunoglobulin conjugated with horseradish peroxidase (Southern Biotech) diluted 1/5000 in washing buffer for 1 h at RT. Then membranes were washed once in washing buffer and three times in PBS. Virus binding was visualized on high-speed X-ray film (OMC; Eastman Kodak, Rochester, N.Y.) by using

an enhanced chemiluminescent substrate (Amersham) according to the manufacturer's recommendations.

CHIKV binding to the total BBM membranes of *Ae. albopictus*

Binding experiments were performed as described previously (Houk et al., 1990). The sucrose gradient purified CHIKV ic. and LR-226V-(E2-211I) viruses were dissolved to 0.1µg/µl in biding buffer (L-15 medium supplemented with 0.75% BSA) and incubated at 4ºC for 5 min followed by centrifugation at 21,000g for 20min at +4 ºC. The supernatant was transferred to a new tube and used in all experiments. The BBM of *Ae. albopictus* was added to 100 µl of CHIKV in binding buffer to a final concentration 0.1, 0.01 and 0 µg/µl, incubated at 4 ºC for 30 min followed by centrifugation at 21,000g for 20 min at 4 ºC. Pellets were washed with 300 µl of binding buffer followed by centrifugation at 21,000g for 5 min at 4 ºC. Pellets were resuspended in 1xSDS-PAGE loading buffer. As a positive control sample, 15 µl of CHIKV was collected before binding to BBM and was mixed with 5 µl of 4xSDS-PAGE loading buffer. This corresponds to 15% of total virus used in analysis. Samples were separated by 10% SDS-PAGE followed by western blot analysis using CHIKV specific antibodies (Powers et al., 2000).

Binding competition experiments

Viruses LR-ApaI-226V and LR-226V-(E2-211I) were mixed 1:1, incubated at 4 ºC for 5 min followed by centrifugation at 21,000g for 20min at 4 ºC. The supernatant was transferred to a new tube and viruses were dissolved to final concentrations of 6.5, 5.5 and 4.5 Log_{10}pfu/ml. 100 µl of dissolved viruses were incubated with 0.1 µg/µl of BBM in binding buffer for 30min at 4 ºC followed by centrifugation at 21,000g for 20min at 4 ºC. Pellets were washed once in binding buffer and resuspended in TRIzol reagent, followed by RNA extraction and RT-PCR analysis as described in Chapter 3.

To analyze CHIKV binding to midguts of *Ae. albopictus,* a blood meal containing 7.5 $Log_{10}TCID_{50}$/ml of a 1:1 mix of LR-ApaI-226V and LR-226V-(E2-211I) viruses was orally presented to *Ae. albopictus*. 15 min after engorgement mosquito midguts were dissected and the engorged blood meal was removed by three consecutive washes in L-15 medium supplemented with 10%FBS. Midguts were lysed in TRIzol reagent, followed by RT-PCR analysis as described in Chapter 3.

Transmission electron microscopy (TEM)

The 41855/LR-GFP-226V and 41855/LR-GFP-226V-(E2-60D/162A) viruses were grown in BHK-21 cells and concentrated to 8.95 $Log_{10}TCID_{50}$/ml by centrifugation through a 20% sucrose cushion. Viruses were resuspended in L-15 medium, mixed with an equal volume of defibrinated blood and orally presented to *Ae. albopictus* mosquitoes. At 0.5 and 1.5 hpi. midguts were dissected and the blood meal was removed by three consecutive washes in PBS, then midguts were fixed in TEM fixative solution comprised of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, 0.03% $CaCl_2$ in 0.05 M cacodylate buffer (pH 7.3). Tissues were stored at 4˚C for no longer than 1 month in the fixative prior to resin embedding. Post-fixation was carried out in 1% OsO_4 in 0.1M cacodylate buffer for 1 hour at room temperature, followed by *en bloc* staining in 1% uranyl acetate in 0.1M maleate buffer for 20 min at 60˚C. Mosquito tissues were dehydrated in an ethanol series: 75% → 95% → 100% →propylene oxide (Polysciences Inc., Warrington, PA) and embedded in PolyBed 812 (Polysciences). For optimal infiltration, tissues were incubated for at least 1 hour in 25%, then 75%, then 100% PolyBed 812 mixed with propidium oxide.

Semi-thin (5 μm) sections were cut on a Reichert-Leica Ultracut S ultramicrotome, and stained with toluidine blue for observation by light microscopy. Light microscopic observations allowed for tissue orientation and gross morphological characterization. Following light microscope analysis, ultra-thin sections (70nm) were cut and placed on 2-3, 150 mesh, 3 mm coated or uncoated copper grids (Electron Microscopy Sciences, Fort Washington, PA), stained with 2% aqueous uranyl acetate and 0.4% Reynolds lead citrate, and examined using FEI CM-100 electron microscope at 60 kV.

Photographs were taken on the electron microscope using 3 ¼" x 4" Kodak 4489 microfine grain film. Negatives were enlarged using a Durst Phototechnik Laborator 1200 (Brixen, Italy) enlarger onto Kodak Professional Plycontrast IV RC black and white photographic paper (Eastman Kodak Company, Rochester, NY). Prints were processed using a Mohr Pro8 processor (Prospect Heights, Illinois). Photographs were scanned and turned into digital files (.TIFF) for labeling using Adobe Photoshop version 6.0.

RESULTS

Introduction of the E1-A226V mutation into the backbone of the Ag41855 strain of CHIKV does not lead to a significant increase in infectivity for *Ae. albopictus* mosquitoes

Previously we demonstrated that the introduction of the single amino acid substitution E1-A226V significantly increases CHIKV infectivity for midguts of *Ae. albopictus* mosquitoes (Chapter 3; Tsetsarkin et al., 2007). To further investigate the effect of this mutation on infectivity of different strains of CHIKV for *Ae. albopictus,* the E1-A226V mutation was introduced into an eGFP expressing i.c. of the Ag41855 strain of CHIKV (designated as p41855-GFP-226A). The Ag41855 strain of CHIKV was chosen because of its close phylogenetic relationship to the strains implicated in the 2006-2007 epidemics, therefore representing an interesting model for studying the evolutionary events that preceded these epidemics. The resultant clone was designated p41855-GFP-226V (Figure 6.1). The specific infectivity values after electroporation of the RNAs produced from p41855-GFP-226A and p41855-GFP-226V were similar - approximately 10^6 pfu/μg of RNA (Table 6.2), with no detectable differences in plaque sizes. Both constructs replicated to similar viral titers following *in vitro* transcribed RNA transfection into BHK-21 cells at 24 and 48 hours post-electroporation (hpe), indicating that introduction of the E1-A226V mutation into the Ag41855 strain does not attenuate this virus in BHK-21 cells (Table 6.2).

The relative infectivities of 41855-GFP-226V and 41855-GFP-226A viruses in *Ae. albopictus* mosquitoes were determined by oral exposure to serial 10-fold viral dilutions. In two independent experiments, the oral infectivities of 41855-GFP-226V and 41855-GFP-E1-226A viruses were not significantly different ($p>0.05$). This demonstrates that for the Ag41855 strain, the E1-A226V mutation does not affect *Ae. albopictus* midgut infectivity (Figure 6.2; Table 6.3). The mean OID_{50} values for 41855-GFP-226V and 41855-GFP-226A were 6.33 and 6.88 $Log_{10}TCID_{50}$/ml respectively (Table 6.3), which

Backbone	Clone name	E1 226 [a]	E2 [b]			Specific infectivity [c]	Titers [d]		Plaque size±SD [e]
			60	162	211		24h	48h	
41855-GFP	41855-GFP-226V	V	G	V	I	$5.0x10^5$	8.95	8.52	3.10 ±0.29
	NG		D	V	I	$5.0x10^5$	7.95	7.21	3.00±0.82
	NG		G	A	I	$4.0x10^5$	6.95	7.71	3.17±0.24
	NG		G	V	T	$8.0x10^5$	7.95	7.95	3.41±0.41
	NG		G	A	T	$4.5x10^5$	ND	8.52	3.33±0.24
	NG		D	V	T	$9.5x10^5$	7.95	7.52	2.83±0.47
	NG		D	A	I	$7.5x10^5$	7.95	7.95	2.83±0.47
	41855/LR-GFP-226V		D	A	T	$10x10^5$	7.95	7.95	3.17±0.24
	41855-GFP-226A	A	G	V	I	$10x10^5$	8.52	7.95	3.33±0.29
	NG		D	V	I	$10x10^5$	ND	7.71	3.00±0.82
	NG		G	A	I	$10x10^5$	7.95	7.52	2.83±0.24
	NG		G	V	T	$8x10^5$	ND	7.95	3.00±0.82
	NG		G	A	T	$10x10^5$	8.52	7.95	3.00±0.82
	NG		D	V	T	$10x10^5$	7.95	7.95	3.00±0.41
	NG		D	A	I	$8.0x10^5$	8,52	7.52	3.33±0.24
	41855/LR-GFP-226A		D	A	T	$6.8x10^5$	ND	7.95	3.10±0.29

Table 6.2 Recovery of the viruses with mutations in the E2 protein after electroporation of *in vitro* transcribed RNA.

a – amino acids at position of E1-226.
b – amino acids at position of E2: 60, 162, 211.
c – Specific infectivity of *in vitro* transcribed RNA.
d – Virus titers at 24 and 48 hpe. expressed as $Log_{10}TCID_{50}$/ml.
e – Plaque size of infectious centers expressed in millimeters ± standard deviation.
h – hours post-electroporation.
NG – the contract name in not given.Blue color corresponds to authentic amino acid at indicated position of strain Ag41855, red color corresponds to authentic amino acid at indicated position of strain LR2006 OPY1.

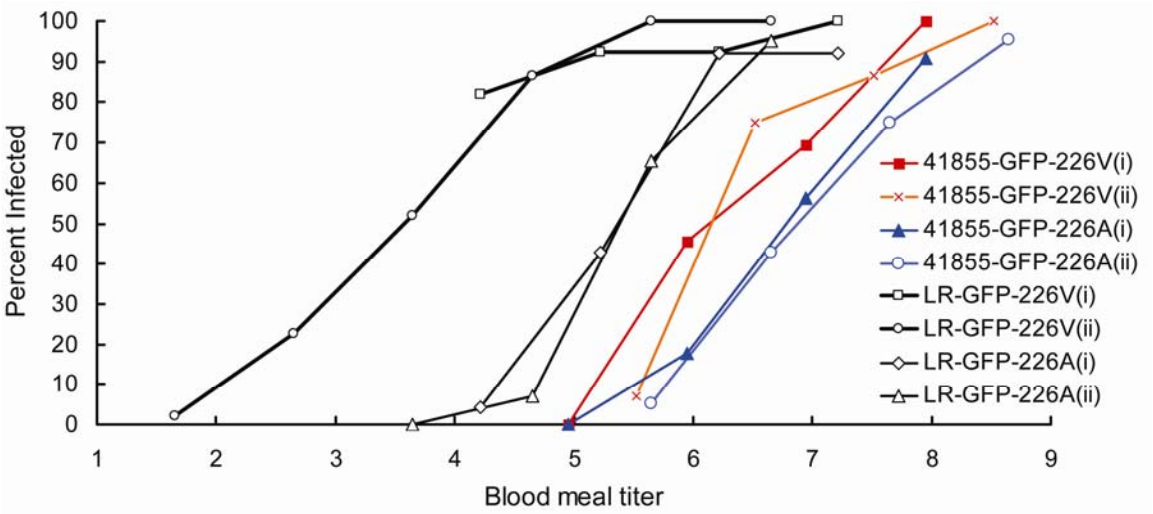


Figure 6.2 Effect of the E1-A226V mutation on infectivity of Ag41855 and LR2006 OPY1 strains of CHIKV to *Ae. albopictus* mosquitoes.

Serial ten-fold dilutions of eGFP expressing CHIKV were made to produce infectious blood meals. The experiments were performed twice for each virus (i and ii).

Backbone	Exp	E1 226	N m	OID_{50} (C_{95})	OID_{50} mean	p
41855-GFP	1	V	67	6.27 (5.82-6.61)	6.33	p>0.05
	2		54	6.38 (5.94-6.74)		
	1	A	78	6.96 (6.57-7.31)	6.88	
	2		53	6.79 (6.40-7.32)		
LR-GFP	Comb	V	261	NG	3.52	p<0.01
	Comb	A	194	NG	5.45	
37997-GFP	Comb	V	260	NG	3.16	p<0.01
	Comb	A	274	NG	5.10	

Table 6.3 Effect of the E1-A226V mutation on infectivity of different strains of CHIKV to *Ae. albopictus* mosquitoes.

Exp – experiment number.
E1-226 – amino acid at position E1-226.
N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
OID_{50} values and confidence intervals were calculated using PriProbit (Version 1.63).
p – comparison of statistical significance of difference in OID_{50} values between viruses with E1-226A and E1-226V mutations.
Comb – combined summary of two independent experiments (Chapter 3).
NG – value is not given.

was significantly higher compared to OID_{50} values of the previously characterized CHIKV strains LR2006 OPY1 and 37997 with either alanine or valine at position E1-226 (Tsetsarkin et al., 2007), suggesting that the Ag41855 strain is significantly more attenuated for *Ae. albopictus* infection when compared with the LR2006 OPY1 and 37997 strains.

The sequence of 41855-GFP-226A was identical to that of the virus stock used for i.c. construction and we previously showed that introduction of the eGFP gene into the backbones of LR2006 OPY1 and 37997 strains of CHIKV does not significantly affect infectivity for *Ae. albopictus* and *Ae. aegypti* mosquitoes (Chater 2; Chater 3; Tsetsarkin et al., 2006; Vanlandingham et al., 2005b). Nevertheless, we wanted to determine whether or not the attenuation observed for 41855-GFP-226V and 41855-GFP-226A in *Ae. albopictus* could reflect artifacts of the i.c. construction, for example introduction of the eGFP gene under control of an additional sub-genomic promoter or incompatibility of viral segments that were combined in the clone-derived virus but that coexist separately as quasispecies in the viral population. We compared infectivity of the stock Ag41855 virus with that of the viruses produced from the Ag41855 and p41855-GFP-226A clones (Table 6.4; Figure 6.1). The OID_{50} values for these viruses were not significantly different ($p>0.1$) indicating that virus produced from the full-length and eGFP-expressing i.c. retained the infection phenotype of the parental virus in *Ae. albopictus*. This also indicated that the lower infectivity observed for 41855-GFP-226A and 41855-GFP-226V viruses for *Ae. albopictus,* as compared with viruses produced from the i.c. of the LR2006 OPY1 and 37997 strains, was attributed to the specific mutation(s) in the genome of Ag41855 strain.

Virus	N m	OID_{50} (mean)	C_{95}	p
Ag41855	67	6.74	6.39-7.17	p>0.1
Ag41855 i.c.	78	6.40	6.06-6.85	
41855-GFP-226A	131	6.88	NG	

Table 6.4 Comparison of oral infectivity of the parental virus and viruses produced from full-length and eGFP expressing i.c. clone of Ag41855 strain in *Ae. albopictus* mosquitoes.

N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
NG – value is not given.

Sequence comparison of Ag41855, LR2006 OPY1 and 37997 strains

	Protein	Ag41855	LR2006 OPY1	37997	Polyprotein
nsP1	326	M	V	M	326 [a]
	391	L	F	L	391 [a]
	488	Q	R	K	488 [a]
nsP2	54	S	N	S	589 [a]
	793	A	V	A	1328 [a]
nsP3	31	D	G	D	1364 [a]
	217	Y	H	Y	1550 [a]
	328	**P**	**Q**	**Q**	1661 [a]
	337	T	I	T	1670 [a]
	358	**P**	**S**	**S**	1691 [a]
	435	R	C	H	1768 [a]
	438	A	V	V	1771 [a]
	449	T	M	A	1782 [a]
	461	**L**	**P**	**P**	1794 [a]
	471	P	S	P	1804 [a]
	524	Stop	R	Stop	1857 [a]
nsP4	75	T	A	T	1938 [a]
	254	T	A	T	2117 [a]
E2	60	**G**	**D**	**D**	385 [b]
	162	**V**	**A**	**A**	487 [b]
	211	**I**	**T**	**T**	536 [b]
	312	T	M	T	637 [b]
	318	M	V	T	643 [b]
	375	S	T	S	700 [b]
	377	V	I	V	702 [b]
	386	V	A	V	711 [b]
6K	8	V	I	A	756 [b]
E1	19	**I**	**V**	**V**	828 [b]
	226	A	V	A	1035 [b]
	284	D	E	D	1093 [b]
	377	**T**	**A**	**A**	1186 [b]

Table 6.5 Genetic difference of Ag41855 and LR2006 OPY1 strains of CHIKV.

a – position in the non-structural polypeptide
b – position in the structural polypeptide
Blue and red colors correspond to amino acids which are the same in LR2006 OPY1 and 37997 strains (Red) but different in Ag41855 strain (Blue)

Several recent phylogenetic analyses of CHIKV have grouped the Ag41855 and LR2006 OPY1 strains into the East/Central/ South African phylogroup (Arankalle et al., 2007; Cherian et al., 2009) suggesting a close evolutionary relationship between them. The 37997 strain is a member of the West African phylogroup, which is the outlier among CHIKV strains. Strain 37997 was therefore used as a reference control to identify positions in the Ag41855 genome responsible for poor infectivity in *Ae. albopictus,* and to determine the sites affecting sensitivity to the E1-A226V mutation in *Ae. albopictus*.

Sequence comparison of the Ag41855 and LR2006 OPY1 strains revealed a total of 202 nucleotide differences (1.7%), encoding 31 amino acid substitutions: 18 in the non-structural and 13 in the structural coding sequence (Table 6.5). The nucleotide sequence of 37997 differed from Ag41855 and LR2006 OPY1 by 14.7%.

A comparison of amino acid sequences for strains Ag41855, LR2006 OPY1 and 37997 (Table 6.5) identified eight positions that are unique in Ag41855, but are the same in both the LR2006 OPY1 and 37997 strains: three in the nsP3 protein (positions 328, 358, and 461), three in the E2 protein (positions 60, 162, and 211) and two in E1 (positions 19 and 377). These data suggest that the unique Ag41855 amino acids could modulate CHIKV infectivity for *Ae. albopictus*. Since numerous previous studies identified the E2 protein as a major determinant of mosquito infectivity for different alphaviruses including Sindbis virus (SINV) and VEEV (Brault et al., 2004b; Brault et al., 2002; Myles et al., 2003; Pierro et al., 2007; Pierro et al., 2008) we first decided to investigate if the mutations at E2 positions 60, 162, and 211 were responsible for the observed attenuation of the Ag41855 strain, and how these mutations related to the insensitivity of this strain to the E1-A226V mutation in *Ae. albopictus*.

Determinants of attenuation of the Ag41855 strain in *Ae. albopictus* mosquitoes

To elucidate genetic determinants of low mosquito infectivity of strain Ag41855, the fragment of 8021-9225 nt. (which corresponds to 152-553 aa. in the structural polypeptide) from the LR2006 OPY1 i.c. containing the E2 60D, 162A, and 211T mutations, was introduced into p41855-GFP-226V (Figure 6.1). Based on specific infectivity and replication data, the chimeric virus 41855/LR-GFP-226V was not attenuated in BHK-21 cells (Table 6.2), indicating that this genome region is interchangeable between the LR2006 OPY1 and Ag41855 strains. The 41855/LR-GFP-

Back bone	Clone name	E1 226	E2			Exp	N m	OID_{50} (C_{95})	OID_{50} mean
			60	162	211				
41855 -GFP	41855-GFP-226V	V	G	V	I	Comb	121	NG	6.33
	NG		D	V	I	1	83	5.51 (5.13-6.13)	5.51
	NG		G	A	I	1	99	6.85 (6.18-9.64)	6.85
	NG		G	V	T	1	125	5.40 (4.94-5.77)	5.40
	NG		G	A	T	1	83	5.57 (5.27-5.83)	5.57
	NG		D	V	T	1	153	3.36 (3.01-3.60)	3.50
						2	105	3.64 (3.31-3.85)	
	NG		D	A	I	1	115	5.52 (5.27-5.80)	5.52
	41855/LR-GFP-226V		D	A	T	1	107	3.78 (2.91-4.08)	3.65
						2	102	3.52 (3.19-3.80)	
	41855-GFP-226A	A	G	V	I	Comb	131	NG	6.88
	NG		D	V	I	1	133	5.69 (5.42-5.92)	5.74
						2	79	5.79 (5.48-6.09)	
	NG		G	A	I	1	75	6.71 (6.44-6.99)	6.71
	NG		G	V	T	1	123	6.51 (6.24-6.78)	6.77
						2	97	7.03 (6.74-7.43)	
	NG		G	A	T	1	98	6.97 (6.68-6.31)	6.97
	NG		D	V	T	1	82	5.48 (5.12-5.79)	5.48
	NG		D	A	I	1	75	5.65 (5.34-5.95)	5.65
	41855/LR-GFP-226A		D	A	T	1	63	5.21 (4.89-5.55)	5.26
						2	134	5.31 (4.96-5.60)	

Table 6.6 Effect of mutations in E2 proteins on infectivity of Ag41855 strain of CHIKV for *Ae. albopictus* mosquitoes.

Exp – experiment number.
N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
Comb – combined summary of two independent experiments.
NG – value is not given.
Blue color corresponds to authentic amino acid at indicated position of strain Ag41855, red color corresponds to authentic amino acid at indicated position of strain LR2006 OPY1.

226V virus was ~1000 times more infectious for *Ae. albopictus* as compared to Ag41855-GFP-226V virus (OID_{50}=3.78) (Table 6.6). The reciprocal chimera LR/41855-GFP-226V containing the 8021-9225 nt fragment of Ag41855 in the backbone of LR-GFP-226V, demonstrated an OID_{50} value similar to that observed for Ag41855-GFP-226V (OID_{50}=6.33), indicating that this region encodes the major determinant(s) for *Ae. albopictus* midgut infectivity in the Ag41855 strain (Table 6.7). Additionally, these data indicate that mutations in the nsP3 and E1 genes of Ag41855 (Table 6.5) probably do not affect the *Ae. albopictus* mosquito infectivity phenotype. They were therefore excluded from further analysis.

Back bone	Clone name	E1 226	E2			Exp	N m	OID_{50} (C_{95})	OID_{50} mean
			60	162	211				
LR-GFP	LR/41855-226V	V	G	V	I	1	135	6.40 (5.98-7.28)	6.40
	NG		G	A	T	1	41	5.38 (5.00-5.91)	5.38
	NG		D	A	I	1	120	5.24 (4.92-5.54)	5.24
	NG		D	V	I	1	107	5.52 (5.27-5.80)	5.52
	NG		G	A	I	1	77	6.33 (5.97-6.92)	6.33
	LR-GFP-226V		D	A	T	Comb	261	NG	3.52

Table 6.7 Effect of mutations in E2 protein on infectivity of LR2006 OPY1 strain of CHIKV for *Ae. albopictus* mosquitoes.

Exp – experiment number.
N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
Comb – combined summary of two independent experiments.
NG – value is not given.
Blue color corresponds to authentic amino acid at indicated position of strain Ag41855, red color corresponds to authentic amino acid at indicated position of strain LR2006 OPY1.

To further investigate the effect of the 8021-9225 region on CHIKV mosquito infectivity in the background of alanine at E1-226, the 8021-9225 fragment from LR2006 OPY1 was introduced into the 41855-GFP-226A virus (Figure 6.1). The OID_{50} for the resultant chimeric virus 41855/LR-GFP-226A was 5.21 $Log_{10}TCID_{50}$/ml (Table 6.6) - not

significantly different to the OID_{50} values for LR-GFP-226A virus (Tsetsarkin et al., 2007). Importantly, introduction of the 8021-9225 genome fragment of LR2006 OPY1 strain into the background of Ag41855 completely restored the enhancing effect of the E1-A226V mutation on infectivity for *Ae. albopictus* to the levels reported for genetic backgrounds of strains LR2006 OPY1 and 37997 (Chapter 3; Tsetsarkin et al., 2007).

Individual roles of the mutations at positions E2 60, 162, and 211 on CHIKV infectivity for *Ae. albopictus* mosquitoes

To further characterize the roles of each of the E2 mutations on CHIKV infectivity for *Ae. albopictus,* point mutations encoding amino acids from the LR2006 OPY1 strain were introduced into the backbone of 41855-GFP-226V and 41855-GFP-226A i.c., either individually or in combination (Table 6.6; Figure 6.1). Specific infectivity of *in vitro* transcribed RNA, plaque size and viral titers produced at 1 and 2 dpe were determined for each of the constructs (Table 6.2). All of the constructs were indistinguishable for these parameters, indicating that these mutations do not cause intermolecular incompatibility with the rest of the viral genome and that the resultant viruses are suitable for testing in *Ae. albopictus*.

Individual introduction of the mutations E2-G60D and E2-I211T into 41855-GFP-226V (viruses 41855-GFP-226V-(E2-60) and 41855-GFP-226V-(E2-211T) respectively) was responsible for a significant increase of viral infectivity for *Ae. albopictus* to similar levels (OID_{50} values: 5.51 and 5.40 respectively). The OID_{50} of 41855-GFP-226V with the E2-V162A mutation [41855-GFP-226V-(E2-162A)] was not significantly different from the OID_{50} for the 41855-GFP-226V virus (Table 6.6). This suggests that E2-V162A likely plays no role in CHIKV infectivity for *Ae. albopictus*. Interestingly, introduction of each of the three mutations individually did not lead to an increase of viral infectivity to the level observed for the triple mutant 41855/LR-GFP-226V, indicating that combinations of at least two mutations are apparently required for the high infectivity phenotype.

Analysis of *Ae. albopictus* midgut infectivity for 41855-GFP-226V, in which two substitutions were introduced into the E2 protein, revealed that a combination of the G60D and I211T mutations is necessary and sufficient to completely restore infectivity of the Ag41855 strain to the same levels as those observed for 41855/LR-GFP-226V and LR-GFP-226V viruses ($p>0.1$) (Table 6.6). The *Ae. albopictus* infectivity of viruses

where the E2-V162A mutation was combined with either the E2-G60D or E2-I211T mutations [viruses: 41855-GFP-226V-(E2-60D/162A) and 41855-GFP-226V-(E2-162A/211T)], was indistinguishable from the infectivity of the 41855-GFP-226V-(E2-60) and 41855-GFP-226V-(E2-211T) viruses (that contained the single mutations in E2-G60D and E2-I211T). This observation further supports the conclusion that position E2-162 does not affect CHIKV infectivity for *Ae. albopictus.* Altogether, these results indicate that there is a strong synergistic effect of the E2-G60D and E2-I211T mutations on CHIKV infectivity for *Ae. albopictus,* when expressed in combination with valine at position E1-226.

To further evaluate the relationships between these different mutations, four additional viruses were constructed in which single and double substitutions at positions E2-60, E2-161 and E2-211 from the Ag41855 strain were substituted into the backbone of LR-GFP-226V (Table 6.7). The OID_{50} values for LR-GFP-226V with individual mutations E2-D60G and E2-T211I (viruses: LR-GFP-226V-(E2-60G) and LR-GFP-226V-(E2-211I)) were indistinguishable as compared to OID_{50} values of the 41855-GFP-226V virus with E2-G60D and E2-I211T mutations expressed individually or in combination with E2-V162A, but were significantly higher as compared with LR-GFP-226V. The OID_{50} value for LR-GFP-226V with both the E2-D60G and E2-T211I mutations [LR-GFP-226V-(E2-60G/211I)] was indistinguishable from the OID_{50} values of 41855-GFP-226V or chimeric virus LR/41855-GFP-226V ($p>0.1$). These data suggest that the specific phenotype(s) associated with the E2 mutations introduced into strain Ag41855 would be retained if these mutations were expressed in other CHIKV strains.

The genome region of the LR2006 OPY1 strain that contained mutations at positions E2-60, E2-162 and E2-211 was also responsible for a significant increase in *Ae. albopictus* midgut infectivity for the 41855-GFP-226A virus. In this regard it was important to investigate the individual roles of these particular mutations in the 41855-GFP-226A virus, and to determine how these roles correlated with the effects of these mutations in the 41855-GFP-226V virus. In contrast to the 41855-GFP-226V, the introduction of the single mutation E2-G60D into 41855-GFP-226A [virus 41855-GFP-226A-(E2-60D)] almost completely restored viral infectivity phenotype to the relatively high levels observed for 41855/LR-GFP-226A and LR-GFP-226A viruses ($p>0.1$) (Table 6.6). However, the E2-I211T mutation led to no apparent effect on CHIKV infectivity for

Ae. albopictus. Analysis of the viruses bearing double mutants in the E2 protein revealed a similar result: viruses containing E2-G60D [41855-GFP-226A-(E2-60D/162A) and 41855-GFP-226V-(E2-60D/211T)] were significantly more infectious for *Ae. albopictus* than the 41855-GFP-226A virus regardless of the second mutations at positions E2-162 and E2-211. The combination of E2-A162V and E2-I211T did not affect the viral infectivity phenotype as compared to 41855-GFP-226A. In the backbone of 41855-GFP-226A, the E2-G60D and E2-I211T mutations had a disproportionate effect on the CHIKV mosquito infectivity phenotype; where E2-G60D exerted the major effect whilst E2-I211T was responsible for only a marginal effect.

CHIKV strain Ag41855 is attenuated in *Ae. aegypti* mosquitoes

Prior to the 2006-2007 outbreaks, *Ae. aegypti* was the principal vector responsible for most urban epidemics of chikungunya (Jupp and McIntosh, 1988). Previously we showed that, in contrast to the situation for *Ae. albopictus* mosquitoes, the E1-A226V mutation does not increase infectivity for *Ae. aegypti* when expressed in the backbones of the LR2006 OPY1 and 37997 strains of CHIKV (Chapter 3; Tsetsarkin et al., 2007). Therefore, the unusual phenotype of the Ag41855 strain in *Ae. albopictus* mosquitoes led us to investigate the mosquito infectivity phenotype of this strain in *Ae. aegypti*.

Backbone	Exp	E1 226	N m	OID_{50} (C_{95})	OID_{50} mean	p
41855-GFP	1	V	112	7.63 (7.35-8.23)	7.71	$p_1<0.05$
	2		82	7.78 (7.46-8.21)		$p_2>0.05$
	1	A	77	6.92 (6.61-7.24)	7.12	
	2		90	7.24 (6.85-7.66)		
LR-GFP	Comb	V	172	NG	6.52	$p_1<0.05$
	Comb	A	156	NG	5.87	$p_2>0.05$
37997-GFP	Comb	V	262	NG	6.47	$p_1<0.01$
	Comb	A	297	NG	5.70	$p_2>0.05$

Table 6.8 Effect of the E1-A226V mutation on infectivity of different strains of CHIKV to *Ae. aegypti* mosquitoes.

Exp – experiment number.
E1-226 – amino acid at position E1-226.
N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
OID_{50} values and confidence intervals were calculated using PriProbit (Version 1.63).
p1 – comparison of statistical significance of difference in OID_{50} values between viruses with E1-226A and E1-226V mutations for experiment 1. p2 – comparison of statistical significance of difference in OID_{50} values between viruses with E1-226A and E1-226V mutations for experiment 2.
Comb – combined summary of two independent experiments (Chapter 3)
NG – value is not given.

The OID_{50} values of 41855-GFP-226A in *Ae. aegypti* were 6.92 and 7.24 $Log_{10}TCID_{50}$/ml, which are significantly higher than the OID_{50} values determined previously for LR-GFP-226A and 37997-GFP-226A viruses ($p<0.01$) (Table 6.8). Mosquito infectivity of the 41855-GFP-226V was significantly lower than that of the LR-GFP-226V and 37997-GFP-226V viruses, indicating that the Ag41855 strain of CHIKV is also attenuated in its ability to infect *Ae. aegypti.* Interestingly, 41855-GFP-226A was slightly more infectious for *Ae. aegypti* than 41855-GFP-226V ($p_1<0.05$ for the first experiment and $p_2>0.05$ for the second). This finding corroborated our previous results showing that CHIKV with the E1-226A residue is slightly more infectious for *Ae. aegypti* mosquitoes (Chapter 3; Tsetsarkin et al., 2007).

Determinants of attenuation of the Ag41855 strain in *Ae. aegypti* mosquitoes

To determine which genomic regions of Ag41855 are responsible for attenuation of Ag41855 in *Ae. aegypti,* the chimeric viruses 41855/LR-GFP-226A and 41855/LR-GFP-226V were tested as described above. These viruses were significantly more infectious for *Ae. aegypti* than 41855-GFP-226A and 41855-GFP-226V viruses ($p<0.01$), with OID_{50} values similar to those of CHIKV possessing either an alanine or a valine at position E1-226 when expressed in the backbone of strains LR2006 OPY1 and 37997 ($p>0.1$) (Table 6.9). These data indicate that the 8021-9225 genome region of Ag41855 contains the major determinant of CHIKV attenuation in *Ae. aegypti.* Further analysis of 41855-GFP-226A and 41855-GFP-226V with different combinations of the single or double mutants at positions E2 60, 162, and 211 revealed that the introduction of the single mutation (E2-G60D) was sufficient to increase viral infectivity of the Ag41855 strain for *Ae. aegypti* to the OID_{50} values attributed to CHIKV strains LR2006 OPY1 and 37997 with either alanine or valine at position E1-226 (Table 6.8; 6.9). Expression of the E2-60D mutation individually or in combination with E2-V162A or E2-I211T [viruses: 41855-GFP-226A-(E2-60D), 41855-GFP-226A-(E2-60D/162A) and 41855-GFP-226A-(E2-60D/E2T)] led to a significant decrease in OID_{50} values as compared with the values determined for the 41855-GFP-226A and 41855-GFP-226V viruses ($p<0.01$). In contrast, introduction of the E2-A162V and E2-I211T mutations individually or in combination [viruses 41855-GFP-226A-(E2-162A), 41855-GFP-226A-(E2-211T) and 41855-GFP-226A-(E2-162A/211T)], resulted in viruses almost indistinguishable from 41855-GFP-

226A and 41855-GFP-226V with respect to their ability to infect *Ae. aegypti* midguts, indicating that these two positions do not play important roles in CHIKV transmitted by *Ae. aegypti*.

Backbone	Clone name	E1	E2			N m	OID_{50}	C_{95}
		226	60	162	211			
41855-GFP	41855-GFP-226V	V	G	V	I	194[a]	7.71[b]	NG
	NG		D	V	I	105	6.18	5.89-6.64
	NG		G	A	I	61	>7.30	ND
	NG		G	V	T	67	>7.52	ND
	NG		G	A	T	73	>7.31	ND
	NG		D	V	T	52	6.31	5.78-7.14
	NG		D	A	I	81	6.42	6.12-6.85
	41855/LR-GFP-226V		D	A	T	114	6.09	5.81-6.43
	41855-GFP-226A	A	G	V	I	167[a]	7.12[b]	NG
	NG		D	V	I	83	6.13	5.83-6.46
	NG		G	A	I	103	>7.52	ND
	NG		G	V	T	50	7.30	7.02-7.96
	NG		G	A	T	73	>7.52	ND
	NG		D	V	T	82	6.20	5.97-6.44
	NG		D	A	I	86	6.27	6.04-6.53
	41855/LR-GFP-226A		D	A	T	93	6.23	5.93-6.52

Table 6.9 Effect of mutations in E2 proteins on CHIKV infectivity for *Ae. aegypti* mosquitoes.

N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} (C_{95}) – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
a – sum of two independent experiments.
b – average of two independent experiments.
NG – value is not given.
ND – value is not determined.
Blue color corresponds to authentic amino acid at indicated position of strain Ag41855, red color corresponds to authentic amino acid at indicated position of strain LR2006 OPY1.

Distribution of the amino acids at E2-60 and E2-211 among characterized CHIKV isolates

Name in phylogeny	Strain Name	Genotype	Location of isolation	Year of isolation	Source of isolate	Passage history	E2-60	E2-211
CAR80v1	DakAr B 16878	ECSA	Central African Region (*Bouboui*)	1980	*An. funestus*	SM-5; Vero-2; C6/36-1	D	I
CARb86v10	CAR256	ECSA	Central African Region	(before 1986)	?	SM-1; (Yaru); C6/36-1	D	I
DRC60v29	LSF5	ECSA	Democratic Republic of Congo	1960?	human	SM1; Vero1; C6/36-1	D	I
LR2006 OPY1	LR2006 OPY1	ECSA	France (*imported from La Reunion*)	2006 (17 Feb)	human	Vero-5; SM-1; C6/36-1	D	T
GAB 07	Gabon 2007	ECSA	Gabon	2007	?	?	D	T
GERb07	Wuerzburg-1	ECSA	Germany (*imported from Mauritius*)	(before Sept 2007)	human	?	D	T
INDI00	IND-00-MH4	ECSA	India	2000	mosquito	C6/36-3; SM-3	D	I
INDI06ap	IND-06-AP3	Asian	India	2006 early	human	C6/36-1	D	T
INDI06ka	IND-06-KA15	Asian	India	2006 early	human	C6/36-2	D	T
INDI06rj	IND-06-RJ1	Asian	India	2006 late	human	C6/36-2	D	T
INDI63	IND-63-WB1	Asian	India	1963	human	SM-6; C6/36-1	D	T
INDI73	IND-73-MH5	Asian	India	1973	human	SM-1; C6/36-1	D	T
INDO85v15	RSU1 (TVP1336)	Asian	Indonesia	1985	human	Vero-2; C6/36-1	D	T
ITA07	ITA07-RA1	ECSA	Italy (*Ravenna*)	2007	?	?	D	T
MAU06	D570/06	ECSA	Mauritius	2006 (March)	human	Vero-1	D	T
NIG64v3	IbH35	W. Afr.	Nigeria (*Ibadan*)	1964	human	SM?; Vero-1; C6/36-1	D	T
NIG65v26	IbAn4824	W. Afr	Nigeria	1965 (13 Apr)	sentinel mouse brain	(NH) SM2; C6/36-1	G	T
PHI85v18	Hu/85/NR/001	Asian	Philippines	1985	human	Vero-2	D	T
REU05csf	06-027	ECSA	La Reunion	2005 (29 Nov)	human	C6/36-1 to 2	D	T
REU05dec	06-049	ECSA	La Reunion (*South, St. Louis*)	2005 (2 Dec)	human	C6/36-1 to 2	D	T

REU05may	05-115	ECSA	La Reunion	2005 (6 May)	human	C6/36-1 to 2	D	T
REU05nov	06-021	ECSA	La Reunion (*South, La Riviere St Louis*)	2005 (28 Nov)	human	C6/36-1 to 2	D	T
SAF56v9	Verseenigin g	ECSA	South African Republic	1956 (April)	human	SM-9; Vero-1; C6/36-1	G	I
SAF76v13	SAH2123	ECSA	South African Republic	1976	human	Mosquito-1; SM-2; C6/36-1	D	T
SAF76v24	AR 18211	ECSA	South African Republic	1976	*Ae. furcifer*	Mosquito-2; SM-3; C6/36-1	D	T
SEN66v2	PM2951	W. Afr	Senegal (*Ndofore*)	1966 (Nov)	*Ae. aegypti*	Sm3; Vero-1; C6/36-1	D	T
SEN83	37997	W. Afr	Senegal (*Kedougou*)	1983 (10 Oct)	*Ae. furcifer*	AP61-1; Vero-2; C6/36-1	D	T
SENb81v23	IPD/A SH 2807	W. Afr	Senegal	(before 1981)	human	SM-3; mosquito-1; C6/36-1	D	T
SEY05	05-209	ECSA	Seychelles	2005 (9 Aug)	human	C6/36-1 to 4	D	T
SLA07v20	SL-CK1	ECSA	Sri Lanka	2007	human	Vero-1; C6/36-1	D	T
TAN53a	Ross (*from GenBank*)	ECSA	Tanzania	1953 (22 Feb)	human	SM-176; Vero-2	D	I
TAN53b	S27 (*from GenBank*)	ECSA	Tanzania (*Liteho, Newala District*)	1953 (22 Feb)	human	(unspecified high-passage)	D	I
TAN53v32	S27	ECSA	Tanzania (*Liteho, Newala District*)	1953 (22 Feb)	human	SM175; Vero1; C6/36-1	D	I
TAN53v33	Ross (*low-psg*)	ECSA	Tanzania (*Liteho, Newala District*)	1953 (22 Feb)	human	SM16; Vero1; C6/36-1	G	I
THAI58v30	TH35	ECSA	Thailand (*Bangkok*)	1958	human	SM21; C6/36-1	D	T
THAI62	AF15561	Asian	Thailand (*Bangkok*)	1962	human	Vero-2;	D	T
THAI95v14	CO392-95	Asian	Thailand (*Bangkok*)	1995	human	LLC-MK2-1; Vero-1; C6/36-1	D	T
Ag41855	Ag41855	ECSA	Uganda (*Mukono District*)	1982	human	SM3; Vero1	G	I

Table 6.10 Summary of virus strains used in phylogenetic analysis.
Genotype: ECSA - Eastern/ Central/South African; W. Afr – West African.
Passage history: SM: suckling mouse; C6/36: *Ae. albopictus* cell line; Vero: African green monkey cell line; RMK: Rhesus monkey kidney cell line; MRC-5: human lung epithelium; AP61: *Ae. pseudoscutellaris* cell line.
? – information is unavailable

The effects of the E2-D60G and E2-T226I on infectivity of CHIKV for *Ae. albopictus* and *Ae. aegypti* that we identified in previous experiments raised an important question regarding the origin of these particular mutations in the genome of the Ag41855 strain, and what evolutionary advantages might be associated with them. To address these questions, we analyzed the distribution of the E2-60G and E2-226I mutations among known CHIKV isolates (Table 6.10) and correlated this distribution with their evolution as determined using phylogenetic relationships.

The genome region encoding the E2-6K-E1 proteins was sequenced or obtained from GenBank and a phylogenetic tree was constructed by the neighbor-joining and maximum parsimony methods followed by bootstrap analysis (Felsenstein, 1985) with 1000 replicates to determine confidence values for the groupings. The phylogeny in Figure 6.10 reproduces the expected 3 major clades [West African, Asian, and East/Central/South African (ECSA)] (Powers et al., 2000), with the strains from the recent outbreak evolving as a monophyletic group from the ECSA clade (Schuffenecker et al., 2006).

The E2-60G residue was present in only four out of 39 CHIKV strains, with no apparent close phylogenetic relationships among them (Figure 6.3, Table 6.10). MaClade character evolution analyses indicated that the aspartic acid residue was ancestral, and the glycine residue evolved convergently 4 times. Although no apparent similarities were detected in the passage histories of the four strains with the glycine residue (Table 6.10), the presence of glutamic acid residue at E2-60 in the IND-00 CHIKV strain, which is almost 100% identical to the Ag41855 strain based on both nucleotide and amino acid sequences and presence of different residues in variants of the 1953 Ross strain with different passage histories suggested that the D60G substitution may have been selected by cell culture or animal passage.

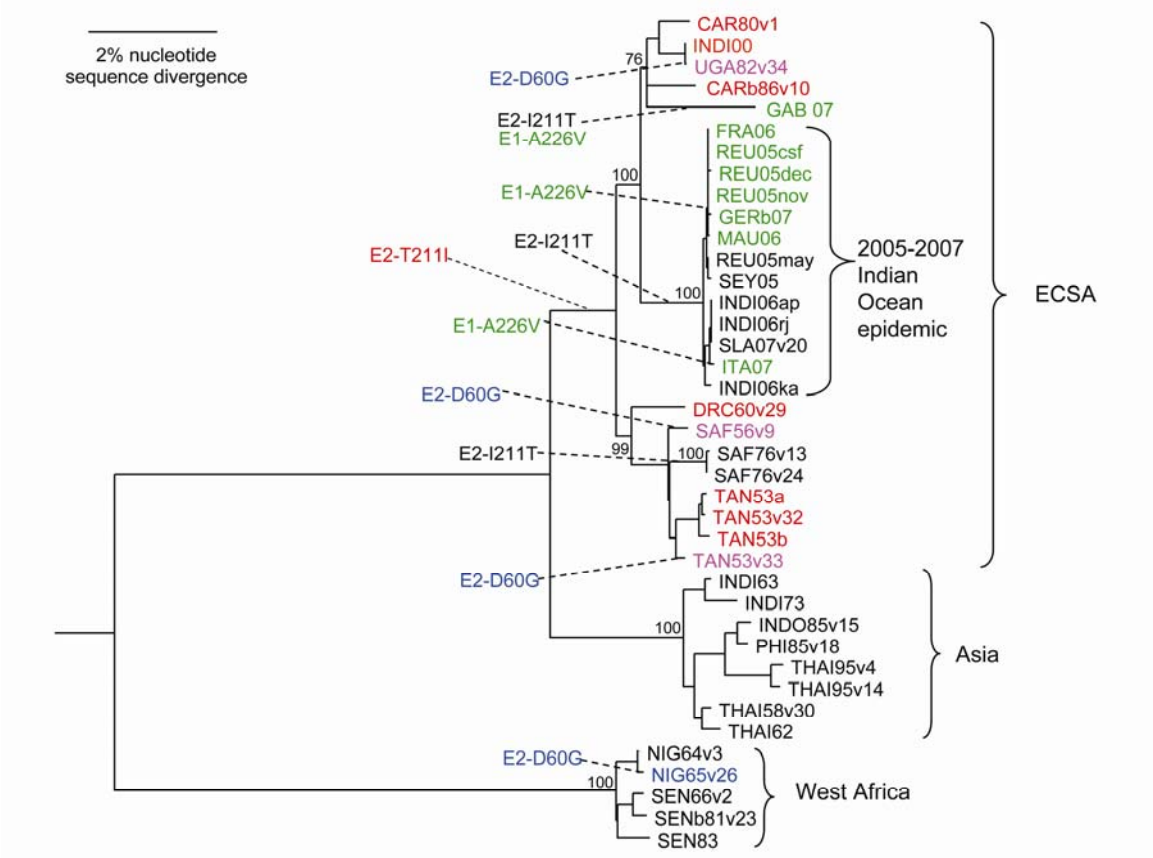


Figure 6.3 Distribution of the amino acids at E2-60, E2-211 and E1-226 among selected CHIKV isolates.

Phylogenetic tree of CHIKV isolates generated using a maximum parsimony algorithm on the 2772 bp complete E2-6K-E1 genes sequence. Bootstrap analysis was performed with 1000 replicates to determine confidence values on the clades within trees. Character evolution analyses was performed using MacClade4 program.
Strains in black – contain E2-60D, E2-211T and E1-226A residues.
Strains in red – contain E2-60D, E2-211I and E1-226A residues.
Strains in green – contain E2-60D, E2-211T and E1-226V residues.
Strains in pink – contain E2-60G, E2-211I and E1-226A residues.
Strains in blue – contain E2-60G, E2-211T and E1-226A residues.

In contrast to E2-60G, the E2-211I had a very different distribution among CHIKV isolates (Figure 6.3). It was present in 10 of 39 strains, all 10 belonging to the ECSA clade. Interestingly, this residue was found to be even more prevalent in this phylogroup among strains isolated before 2005 as compared to E2-211T. These data suggest that the E2-211I might be important for adaptation of CHIKV to some particular conditions prevalent in these regions, for example specific vectors or vertebrates involved in the sylvatic transmission cycle. Within the ECSA clade, E2-211T was found in all

isolates from the 2005-2007 CHIKV outbreak, and in two more strains isolated in 1976 from the South African Republic (Figure 6.3; Table 6.10). Character analyses indicated that the E2-I211T substitution probably occurred convergently on three separate occasions within the ECSA clade, leading to South African 1976 strains, Indian Ocean 2005-2007 strains and Gabonese 2007 strain (Figure 6.3). This conclusion is supported by the presence of the E2-211I residue in two strains from Comoros isolated in 2005 (Kariuki Njenga et al., 2008) belonging to the Indian Ocean clade. The sequences for these strains are unavailable in GenBank which precluded us from including them in our phylogenetic analysis. Finally, as determined previously (de Lamballerie et al., 2008; Schuffenecker et al., 2006), our analyses indicated at least three convergent E1-A226V replacements during the recent epidemics in the Indian Ocean and India (Figure 6.3).

Role of mutations at positions E2-60 and E2-211 on CHIKV replication in cell culture

The growth of CHIKV with different mutations at positions E2-60 and E2-211 was compared in BHK-21 and C6/36 cells (Figure 6.4). The E2-60D and E2-211T residues were introduced into non-eGFP expressing virus 41855-226V that has authentic for Ag41855 strain residues E2-60G and E2-211I. In BHK-21 the residue E2-211T was found to promote the growth of CHIKV as compared to viruses with E2-211I residue (Figure 6.4A); the titers of 41855-226V-(E2-211T) were significantly higher ($p<0.05$ stuident`s t test) as compared to 41855-226V and 41855-226V-(E2-60D) viruses at 6 and

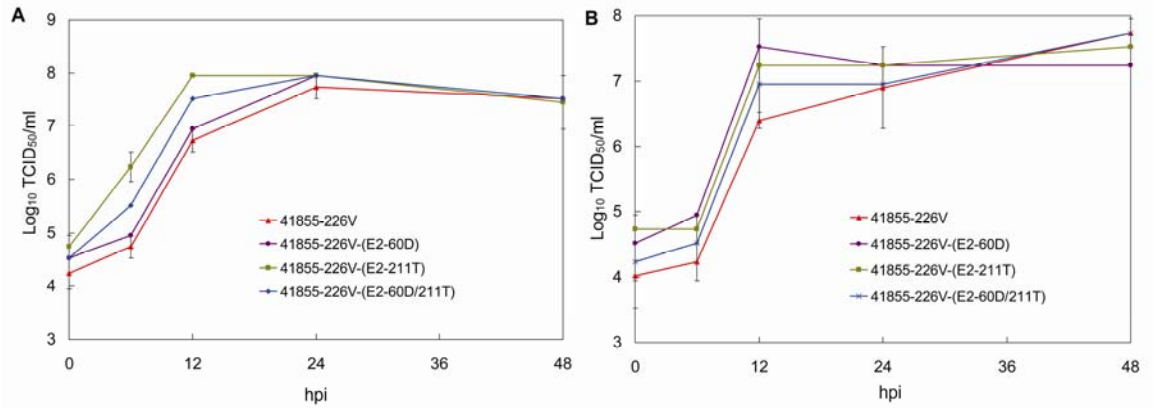


Figure 6.4 Effect of mutations at E2-60 and E2-211 on growth of CHIKV in BHK-21 (A) and C6/36 (B) cells.

Cells were infected at an MOI of 1.0. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of 2 independent experiments.

12 hpi. The E2-60D has a slight negative effect on CHIKV replication; the titer of 41855-226V-(E2-211T) was higher than that of 41855-226V-(E2-60D/211T) at 6 and 12 hpi but the difference was not statistically significant ($p>0.05$). In C6/36 cells (Figure 6.4 B) the residues E2-60D and E2-211T have some positive effect on CHIKV replication, but the differences in titers were not statistically significant ($p>0.05$).

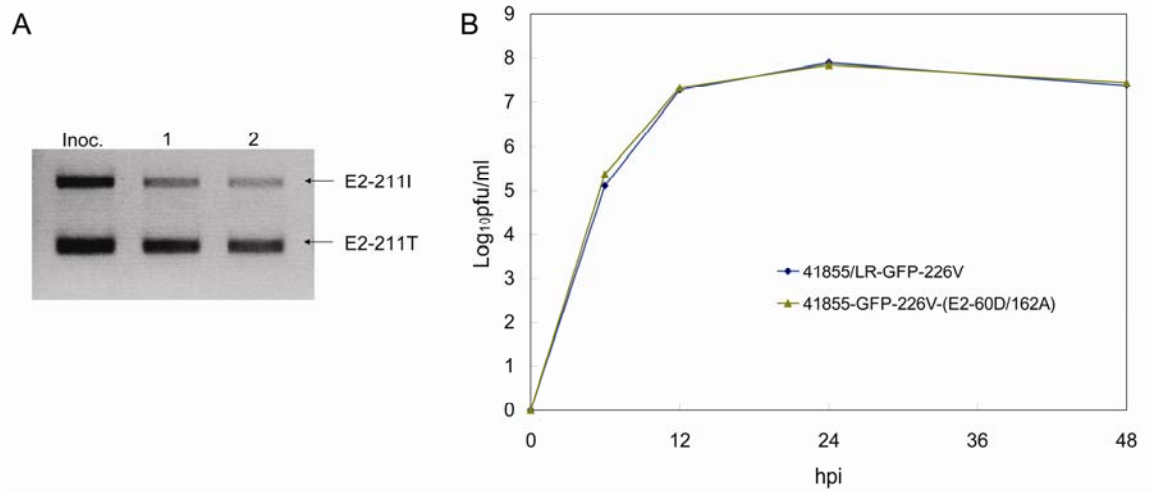


Figure 6.5 Effect of threonine and isoleucine residues at E2-211 on growth of CHIKV in infected (A) and electroporated (B) BHK-21.
A – LR-ApaI-226V and LR-226V-(E2-211I) were mixed 1:1 and used to infect BHK-21 cells at an MOI of 1.0. At 2 dpi cell culture supernatant was harvested for RNA extraction, followed by RT-PCR analysis as described in Chapter 3. Inoc. – initial ratio of LR-ApaI-226V and LR-226V-(E2-211I) RNA used for infection. 1 and 2 - results of 2 independent experiments.
B – 10μg of RNA derived form p41855/LR-GFP-226V and p41855-GFP-226V-(E2-60D/162A) constructs were electroporated into 10^7 BHK-21 cells. Cell culture supernatants were collected at indicated times post electroporation, and titrated by plaque assay on Vero cells.

The positive effect of the E2-211T residue on CHIKV replication in BHK-21 cells was also observed in the competition experiment. The E2-T211I mutation was introduced into CHIKV-LR and the resultant virus LR-226V-(E2-211I) was mixed with an equal amount of LR-ApaI-226V, which has a E2-211T residue. The viruses were used to infect BHK-21 cells and at 48 hpi, viral RNA was extracted from cell culture supernatant and processed as described in Chapter 3 (Figure 6.5 A). The RNA derived from LR-ApaI-226V was markedly more predominant at 48 hpi as compared with RNA of LR-226V-(E2-211I) virus, which is in agreement with growth curve results (Figure 6.4 A). Interestingly, the growth of CHIKV with either E2-211I or E2-211T was indistinguishable if *in vitro* transcribed RNA was directly electroporated into BHK-21 cells (Figure 6.5 B), indicating that residues at position E2-211 probably play a role in the

CHIKV replication cycle at the steps preceding release of virus RNA into the cytoplasm or in virus maturation.

The mutations at E2-211 might affect the stability of the E2-E1 heterodimers, which would differentially affect fusion properties of the CHIKV. Thus it has been shown that the mutation E2-D216G can rescue a PE2 cleavage mutant of SINV by disrupting the E2-E1 heterodimer stability under acidic conditions (Smit et al., 2001). To address this question, the ability of CHIKV with either isoleucine or threonine residues at E2-211 to trigger a cell-to-cell fusion reaction was compared in BHK-21 and C6/36 cells (Figure 6.6). BHK-21 and C6/36 cells were infected with 41855/LR-GFP-226V, 41855-GFP-226V-(E2-60D/162A), 41855/LR-GFP-226A and 41855/LR-GFP-226A-(E2-60D/162A) viruses and cell-cell fusion was triggered by incubation of the cells in medium with different pH as described in Chapter 5. Viruses that differ only by mutations at E2-211 had identical pH thresholds of fusion reaction in both BHK-21 and C6/36 cells. However, substitutions at position E1-226 markedly modulated the CHIKV pH threshold in both BHK-21 and C6/36 cells. Additionally, viruses with isoleucine at E2-211 had a very similar sensitivity to the inhibitory effects of NH_4Cl as compared to those with a threonine residue (data not shown). These data indicate that mutations at E2-211 do not affect fusogenic properties of CHIKV, and probably function at the level of interaction of CHIKV with its cell surface receptor.

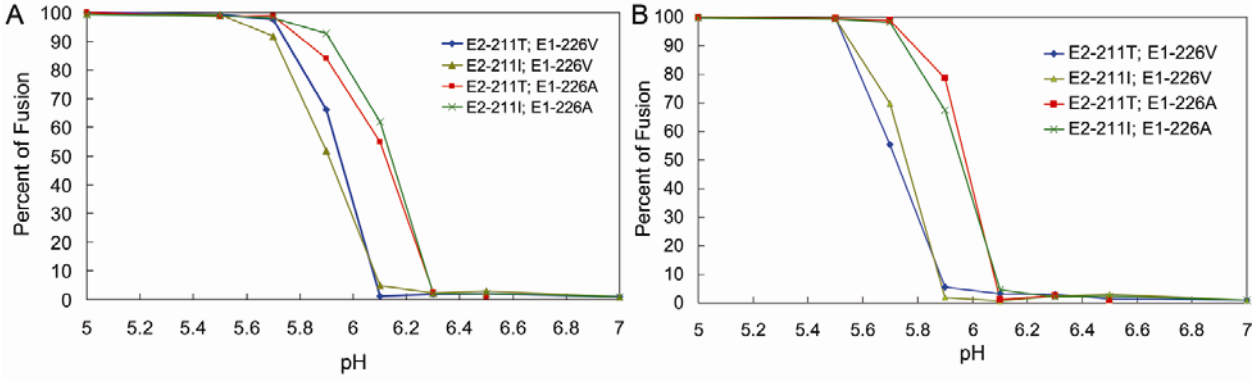


Figure 6.6 Effect of threonine and isoleucine residues at E2-211 on CHIKV induced cell-cell fusion.

BHK-21(A) and C6/36 (B) cells infected with 41855/LR-GFP-226V (Blue), 41855-GFP-226V-(E2-60d/211A) (Brown), 41855/LR-GFP-226A (Red) and 41855-GFP-226A-(E2-60d/211A) (Green) viruses were incubated for 2 min with L-15 medium which pH was previously adjusted to desired values. The reaction was abrogated by replacement of the fusion medium with 0.5 ml of standard L-15 media. Cells were incubated for 3h at 37 ºC (BHK-21) or 28 ºC (C6/36) to allow polykaryons to develop. Percent of fusion was calculated as (1-c/n)x100%, where c is a number of eGFP expressing cells, n is number of nuclei (n≥70). Experiments were performed in duplicates for each virus.

Effect of mutations at positions E2-60 and E2-211 on CHIKV interaction with membrane proteins from C6/36 cells and midguts of *Ae. albopictus* and *Ae. aegypti* mosquitoes

Differential infectivity of CHIKV associated with mutations at position E2-60 and E2-211 in *Ae. albopictus* and *Ae. aegypti* led us to hypothesize that these mutations might influence the ability of CHIKV to interact with specific membrane-associated proteins from midguts of these mosquito species. Membrane proteins from C6/36 cells and from brush border membranes from midguts of *Ae. albopictus* and *Ae. aegypti* were isolated and separated by 10% SDS-PAGE under non-reducing conditions. Proteins were transferred to nitrocellulose membranes and probed with CHIKV containing different combinations of mutations at E2-60 and E2-211 (Figure 6.7). All tested viruses showed no interaction with proteins isolated from BBM of *Ae. aegypti*. Viruses 41855-226V and 41855-226V-(E2-60D) which contain isoleucine at E2-211 strongly interacted with protein of ~100 kDa from BBM of *Ae. albopictus*. Viruses 41855-(E2-211T) and 41855-226V-(E2-60D/211T) which contain threonine at E2-211 showed no interaction with this protein. To confirm that an isoleucine residue at E2-211 is responsible for interaction with the ~100 kDa protein from BBM of *Ae. albopictus,* a E2-T211I mutation was introduced into CHIKV-LR and 37997-226A viruses (Figure 6.8). The resultant viruses LR-226V-(E2-211I) and 37997-226A-(E2-211I) showed strong interactions with a ~100 kDa protein; however, the CHIKV-LR and 37997-226A did not interact with it. These data also indicate that amino acids at position E1-226 most likely do not influence CHIKV interaction with~100 kDa protein. Thus, LR-226V-(E2-211I) and 37997-226A-(E2-211I) have valine and alanine at E1-226 respectively, however, both viruses efficiently interacted with the ~100 kDa protein. CHIKV also interacted with 30 and 46 kDa proteins from C6/36 cells, but there is no association between particular residues at E2-60 and E2-211, and the ability of CHIKV to interact with these proteins. Altogether these data indicate that mutations at E2-211 can significantly change the ability of CHIKV to interact with proteins from BBM of mosquito cells.

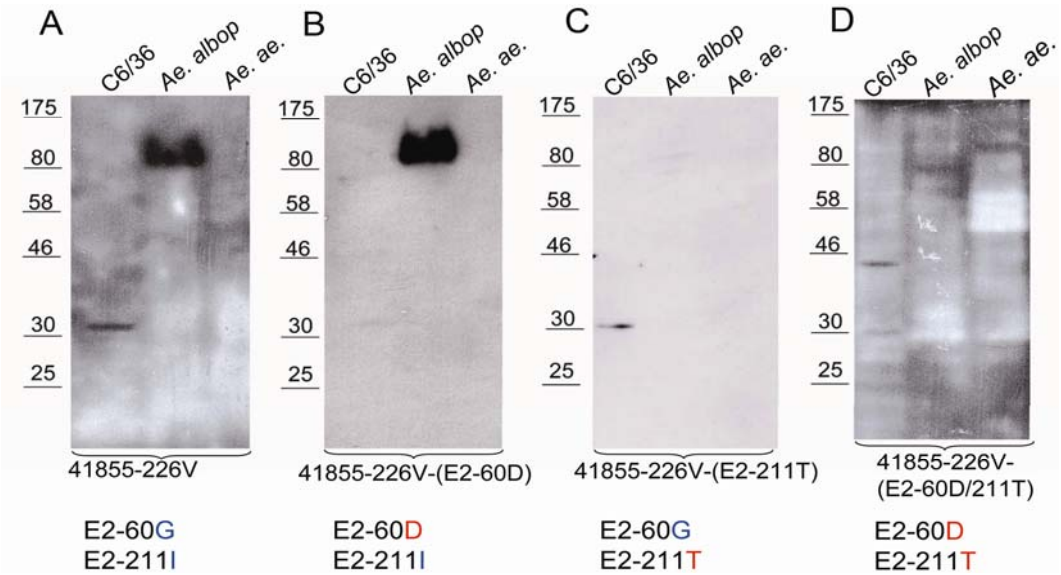


Figure 6.7 Effect of mutations at E2-60 and E2-211 on CHIKV binding to membrane proteins from C6/36 cells and midguts of *Ae. albopictus* and *Ae. aegypti* mosquitoes.

15 µg of membrane proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and were probed with 41855-226V (**A**), 41855-226V-(E2-60D) (**B**), 41855-226V-(E2-211T) (**C**) and 41855-226V-(E2-60D/211T) (**D**) viruses. Binding was visualized by incubation of the membranes with mouse anti-CHIKV antibodies (Powers et al., 2000) followed by incubation with goat anti-mouse HRP-labeled antibodies.

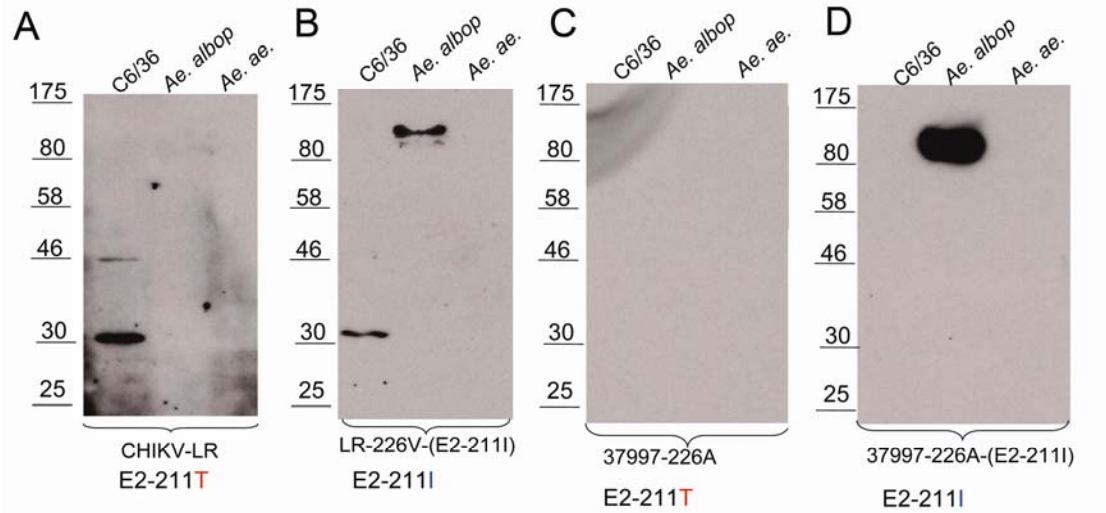


Figure 6.8 Effect of mutations at E2-211 on binding of CHIKV strain LR2006-OPY1 (A;B) and 37997 (C;D) to membrane proteins from C6/36 cells and midguts of *Ae. albopictus* and *Ae. aegypti* mosquitoes.

Membrane proteins on nitrocellulose membrane were probed with CHIKV-LR (**A**), LR-226V-(E2-211I) (**B**), 379979-226A (**C**) and 37997-226A-(E2-211I) (**D**) viruses. Binding was visualized by incubation of the membranes with mouse anti-CHIKV antibodies (Powers et al., 2000) followed by incubation with goat anti-mouse HRP-labeled antibodies.

To get better resolution of the ~100 kDa protein band, BBM from *Ae. albopictus* were separated by 8% SDS-PAGE and probed with LR-226V-(E2-211I) virus (Figure 6.9). ~100 kDa band was found to consist of two bands ~110 kDa and ~130 kDa.

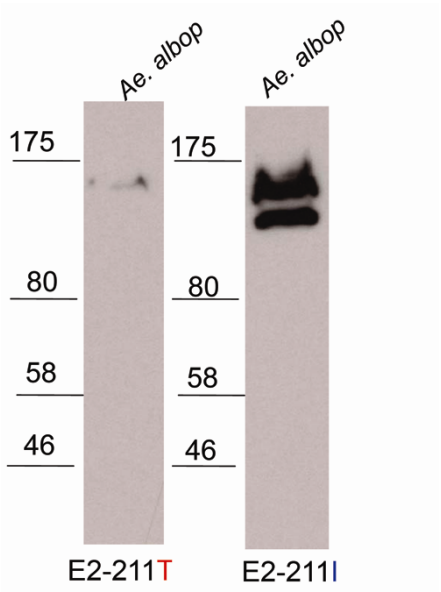


Figure 6.9 ~100 kDa band consists of two bands ~110 kDa and ~130 kDa.

Ae. albopictus midgut membrane proteins were separated by 8% SDS-PAGE and probed with CHIKV-LR or LR-226V-(E2-211I) viruses.

Effect of mutations at E2-211 on CHIKV interaction with BBM from midguts of *Ae. albopictus* mosquitoes

To investigate if the E2-211I residue is also responsible for more efficient CHIKV binding to the total BBM membranes of *Ae. albopictus*, CHIKV with either E2-211I or E2-211T were incubated with BBM in the binding buffer comprised of L-15 medium supplemented with 0.75% BSA followed by precipitation of BBM/virus complexes. Pellets were resuspended in 1xSDS-PAGE loading buffer and separated by 10% SDS-PAGE followed by Western blot analysis with CHIKV specific antibodies (Figure 6.10A). Surprisingly, no marked differences were found between the ability of CHIKV with E2-211I or E2-211T residues [CHIK-LR and LR-226V-(E2-211I)] to interact with BBM. In the presence of 0.1 and 0.01 µg/µl of BBM in suspension, the majority of CHIKV is precipitated by centrifugation, however, in the absence of BBM both viruses remain in solution. These results were also supported by the binding competition experiments: LR-ApaI-226V (which has a threonine at E2-211) and LR-226V-(E2-211I) were mixed 1:1 and incubated with 0.1 µg/µl of BBM in binding buffer followed by centrifugation at 21,000g. Pellets were washed once in binding buffer and resuspended in TRIzol reagent, followed by RNA extraction and RT-PCR analysis (Figure 6.10B). The ratio of viruses in the CHIKV/BBM pellets remained unchanged for all virus concentrations tested as compared to the initial ratio of the viruses in the inoculum. These data suggest that CHIKV with an E2-211T residue probably interacts with some BBM protein(s) that cannot be visualized using VOPBA.

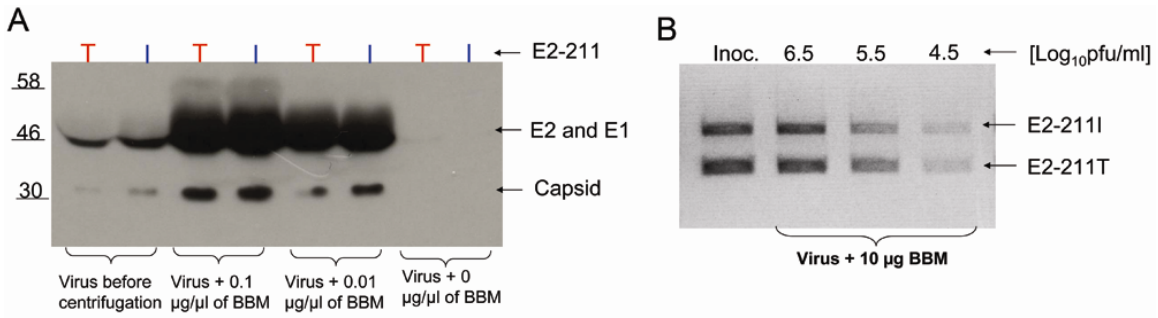


Figure 6.10 Effect of mutations at E2-211 on binding of CHIKV to BBM fraction of *Ae. albopictus.*

A – CHIKV (0.1 µg/µl) with E2-211T or E2-211I residues were incubated with different amount of BBM at 4 ºC for 30 min, followed by centrifugation at 21,000g for 20min at 4 ºC. Precipitated fraction was separated by SDS-PAGE followed by western blot analysis using CHIKV specific antibodies (Powers et al., 2000). The fraction "virus before centrifugation" corresponds to 15% of total virus used in the experiment.
B – LR-ApaI-226V and LR-226V-(E2-211I) were mixed 1:1, and diluted to final concentrations of 6.5, 5.5 and 4.5 Log_{10}pfu/ml. Virus was incubated with 0.1 µg/µl of BBM in for 30min at 4 ºC followed by centrifugation at 21,000g for 20min at 4 ºC. Pellets were resuspended in TRIzol reagent, followed by RNA extraction and RT-PCR analysis as described in Chapter 3.

To test the ability of LR-ApaI-226V and LR-226V-(E2-211I) to interact with native midgut epithelial cells of *Ae. albopictus,* the mosquitoes were orally presented with 7.5 $Log_{10}TCID_{50}$/ml of a 1:1 mix of these viruses. At 15 min after engorgement, mosquito midguts were dissected and engorged blood meal was removed by three consecutive washes in L-15 medium supplemented with 10%FBS. Midguts were lysed in TRIzol reagent, followed by RT-PCR analysis (Figure 6.11A). The ratio of RNA derived from LR-ApaI-226V and LR-226V-(E2-211I) viruses bound to midguts remained

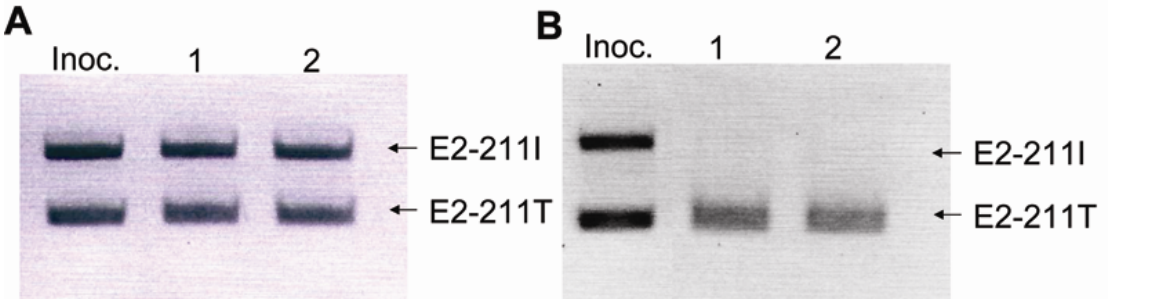


Figure6.11 Effect of mutations at E2-211 on binding of CHIKV to midgut cells of *Ae. albopictus.*

Blood meal containing 7.5 $Log_{10}TCID_{50}$/ml of 1:1 mixed LR-ApaI-226V and LR-226V-(E2-211I) viruses, was orally presented to *Ae. albopictus*.
A – 15 min after engorgement mosquito midguts were dissected and lysed in TRIzol reagent, followed by RT-PCR analysis as described in Chapter 3.
B – CHIKV RNA was extracted from total mosquito body at 7 dpi, and analyzed by RT-PCR analysis as described in Chapter 3.
Inoc. – initial RNA ratio of LR-ApaI-226V and LR-226V-(E2-211I) viruses used for infection.
1 and 2 - ratio of LR-ApaI-226V and LR-226V-(E2-211I) in two independent replicas of the 12 midguts (A) or 12 mosquitoes bodies (B) per replica.

unchanged as compared to initial RNA ratio. This indicates that mutations at E2-211 do not change the general ability of CHIKV to bind to midgut cells of *Ae. albopictus*. Interestingly, when the same engorged mosquitoes were analyzed at 7 dpi the only virus RNA detected in the infected mosquitoes belonged to the LR-ApaI-226V virus (Figure 6.11B), which is in agreement with mosquito infectivity data (Table 6.6; 6.7), indicating that residue E2-211I significantly decreases the fitness of the virus to replicate in *Ae. albopictus* when it is expressed with E1-A226V mutation.

We also analyzed effect of different residues at E2-211 on CHIKV interaction with midgut epithelial cells of *Ae. albopictus* using TEM. The 41855/LR-GFP-226V (has a E2-211T residue) and 41855/LR-GFP-226V-(E2-60D/162A) (has a E2-211I residue) viruses were grown in BHK-21 cells and concentrated to 8.95 $Log_{10}TCID_{50}$/ml by centrifugation through a 20% sucrose cushion. Viruses were resuspended in L-15 medium, mixed with an equal volume of defibrinated blood and orally presented to *Ae. albopictus* mosquitoes. At 0.5 and 1.5 hpi. midguts were dissected and the blood meal was removed by three consecutive washes in PBS and midguts were fixed in TEM fixative solution. Both viruses were found to interact with microvilli of midgut epithelial cells with relatively equal efficiency (Figure 6.12) and no interaction was observed between these viruses and basal lamina (data not shown). These data support my data that different mutations at E2-211 do not affect the overall ability of CHIKV to bind to BBM of *Ae. albopictus*. Interestingly, CHIKV with the E2-211T residue (41855/LR-GFP-226V) was found more often in contact with the plasma membrane of epithelial cells between the microvilli as compared to CHIKV with the E2-211I [41855/LR-GFP-226V-(E2-60D/162A)]. CHIKV with the E2-211I residue was usually associated with the apical tip of microvilli, and only rarely was found in the basal part of microvilli or plasma membrane (Figure 6.13).

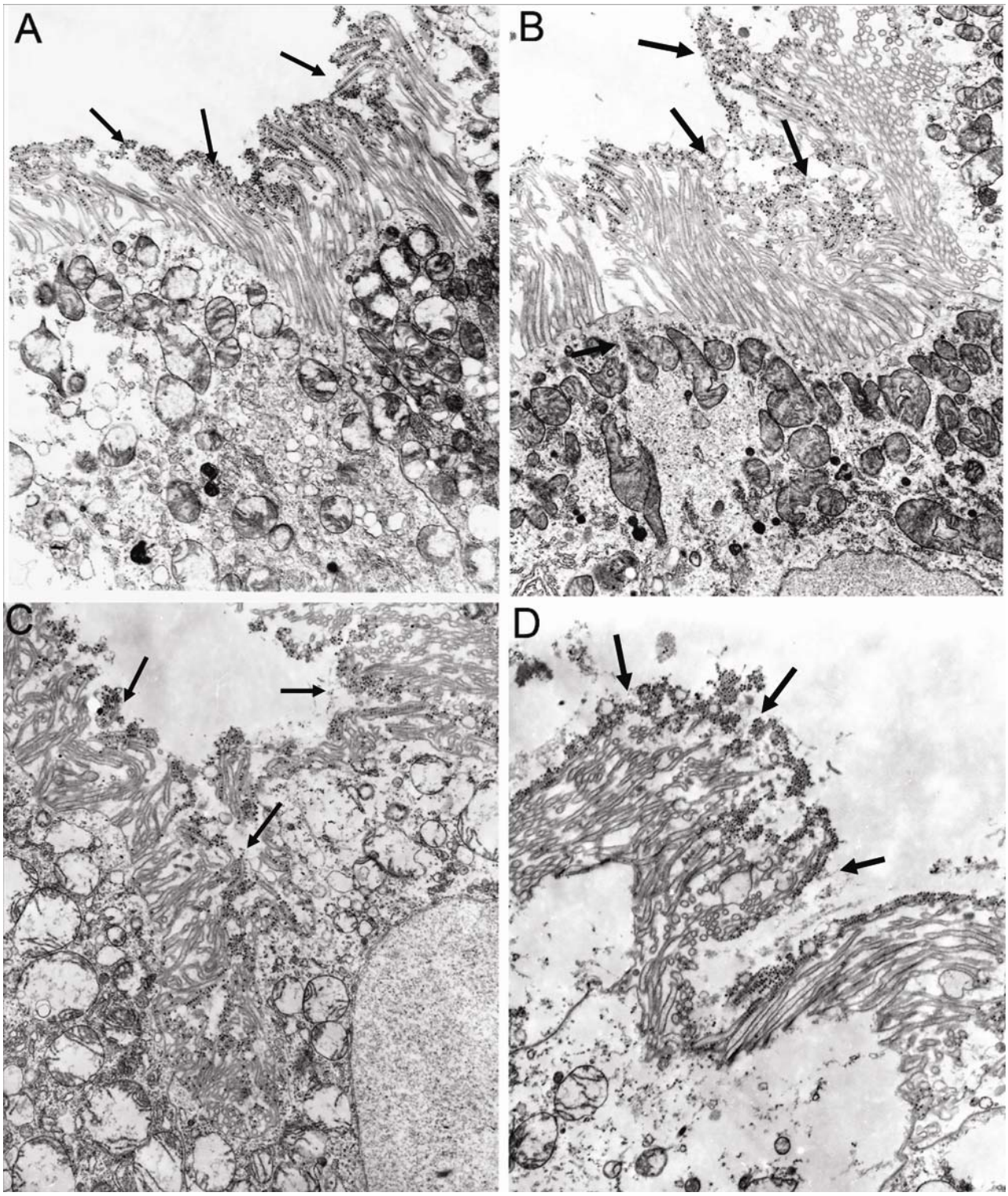


Figure 6.12 Effect of mutations at E2-211 on binding of CHIKV to midgut of *Ae. albopictus.*

Ae. albopictus were orally presented with 8.95 $Log_{10}TCID_{50}$/ml of 41855/LR-GFP-226V (A and C) or 41855/LR-GFP-226V-(E2-60D/162A) (B and D) viruses. Midguts were dissected at 0.5 (A and B) and 1.5 (C and D) hpi and analyzed by TEM. Arrows indicate the CHIKV bound to microvilli of midgut epithelial cells.

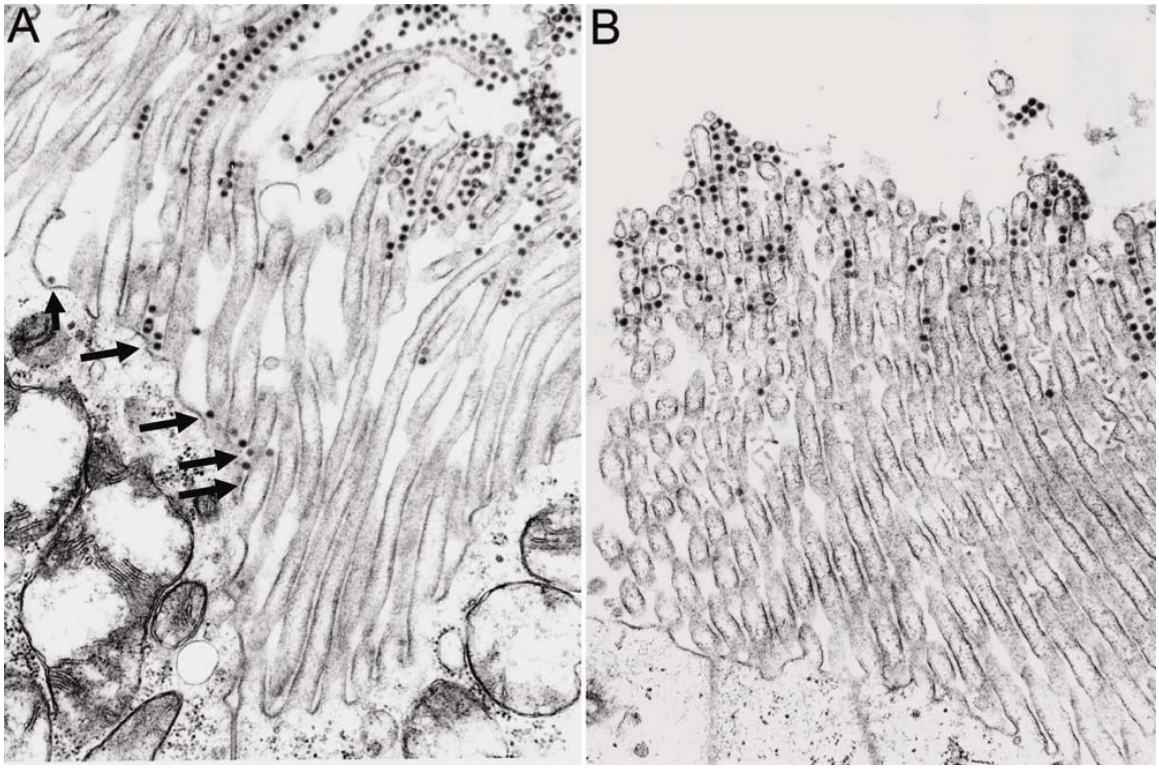


Figure 6.13 Effect of mutations at E2-211 on binding of CHIKV to midgut of *Ae. albopictus*.

Ae. albopictus were orally presented with 8.95 $Log_{10}TCID_{50}$/ml of 41855/LR-GFP-226V (A) or 41855/LR-GFP-226V-(E2-60D/162A) (B) viruses. Midguts were dissected at 0.5 hpi and analyzed by TEM. Arrows indicate the CHIKV contacting with apical plasma membrane of midgut epithelial cells.

The E1-A226V mutation does not lead to an increase in infectivity of VEEV, ONNV and MAYV to *Ae. albopictus* mosquitoes

In this chapter I demonstrated that the effect of the epidemic E1-A226V mutation on CHIKV infectivity for *Ae. albopictus* is not universal, but is dependent on a specific combination of mutations in other genome regions. Based on this observation I

221					E1-226						232	
L	Q	R	P	A	A	G	T	V	H	V	P	CHIKV (Ag41855)
L	Q	R	P	A	A	G	A	I	H	V	P	ONNV (Sg650)
L	A	R	P	A	A	G	N	I	H	V	P	Mayaro virus
L	Q	R	P	K	A	G	A	I	H	V	P	VEEV (ZPC-738)

Figure 6.14 Amino acid alignment of 221-232 regions of CHIKV, ONNV, MAYV and VEEV.

Polymorphic positions are indicated in gray. Position E1-226 is indicated in red.

Plasmid[a]	E1[b]		Specific infectivity [c]	Titers [d]	
	225	226		24h	48h
pMayaro-GFP	A	A	$4.5x10^5$	7.95	7.95
pMayaro-GFP-226V	A	V	$7.5x10^5$	ND	7.52
pONN-GFP	A	A	$9.5x10^5$	5.52	6.52
pONN-GFP-226V	A	A	$10.0x10^5$	5.52	6.52
PM1-738/GFP	K	A	$5.0x10^5$	7.95	7.52
PM1-738/GFP-226V	K	V	$<1.0x10^3$	2.95	5.52
PM1-738/GFP-225A226V	A	V	$10.0x10^5$	ND	7.71

Table 6.10 Recovery of the viruses with mutations in E1 protein after electroporation of *in vitro* transcribed RNA.

a – plasmid used for *in vitro* transcription
b – amino acids at position of E1: 225, 226.
c – Specific infectivity of *in vitro* transcribed RNA (pfu/1µg RNA).
d – Virus titers at 24 and 48 hpe expressed as $Log_{10}TCID_{50}$/ml.
h – hours post-electroporation.
ND – value is not determined.

hypothesized that the E1-A226V mutation most likely would not have a significant impact on *Ae. albopictus* infectivity for viruses other than CHIKV. To test this hypothesis I introduced E1-A226V mutation into the backbone of the eGFP-expressing i.c. of VEEV subtype ID, MAYV and ONNV. All of these viruses have an authentic alanine residue at E1-226 (Figure 6.14). Introduction of the E1-A226V mutation into ONNV and MAYV did not lead to marked attenuation of these viruses in cell culture (Table 6.10): specific infectivity values and virus titers post-electroporation of pONNV-GFP-226V and Mayaro-GFP-226V constructs were similar to parental constructs. Interestingly, introduction of the single mutation E1-A226V into the backbone of VEEV was lethal for the virus (PM-GFP-226V in Table 6.10), however, simultaneous substitutions E1-K225A and E1-A226V resulted in construct (PM1-738/GFP-225A226V) that had identical to the parental viral specific infectivity values and virus titers post-electroporation.

The relative infectivities of virus pairs: ONNV-GFP and ONNV-GFP-226V, Mayaro-GFP and Mayaro-GFP -226V, PM1-738/GFP and PM1-738/GFP-225A226V in

Ae. albopictus mosquitoes were determined as described previously (Chapter 3). For all analyzed viruses, the mutation E1-A226V (or in case of VEEV the double mutation E1-K225A and E1-A226V) did not lead to a significant ($p>0.05$) increase in OID_{50} values (Table 6.11). These data indicate that possible adaptation of alphaviruses other than CHIKV to *Ae. albopictus* mosquitoes would require mutation(s) in the genome region different to those described for CHIKV.

Virus name	226	N m	OID_{50}	C_{95}	p value
ONN-GFP	A	84	6.50	6.25-6.99	p>0.05
ONN-GFP-226V	V	85	5.90	5.60-6.30	
Mayaro-GFP	A	82	6.30	6.04-6.59	p>0.05
Mayaro-GFP-226V	V	77	6.76	6.42-7.42	
PM1-738/GFP	A	70	7.00	6.66-7.43	p>0.05
PM1-738/GFP-225A226V	V	74	7.39	7.09-8.21	

Table 6.11 Effect of the E1-A226V mutation on infectivity of different alphaviruses to *Ae. albopictu* mosquitoes.

N m – number of mosquitoes used to estimate OID_{50} value.
OID_{50} and C_{95} – oral infectious dose 50 and 95% confidence intervals are expressed as $Log_{10}TCID_{50}$/ml.
p – comparison of statistical significance of difference in OID_{50} values between viruses with E1-226A and E1-226V mutations.

DISCUSSION

In this work we performed a detailed investigation of the genetic factors responsible for the relatively low infectivity of the CHIKV Ag41855 strain for *Ae. albopictus* and *Ae. aegypti* mosquitoes. Comprehensive analyses identified amino acid residues at E2-60 and E2-211 that modulate the role of the E1-A226V mutation that first arose during the Indian Ocean epidemic (de Lamballerie et al., 2008; Schuffenecker et al., 2006) on CHIKV infectivity in these vectors. Individual expression of the E2-G60D or E2-I211T mutation in the 41855-GFP-226V virus had an identical effect on the OID_{50} for *Ae. albopictus,* and their combined expression increased infectivity of strain

Ag41855, into which the E1-A226V mutation was introduced, to the level characteristic for strains LR2006 OPY1 and 37997. However, expression of only E2-G60D (but not E2-I211T) was necessary and sufficient to elevate the infectivity of the Ag41855 strain with an alanine at E1-226for *Ae. albopictus*. When considered together, as explained below, these data provide new and important insights into the roles played by E2-G60D and E2-I211T mutations in determining CHIKV infectivity for *Ae. albopictus* mosquitoes.

The mutation E2-G60D significantly increases Ag41855 infectivity for *Ae. albopictus* when expressed with either alanine or valine at E1-226. This indicates that position E2-60 is important for vector infectivity of CHIKV, but it does not specifically affect the previously observed phenotypes reported for viruses with the E1-A226V mutation. This conclusion was further supported by the results obtained from *Ae. aegypti* infectivity experiments. In this mosquito, the E1-A226V mutation does not increase CHIKV infectivity (Chapter 3; Tsetsarkin et al., 2007) and therefore this mosquito vector can be used as a species-specificity control. In *Ae. aegypti,* expression of the E2-G60D alone was necessary and sufficient to increase Ag41855 infectivity to that of the LR2006 OPY1 and 37997 levels. Interestingly, the E2-60G residue was found only in three other CHIKV strains, none of which share close phylogenetic relationships. More importantly, the IND-00 CHIKV strain, which is almost 100% identical to the Ag41855 strain based on both nucleotide and amino acid sequences, has a glutamic acid residue at E2-60. This suggests that acquisition of E2-D60G occurs sporadically, possibly during propagation of the virus under laboratory conditions. Altogether, accumulated data suggests that the E2-60 residue affects some basic mechanism used by CHIKV to infect its vector. Disruption of this mechanism exerts a strong inhibitory effect on CHIKV infectivity to both *Ae. albopictus* and *Ae. aegypti,* and therefore this mutation should be quickly eliminated from naturally transmitted strains.

To my knowledge, this is the first report incriminating alphavirus position E2-60 as a major determinant of mosquito infectivity. Interestingly, a recent study demonstrated that introduction of two mutations; E2-H55Q and E2-K70E from the TR339 SINV strain into the TE/5’2J of SINV increased infectivity for *Ae. aegypti,* both independently and in combination (Pierro et al., 2007). These mutations are located in close proximity to, and share similar properties with E2-G60D; they both decrease the net positive charge of the

E2 protein, indicating that their mechanism of action in SINV and CHIKV may be the same. Currently, the particular roles of mutations that increase the charge in this region of the E2 protein are uncertain. Previous studies demonstrated that the E2-Q55H substitution leads to increased SINV neurovirulence in mice (Levine and Griffin, 1993), and also increases binding to neuroblastoma cells. However, E2-E70K was shown to reduce neurovirulence in neonatal mice (McKnight et al., 1996). It would be interesting to investigate the possible association between mutations at E2-60 with the rare neurological complications and fatalities that were reported for the first time during the recent chikungunya epidemics (Arpino et al., 2009). Both mutations E2-Q55H and E2-E70K were also found to be involved in increased SINV binding to heparan sulfate (HS) (Levine and Griffin, 1993; Smit et al., 2002) which is in agreement with the hypothesis that the E2-D60G substitution occurred in the Ag41855 strain as a result of adaptation of the virus to sulfated proteoglycans abundantly expressed on the cell surface of BHK-21 and Vero cells. Although, to our knowledge there are no examples of HS adaptation due to mutations that lead to loss of a negative charge in the E2 protein for any alphaviruses, it has been shown that an E to G mutation at position 122 of the E protein of tick-borne encephalitis virus (TBEV) (a member of family *Flaviviridae*) mediates adaptation of TBEV to BHK-21 cells via increasing virus binding to HS (Mandl et al., 2001). Interestingly, growth kinetics of 41855-226V-(E2-211T) and 41855-226V-(E2-60D/211T) demonstrated that the E2-60G residue has some positive effect on CHIKV replication in BHK-21 cells, which is in agreement with the hypothesis of cell culture adaptation, although the differences in viral titers were not statistically significant ($p>0.05$).

In contrast to the E2-G60D, the mutation E2-I211T significantly increases infectivity of the Ag41855 strain for *Ae. albopictus* only when expressed together with the E1-226V mutation. If CHIKV has the pre-2005 E1-226A, then the substitution E2-211T has almost no effect on infectivity for *Ae. albopictus* compared with the E2-211I variant. This indicates that the E2-I211T mutation is responsible for specific modulation of CHIKV infectivity for *Ae. albopictus*. The results of CHIKV infectivity for *Ae. aegypti* further support this conclusion. In *Ae. aegypti*, the E1-A226V mutation does not increase CHIKV infectivity (Tsetsarkin et al., 2007) and in the current study, the E2-I211T mutation did not affect infectivity for these mosquitoes. Altogether, these mosquito

infectivity data indicate that the E1-A226V would not give a selective advantage to the CHIKV strains possessing E2-211I with respect to transmissibility by *Ae. albopictus*. Such viruses would probably not be selected in nature. This conclusion is in agreement with our phylogenetic analyses. It has been shown that the E1-A226V mutation appeared independently at least three times in strains of CHIKV transmitted by *Ae. albopictus* (de Lamballerie et al., 2008), but it is important to note that all of these CHIKV isolates had threonine at position E2-211.

Phylogenetic analysis of the distribution of the E2-211I mutation revealed substantial differences compared to the E2-60G mutation. E2-211I was present in the majority of pre-2005 CHIKV isolates in the ECSA complex, suggesting that it might play an important role for maintenance of CHIKV in the enzootic African cycle involving wild non-human primates and forest-dwelling *Aedes* spp. mosquitoes. Based on isolation frequencies, the main sylvatic vectors of CHIKV in Africa are probably *Ae. furcifer-taylori*, *Ae. africanus* and *Ae. luteocephalus* (Jupp and McIntosh, 1988), with *Ae. furcifer-taylori* more important in southern and western Africa (Diallo et al., 1999; McIntosh et al., 1977) and *Ae. africanus* more important in central regions (Jupp and McIntosh, 1988; McCrae et al., 1971; Weinbren et al., 1958). Since the E2-211I mutation was predominantly found in the CHIKV strains isolated in central Africa, it is possible that this mutation might give a selective advantage to CHIKV transmitted by *Ae. africanus*. The involvement of the particular species of non-human primates as vertebrate hosts for CHIKV in central Africa is yet to be analyzed.

I cannot exclude the possibility that the observed predominance of E2-211I among CHIKV strains within the ECSA complex arose not due to some selective advantage but rather due to a founder effect. It is possible that the ancestral progenitor of these viruses had an isoleucine at E2-211 and that the absence of selective pressure directed at this position led to fixation of this residue in the viral population. It is also possible that CHIKV strains with T or I at position E2-211 coexist in nature, and changes at this position occur due to some, as of yet unidentified, conditions. In this case, the apparent prevalence of E2-211I among ECSA strains isolated before 2005 could be an artifact resulting from the limited numbers of CHIKV strains available for analysis. This hypothesis is supported by recent studies of CHIKV evolution on Comoros and Reunion islands (Kariuki Njenga et al., 2008; Schuffenecker et al., 2006). It was shown that the

strains of CHIKV that caused the 2005-2006 outbreak on Reunion were almost identical to those isolated during the 2005 outbreak on Comoros island. Two out of three sequenced isolates from Comoros have E2-211I and one has E2-211T, indicating that both variants were simultaneously circulating in the region (Kariuki Njenga et al., 2008). Interestingly, *Ae. aegypti* was likely the only vector involved in this initial epidemic (Kariuki Njenga et al., 2008), which is consistent with my observation that the E2-I211T mutation does not affect CHIKV infectivity for this vector. The precise distribution of E2-211I and E2-211T residues among early strains (prior to the appearance of the E1-A226V mutation) from the 2005-2006 outbreak on Reunion is unknown. However, the presence of only E2-211T in three of three sequences with the E1-226A circumstantially indicates that E2-211T was probably the predominant variant on Reunion islands.

Position E2-211 is located within, or in close proximity to, the sites that have previously been shown to harbor genetic determinants of host specificity for several alphaviruses. Substitution E2-S218N was responsible for increased VEEV subtype IE infectivity to *Ae. taeniorhynchus* mosquitoes (Brault et al., 2004b). The deletion of E2-200-220 in SINV significantly decreases infectivity of the strain MRE16 to *Ae. aegypti* (Myles et al., 2003). Mutations at E2-T213R of VEEV (Anishchenko et al., 2006) and E2-T219A of Ross River virus (RRV) (Burness et al., 1988) were also shown to be responsible for adaptation of these viruses to a new host species. Long ago, it was suggested that this region of the E2 protein constitutes a cell-receptor binding domain (Strauss and Strauss, 1994) and mutations here might affect mosquito infectivity by disruption of the proper interactions of alphavirus with their receptor(s) expressed on midgut cells (Myles et al., 2003). The atomic structure of E2 has not been solved for any alphavirus, but several lines of evidence indicate that the regions around position E2-211 are exposed on the virion surface and are involved in interactions with cellular receptors (Strauss and Strauss, 1994). Analysis of SINV escape mutants resistant to six neutralizing monoclonal antibodies (MAb) identified that all changes occurred between residues 183-216 of the E2 protein, suggesting that this region constitutes prominent antigenic domain(s) that interact directly with neutralizing antibodies (Strauss et al., 1991). In an alternative approach using λgt11 clones expressing parts of E2, the same 183-216 aa. region was found to interact with five MAbs reactive to E2 protein (Wang and Strauss, 1991). Anti-idiotypic antibodies produced to one of the MAbs (MAb49) that was used by

(Strauss et al., 1991; Wang and Strauss, 1991) blocked SINV binding by up to 50% and were capable of immunoprecipitating a 63kD protein from chicken cell's plasma membranes. Interestingly, the SINV escape mutant for MAb49 has a single aa. substitution E2-R214P (Wang et al., 1991a). Cryoelectron microscopy of RRV complexed with the Fab fragment of MAb T10C9, which binds in the vicinity of the E2-216 residue, revealed that the binding region is located at the outermost tip of the E2 glycoprotein (Smith et al., 1995). Cryoelectron microscopy followed by image reconstruction of HS-adapted RRV revealed that HS binds in the same outermost region of the E2 glycoprotein as MAb T10C9 (Zhang et al., 2005). The E2-N218K mutation, which was responsible for adaptation of RRV to HS binding, was also responsible for resistance of the virus to neutralization by MAb T10C9 (Heil et al., 2001). Interestingly, the E2-N218K mutation was originally selected for replication of RRV in chicken embryo fibroblast cells and was shown to attenuate the virus in 1-day old mice (Kerr et al., 1993). This demonstrates that changes in this region of the E2 glycoprotein expand the host range of RRV in cell culture by allowing virus to interact with cell surface HS moieties.

Considering the evidence for the involvement of mutations in the region around position E2-211 in receptor binding and in adaptation to new host species, I believe that the simplest explanation for the specific effects that I have observed for the E2-211I mutation on CHIKV infectivity for *Ae. albopictus* mosquitoes is that this substitution disrupts the ability of CHIKV to interact with a particular receptor on the midgut epithelial cells of *Ae. albopictus*. In agreement with this hypothesis are results of VOPBA experiments (Figures 6.7; 6.8). Thus, I showed that mutations at position E2-211 can significantly alter the ability of CHIKV to interact with proteins from BBM of *Ae. albopictus* midgut cells. Only CHIKV expressing E2-211I residue was able to bind to 110 and 130kDa proteins from BBM, and no proteins were found to interact with CHIKV expressing E2-211T residue. The identity of 110 and 130kDa proteins and biological role of their interactions with CHIKV remain to be determined. Interestingly, the experiments of direct CHIKV binding to the total BBM extracts or native midguts cells of *Ae. albopictus* and TEM analysis showed no marked differences between binding ability of CHIKV with either isoleucine or threonine at position E2-211. This indicates that not all proteins from *Ae. albopictus* that are involved in interactions with CHIKV *in vivo* can be

elucidated using VOPBA experiments. Thus, based on these results it is possible to assume that CHIKV with an E2-211T residue might interact with some proteins that are not recognized by CHIKV with the E2-211I residue. This protein(s) serving as the receptor might therefore be responsible for targeting of CHIKV to the specific endosomal compartments/domains with a unique lipid composition that favors fusion of CHIKV possessing valine at E1-226, as compared to alanine. If this pathway is blocked by the E2-211I mutation then CHIKV may infect *Ae. albopictus* mosquitoes using an alternative receptor(s) that targets the virus into different endosomes, in which the presence of the E1-A226V does not result in differential infectivity. This hypothesis is in agreement with my observation that virus with E2-211T residue was found more often interacting with apical part of plasma membrane of *Ae. albopictus* (where CHIKV internalization most likely to occur) as compared to CHIKV with E2-211I residue (Figure 6.13). It is also tempting to suggest that, in *Ae. aegypti* mosquitoes, only the second (alternative) pathway is available for CHIKV infection, thereby making this species insensitive to the E1-A226V mutation.

The role of different receptors in targeting of the same ligand to the different endosomal compartment has been clearly demonstrated for anthrax toxin. Anthrax toxin consists of three subunits which self-assemble into toxic complex after one of these proteins, protective antigen (PA), binds to its receptors [tumor endothelial marker 8 (TEM8) or capillary morphogenesis protein 2 (CMG2)] on the surface of the cells and is internalized by clathrin-mediated endocytosis. Following endosomal acidification PA dissociates from its receptors and inserts into the lipid bilayer forming transmembrane pore. It has been shown that if PA interacts with TEM8, the pore formation occur at the mild pH of early endosomes, however, interaction of PA with the second receptor CMG2 leads to pore formation in the more acidic pH of late endosomes (Rainey et al., 2005). It also has been proposed that dissociation of PA from the CMG2 receptor requires higher pH for pore formation than that of the PA-TEM8 complexes because PA has a higher affinity to CMG2 than to TEM8 protein (Scobie et al., 2005; Wigelsworth et al., 2004). However, a recent study challenged this hypothesis showing that the affinity of PA for CMG2 and TEM8 expressing cells are similar (Liu et al., 2007). It is possible to suggest that CHIKV interactions with different proteins from midgut cells of *Ae. albopictus* due

to mutations at E2-211 could have similar consequences on determining the location of the fusion step within endosomal compartments.

An alternative explanation of the effects of different residues at E2-211 on CHIKV infectivity to *Ae. albopictus* is that these mutations might affect the stability of the E2-E1 heterodimers, which would differentially affect fusion properties of the CHIKV with E1-226A or E1-226Vresidues. Thus, it has been shown that the mutation E2-D216G (which is located in close proximity to position E2-211) can rescue a PE2 cleavage mutant of SINV by disrupting the E2-E1 heterodimer stability under acidic condition (Smit et al., 2001). However, my experiments demonstrate that there are no significant differences in the pH threshold for membrane fusion (Figure 6.6) or cholesterol dependence of CHIKV containing E2-211T or E2-211I (data not shown). These data indicate that these mutations probably affect steps in CHIKV cells entry preceding membrane fusion.

The observation that mutations in the E2 protein can significantly alter the sensitivity of CHIKV to effects of the mutations at E1-226 also raised the question of whether or not the E1-A226V mutation will have the same effect on infectivity of other alphaviruses to *Ae. albopictus*. For several alphaviruses including: EEEV (Mitchell et al., 1992; Scott et al., 1990), VEEV (Beaman and Turell, 1991; Fernandez et al., 2003), RRV (Mitchell and Gubler, 1987; Mitchell et al., 1987) and Mayaro virus - MAYV (Smith and Francy, 1991) *Ae. albopictus* could serve as competent vector, and the majority of known alphaviruses have alanine at E1-226. Thus, it is possible that the E1-A226V mutation may cause the same increase in mosquito infectivity which was observed for CHIKV. However, the analysis of the effect of the E1-A226V mutation on VEEV, MAYV and ONNV infectivity showed that this mutation is insufficient to significantly increase virus infectivity to this mosquito. In fact, the infectivity of VEEV and MAYV with the E1-A226V mutation was even slightly lower than infectivity of these viruses having alanine at E1-226. Therefore, the possible adaptation of alphaviruses to *Ae. albopictus* mosquitoes would require mutation(s) in a genome region other than E1-226. The possible explanation of these results is that due to high genetic divergence of VEEV, MAYV and ONNV from CHIKV they cannot interact with correct cell surface receptor on midgut cells of *Ae. albopictus* which is responsible for manifestation of positive effect

of E1-A226V mutation. However, we also cannot exclude the possibility that other mechanisms might be involved in this process.

CONCLUSIONS

Additional studies are required to investigate the precise molecular mechanisms responsible for the observed, unique roles of the substitutions at position E2-60 and E2-211 of CHIKV. The widespread and increasing distribution of *Ae. albopictus* (Benedict et al., 2007; Gratz, 2004) represents a potential threat with respect to the spread and establishment of CHIKV in other tropical and temperate regions. The current study reveals that the mosquito species-specificity of CHIKV, and potentially of other important human and animal pathogens, can be influenced by multiple genes that can act synergistically. Understanding the complex virus-vector interactions and their underlying mechanisms is critical to enhancing our capacity to assess the risks of epidemic emergence. Furthermore, understanding these interactions may also reveal targets that can be exploited for the design of antiviral strategies to modify viral infectivity/attenuation and identify cellular molecules and pathways involved in the infection process.

CHAPTER 7: CONCLUSIONS

In the past few years Chikungunya virus has caused its largest outbreak in documented history, affecting parts of Africa, the Indian Ocean islands, India, and Europe, which rapidly has elevated this arbovirus to the status of a major global health problem (Simon et al., 2008). The unique feature of this epidemic was the involvement of a previously unrecognized vector species, *Ae. albopictus,* as a critical contributor facilitating spread of the virus in the affected areas. Therefore, it has been proposed that during this epidemic CHKIV might have accumulated a specific mutation(s) which would ensure more efficient virus transmission by this mosquito species (Schuffenecker et al., 2006). The aim of this dissertation was to develop an infectious clone (i.c) from an isolate of CHIKV collected during the epidemic, to use this clone as a tool to characterize the genetic factors responsible for adaptation of CHIKV to the epidemic vector - *Ae. albopictus* mosquitoes, and to investigate the underlying mechanism of action associated with these factors.

Characterization of the genetic determinants responsible for adaptation of CHIKV to a new vector *Ae. albopictus* is critical to improving our understanding of CHIKV transmission dynamics in the areas lacking the typical CHIKV vector – *Ae. aegypti*. This information will enhance our ability to assess and predict the potential of CHIKV to invade and emerge in new geographic areas, and therefore is important for the development of effective vector control strategies directed towards specific arthropod vector species. Delineation of the molecular mechanism responsible for adaptation of the arbovirus to its vector species, may also help us to understand general principles governing the transmission process and in doing so, perhaps gain insight into counteracting strategies which will lessen the efficiency of virus transmission by arthropods.

The central goal of this dissertation project was to determine what viral genetic factors define the process of CHIKV adaptation to *Ae. albopictus* mosquitoes, and to investigate the molecular mechanisms of how these factors affect fitness of CHIKV in this mosquito species. The first step toward the achievement of this goal was to develop the infectious cDNA clones based on the epidemic strain of CHIKV (LR2006 OPY1) isolated during the 2005-2006 CHIKV epidemic on Reunion Island. This epidemic was the first well-documented example of a chikungunya outbreak where the vector

responsible for virus transmission was not *Ae. aegypti,* but instead was the previously unrecognized vector species, *Ae. albopictus* (X. de Lamballerie, personal communication)(Delatte et al., 2008). Therefore it was hypothesized that the CHIKV strains circulating on the island might have undergone an adaptation to this new vector. Thus, the complete genome of the LR2006 OPY1 strain was sequenced revealing the presence of an E1-A226V mutation, which was suspected, might be involved in CHIKV adaptation to *Ae. albopictus* (Schuffenecker et al., 2006). The nucleotide sequence of the infectious clone (designated as pCHIKV-LR i.c.) produced as part of this dissertation research, and reported in the jounal Vector-Borne and Zoonotic Diseases (Tsetsarkin et al., 2006) was identical to the sequence of the LR2006 OPY1 strain except for one silent mutation at position 7738 C→A (Capsid-P57P). However, virus produced from the CHIKV-LR i.c has retained the phenotypes of the original isolate in both cell culture and in *Ae. aegypti* and *Ae. albopictus* mosquitoes indicating that the 7738 C→A silent mutation and possible other artifacts of the i.c. construction such as incompatibility of viral segments that were combined in the clone-derived virus (but that coexist separately as quasispecies in the original virus) do not significantly affect the phenotype of the clone-derived virus. Infectious clones that express the enhanced green fluorescent protein (eGFP) gene from either a 3' or 5' promoter were also produced and characterized in cell culture and in *Aedes* mosquitoes. The virus produced from CHIKV-LR 5'GFP i.c. was markedly more stable than CHIKV-LR 3'GFP during serial passage in C6/36 and BHK-21 cells. It also infected *Ae. aegypti* and *Ae. albopictus* mosquitoes at a rate similar to that observed for both the original LR2006 OPY1 virus, and virus derived from pCHIKV-LR i.c. However, virus produced from CHIKV-LR 3'GFP i.c. only infected *Ae. albopictus* mosquitoes, but failed to infect *Ae. aegypti*. Overall I concluded that, pCHIKV-LR i.c. and pCHIK-LR 5'GFP infectious clones were suitable for investigation of genetic factors influencing CHIKV fitness in the mosquito vector.

One of the interesting features of the chikungunya epidemic on Reunion Island was the detection of an alanine to valine mutation at position 226 in the E1 envelope glycoprotein among viral isolates obtained during the outbreak (Schuffenecker et al., 2006). To test the hypothesis that the E1-A226V mutation in the epidemic CHIKV (strain LR2006 OPY1) was responsible for CHIKV adaptation to *Ae. albopictus* mosquitoes, the viral infectivity, dissemination, and transmission of CHIKV were compared in *Ae.*

albopictus (Galveston strain), the species implicated in the epidemic, and the recognized typical vector *Ae. aegypti* (Higgs white-eyed Rexville D strain). Using viral infectious clones of Reunion (LR2006 OPY1) and West African (37997) strains of CHIKV, into which either the E1-226A or the E1-226V residues were engineered, I demonstrated that the E1-A226V mutation was directly responsible for a significant increase in CHIKV infectivity for *Ae. albopictus*, and led to more efficient viral dissemination into mosquito secondary organs and transmission to suckling mice. These findings demonstrate that the E1-A226V mutation can act through multiple phenotypic effects that confer CHIKV adaptation to transmission by *Ae. albopictus,* and provide a plausible explanation of how this mutant virus caused an epidemic in a region lacking the typical vector. This work was published in PloS.Pathogens journal (Tsetsarkin et al., 2007). Similar results confirming the role of E1-A226V mutation were provided by Vazeille et al. (2007) who studied interactions of the Indian Ocean 05.115 and 06.21strains of CHIKV with *Ae. albopictus* mosquitoes collected from Reunion and Mayotte islands. Thus, Vazeille et al. (2007) showed that CHIKV with an E1-226V residue better replicates in the midguts, and disseminates more efficiently into salivary glands, of *Ae. albopictus* mosquitoes as compared to CHIKV with an E1-226A residue. This indicates that the effects of the E1-A226V mutation on CHIKV fitness in *Ae. albopictus* observed in our study would be probably common for different geographical strains of this mosquito species. Later studies of the CHIKV outbreaks maintained by *Ae. albopictus*-human transmission cycle independently demonstrated convergent acquisition of E1-A226V mutations in the unrelated CHIKV lineages which additionally supports my findings (Cherian et al., 2009; de Lamballerie et al., 2008; Kumar et al., 2008; Pages et al., 2009; Schuffenecker et al., 2006). These studies altogether provide compelling evidence incriminating the E1-A226V mutation as the major determinant of CHIKV adaptation to *Ae. albopictus* mosquitoes.

Interestingly, the analysis of effects of the E1-A226V mutation on the fitness of CHIKV in *Ae. aegypti* mosquitoes showed that in contrast to the situation observed for *Ae. albopictus*, this mutation caused a marginal decrease in the ability of CHIKV to infect the *Ae. aegypti* midgut, although it had no effect on viral dissemination, and was associated with a slight increase in transmission by *Ae. aegypti* to suckling mice in competition experiments. These findings may explain why the E1-226V residue was not

detected in the genome of CHIKV strains collected during previous (before 2006) outbreaks where *Ae. aegypti* was the vector and the predominance of E1-226A viruses during the 2006 CHIKV epidemic in India, in which *Ae. aegypti* is considered to be the main vector species (Arankalle et al., 2007).

The synergistic effects of increased infectivity and faster dissemination (i.e. shorter extrinsic incubation period) that I identified for the E1-A226V virus in *Ae. albopictus* would accelerate virus transmission to a naïve human population, and as a result, would likely have contributed to initiating and sustaining the 2005-2006 CHIKV epidemic on Reunion island. However, the precise role played by the E1-A226V mutation at the different steps of the infection process remained unknown. The growth kinetics of LR-ApaI-226V and LR-226A viruses in bodies of orally infected *Ae. albopictus* mosquitoes suggested that the E1-A226V mutation exerts its effect very early after infection. The titers of these viruses in *Ae. albopictus* were statistically different at 1 dpi, but were similar at later time points (Figure 3.11). Comparison of the effects of A and V residues at E1-226 on CHIKV dissemination in, and transmission by, orally and intrathoracically infected *Ae. albopictus* mosquitoes demonstrated that an increase in midgut infectivity/replication is the most important phenotypic effect of the E1-226V mutation, which subsequently determines other phenotypic manifestations such as increased dissemination and transmission of the virus by *Ae. albopictus*. I concluded that more efficient infectivity of the midgut cells associated with the E1-A226V mutation leads to more efficient virus replication in this mosquito vector, more rapid dissemination of the virus into salivary glands and more efficient transmission. These findings also justify my primary interest in CHIKV infectivity of midgut cells of *Ae. albopictus.*

The next important objective of the research was to determine the underlying molecular mechanism of action of the E1-A226V mutation on the CHIKV infectivity for *Ae. albopictus* midgut cells. Ideally these studies require a cell culture model which mimics the effect of this mutation *in vitro*. Unfortunately, there are no available cell culture systems based on the midgut epithelial cells for any mosquito species. Also, the E1-A226V-associated increase in infectivity cannot be reproduced using *Ae. albopictus*-derived C6/36 cells, nor mammalian BHK-21 or Vero cells. In these circumstances we hypothesized that an acceptable alternative approach could be the characterization of particular parameters of the infection process *in vitro* for CHIKV, which differ between

CHIKV with E1-226A or E1-226V residues. The approach depended on the generation of a panel of viruses with mutations that specifically modulate the virus phenotype with respect to this parameter. Therefore using these viruses we could investigate correlation between this particular parameter *in vitro* and mosquito infectivity phenotype *in vivo*.

Since previous studies with Semliki Forest virus (SFV) had demonstrated a relationship between sequence at position E1-226 with viral dependence on cholesterol during cell infection (Vashishtha et al., 1998), we used cholesterol depleted C6/36 cells and demonstrated that the E1-A226V mutation is associated with an increase in cholesterol-dependency of CHIKV for growth and cells entry. To correlate phenotypes of cholesterol-dependency and mosquito infectivity we performed a detailed mutagenesis analysis of position E1-226. We found that CHIKV can tolerate significant variability at this position, and, depending on the residue at E1-226, CHIKV infectivity to *Ae. albopictus* mosquitoes can be classified into three groups: highly infectious (residues: V, I, M and L); intermediately infectious (residues: H and P); and poorly infectious (residues: A, S, T, G, F and D). Analysis of CHIKV with specific mutations at E1-226 indicated that all residues which confer high infectivity for *Ae. albopictus* were also responsible for an increase in cholesterol-dependence of CHIKV, however, there was no apparent correlation between cholesterol-dependence and mosquito infectivity phenotypes for virus containing residues responsible for intermediate and low *Ae. albopictus* infectivity. To investigate if increased CHIKV dependence for cholesterol is directly necessary for increased infectivity to *Ae. albopictus* mosquitoes, mutations in genome region other than position E1-226 (which we showed to confer the cholesterol-independent phenotype [namely; double mutation (E1-A66S and E1-D70N) or E1-Q222H)] were introduced into the LR-GFP-226V virus. Resultant viruses were then tested in *Ae. albopictus*. Interestingly, using these mutants we also observed that there was no apparent mechanistic correlation between cholesterol-dependence and mosquito infectivity phenotypes. The simplest explanation for these effects is that cholesterol dependence and infectivity to *Ae. albopictus* are two independent phenotypic effects of the same mutations at E1-226. Alternatively, CHIKV dependence for cholesterol might be controlled at several steps of infectious process such as E2-E1 dissociation, a hypothetical initial lipid-sensing step, acid-specific epitope exposure and/or homotrimerazation of E1, fusion peptide insertion and fusion. Thus, cholesterol-

independent viruses (for example LR-226A and LR-226S) do not relay on cholesterol at each of these steps, however, cholesterol-dependent viruses may require cholesterol at the different (not overlapping) steps, which are not necessarily connected to *Ae. albopictus* infectivity.

In this study the cholesterol-dependence of particular mutations in CHIKV was measured using the cholesterol-depleted C6/36 cells, however, cholesterol depletion could also induce some changes in membrane lipid composition, lipid distribution, or other properties of the cell membrane that could affect its biological functions (Chatterjee et al., 2000). Although, it was shown that phospholipid composition and fatty acyl chain composition of such sterol-depleted insect cells do not appear significantly altered (Silberkang et al., 1983). It is also possible that E1-A226V mutation modulates CHIKV sensitivity to some lipids other than cholesterol, for example sphingolipid, which also can be connected to CHIKV infectivity to *Ae. albopictus*. Thus it was shown that mutations E1-L44F and E1-V178A of SFV control not only cholesterol but also sphingolipid dependence of the virus (Chatterjee et al., 2002). Therefore I believe that it will be interesting to characterize the effect of the available E1 protein mutants *in vitro* using a liposome system consisting of different lipids, including sphingolipid.

I also showed that the E1-A226V mutation increases the pH dependency of the CHIKV fusion reaction, however, subsequent genetic analysis failed to support a mechanistic association between CHIKV dependency for pH and the mosquito infectivity phenotypes. This and previous data of the role of mutations at E1-226 in controlling cholesterol dependence of CHIKV, indicates that the E1-A226V mutation probably acts at the different steps of CHIKV life cycle affecting multiple functions of the virus.

Interestingly, the common feature of residues at E1-226 that ensures high infectivity phenotype of CHIKV in *Ae. albopictus* is that they all belong to a class of non-polar aliphatic amino acids. Analysis of 3 dimensional models of the E1 protein of CHIKV based on the E1 structure of SFV suggested that interactions between methyl (CH_3-) and/or methylene (-CH_2-) groups of the aliphatic residues at E1-226 with methylene groups at E1-86P located in fusion loop, might be connected to our observed phenotypic effect of the E1-226 mutations in *Ae. albopictus*. The current model of the alphaviral membrane fusion process postulates that upon exposure to low pH and E2-E1 heterodimer dissociation, the fusion loop and/or adjacent ij loop (encompassing position

E1-226) act as "sensors of lipid composition" of target membrane which regulate the stable insertion of the fusion loop into the membrane of endosomes (Chatterjee et al., 2000; Gibbons et al., 2004a; Kielian et al., 2000). Therefore, the mutations that modulate interactions between the fusion and ij loops may act by affecting this "lipid sensor" step. Alternatively, it also is possible that mutations at the tip of the ij loop (E1-226) may act independently of the fusion loop by changing over conformation of the E1 protein which may affect E1 "lipid sensing" properties. Future work is required to more precisely characterize the biochemical events taking place during the "lipid sensing" step of the fusion process and to define the role of mutations at E1-226 in this process.

My analysis of the effect of E1-A226V on infectivity of different alphaviruses other than CHIKV (VEE, SINDV, MAYV) to *Ae. albopictus* demonstrated, that this mutation is not sufficient to significantly increase viral infectivity for this mosquito species. I therefore conclude that possible adaptation of alphaviruses other than CHIKV to *Ae. albopictus* mosquitoes would probably require mutation(s) in genome regions other than at position E1-226. Moreover, although I showed that the E1-A226V mutation can significantly change the ability of CHIKV to infect *Ae. albopictus* midgut cells when expressed in the background of distantly related CHIKV strains LR2006 OPY1 and 37997, the introduction of the E1-A226V mutation into the background of an i.c. derived from the Ag41855 strain (isolated in Uganda in 1982) does not significantly increase CHIKV infectivity for *Ae. albopictus.* Detailed molecular genetic investigation identified amino acid residues at E2-60 and E2-211 that modulate the role of the E1-A226V mutation that first arose during the Indian Ocean epidemic. Thus, the glycine at E2-60 (which is authentic for Ag41855 strain) was an important determinant of CHIKV infectivity for both *Ae. albopictus* and *Ae. aegypti,* but only moderately influenced the effect of the E1-A226V mutation in *Ae. albopictus.* Analysis of distribution of E2-D60G mutation among CHIKV isolates, indicated that this residue is present in 4 out of 39 strains with no apparent close phylogenetic relationships among them. We therefore suggested that the D60G substitution may have been selected by cell culture or animal passage. In agreement with hypothesis are observations that mutations that increase the positive charge of E2 proteins are often associated with adaptation to binding to heparan sulfate (HS) which is abundantly expressed on the cell surface of BHK-21 and Vero cells (Bernard et al., 2000; Byrnes and Griffin, 2000; Klimstra et al., 1998). Additionally, I

demonstrated that the E2-60G residue has some positive effect on CHIKV replication in BHK-21 cells. However, additional studies are required to definitely demonstrate the role of E2-60G mutation in CHIKV interaction with HS. Our data related to these studies were accepted for publication in PloS.ONE jornal.

In contrast to the E2-60G, the residue E2-211I significantly decreases infectivity of the Ag41855 strain for *Ae. albopictus* only when expressed together with the E1-226V mutation. Interestingly, if CHIKV has the pre-2005 ancestral E1-226A residue, then the substitution E2-211I has almost no effect on infectivity for *Ae. albopictus* compared with the E2-211T variant. Additionally, CHIKV infectivity for *Ae. aegypti* was not influenced by the E2-T211I mutation. This indicates that the E2-T211I mutation is responsible for very specific modulation of CHIKV infectivity for *Ae. albopictus* depending on the particular amino acid at E1-226. The occurrence of the E2-211I residues among CHIKV isolates was also analyzed, revealing a high prevalence of E2-211I among strains belonging to the East Central South African (ECSA) clade. This suggests that the E2-211I might be important for adaptation of CHIKV to some particular conditions prevalent in areas occupied by ECSA strains, possibly related to the sylvatic vectors of CHIKV such as *Ae. africanus*. Considering that the process of CHIKV emergence from enzootic cycle into the rural and urban areas has not been sufficiently studied, I believe that it will be very interesting to investigate the role of mutations discovered here on the evolution of CHIKV. Another important conclusion of mosquito infectivity data is that the E1-A226V mutation would not give a selective advantage to the CHIKV strains possessing E2-211I with respect to transmissibility by *Ae. albopictus*, and therefore such viruses would probably not be selected in nature. Considering that before 2005 residue E2-211I was the predominant among CHIKV strains in the ECSA clade we believe that prior to acquisition of the E1-A226V mutation, CHIKV strains should have additionally incorporated the E2-I211T mutation. However, the evolutionary factors that would promote this transition remain unknown.

The molecular mechanism explaining the role of the mutation at position E2-211 in modulating the effect of E1-A226V mutation is unknown, however, several pieces of evidence suggest that mutations at position E2-211 might be involved in modulation of CHIKV's interaction with cell surface receptor(s). Thus, data from the literature indicate that position E2-211 is located within the region which constitutes a cell-receptor binding domain of several alphaviruses, as revealed from studies of antibody escape mutants of SINV and HS binding mutants of Ross River virus (RRV) (Heil et al., 2001; Smith et al., 1995; Strauss et al., 1991; Strauss and Strauss, 1994; Wang et al., 1991a; Wang and Strauss, 1991). Cryoelectron microscopy followed by image reconstruction revealed that the region around position E2-211 is exposed on the virion surface and is located at the outermost tip of the E2 glycoprotein (Figure 7.1).

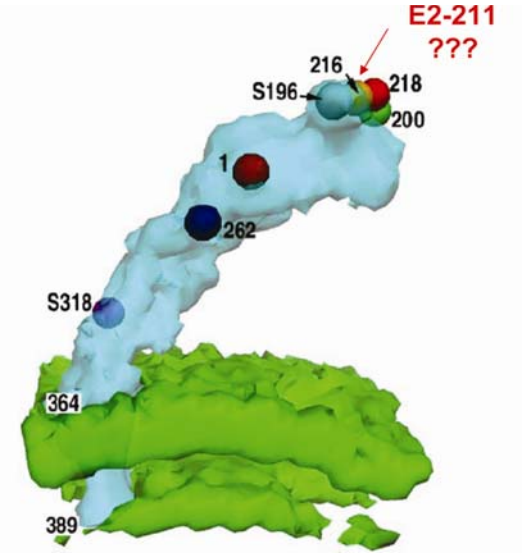


Figure 7.1 Approximate locations of several residue spheres on a single E2 molecule.

RRV residues 200, 216, and 218, and SINV glycosylation site 196 cluster are located at the tip of E2. Adapted from (Zhang et al., 2005)

I showed that the E2-T211I mutation significantly attenuates the growth of CHIKV in BHK-21 cells after standard infection procedures, however, this mutation had no effect on CHIKV replication in the BHK-21 cells that were electroporated with *in vitro* transcribed RNA (i.e omitting the cell entry step). This indicates that residues at position E2-211 probably play a role in CHIKV replication cycle at the steps preceding release of virus RNA into the cytoplasm. Interestingly, my data indicate that mutations at E2-211 do not affect the fusogenic properties of CHIKV, which indirectly suggests that these mutations function at the level of interaction of CHIKV with its cell surface receptor. Moreover, we directly showed that mutations at position E2-211 can significantly alter the ability of CHIKV to interact with proteins from brush border membranes (BBM) derived from *Ae. albopictus* midgut cells. Only CHIKV expressing the E2-211I residue was able to bind to as yet unidentified, 110 and 130kDa proteins from the BBM. No such proteins were found that would interact with CHIKV expressing

the E2-211T residue. Additionally, although the experiments examining the direct binding of CHIKV to the total BBM extracts or native midgut cells of *Ae. albopictus* did not reveal difference between T and I residues at E2-211, the virus with the E2-211T residue was found to interact more often with apical part of plasma membrane of *Ae. albopictus* (where CHIKV internalization is most likely to occur) as compared to CHIKV with the E2-211I residue. This finding also supports the role of the mutation at E2-211 in modulating the capacity of CHIKV to bind to the particular proteins expressed on the midgut cell surface.

Considering the evidence for the involvement of mutations at position E2-211 in receptor binding, we believe that the simplest explanation for the specific effects of the E2-211I mutation on CHIKV infectivity for *Ae. albopictus* mosquitoes, that we have observed, is that this substitution might disrupt the ability of CHIKV to interact with a particular receptor on the midgut epithelial cells of *Ae. albopictus*. This protein(s) serving as receptor might be responsible for targeting of CHIKV to the specific endosomal compartments/domains with a unique lipid/protein composition that favors fusion of CHIKV possessing valine at E1-226, as compared to alanine. If this pathway is blocked by the E2-211I mutation then CHIKV may infect *Ae. albopictus* mosquitoes using an alternative receptor(s) that targets virus into different endosomes, in which the presence of the E1-A226V does not result in differential infectivity. It is also tempting to suggest that, in *Ae. aegypti* mosquitoes, only the second (alternative) pathway is available for CHIKV infection, thereby making this species insensitive to the E1-A226V mutation.

After endocytosis alphaviruses are delivered into morphologically complex early endosomes. Although they have usually been considered to be a homogeneous population, there is increasing evidence that viruses and other endocytosed cargo are selectively targeted to specific populations of endosomes depending (at least partially) on the receptor which is involved in endocytosis (Kirkham et al., 2005; Lakadamyali et al., 2006; Le Blanc et al., 2005; Pons et al., 2008). Thus for example, influenza virus is targeted to the population of early endosomes that rapidly relocates the viruses via microtubule-mediated transport to the perinuclear region where fusion occurs (Lakadamyali et al., 2003). Additionally, the role of different receptors in targeting of the same ligand to the different endosomal compartment has been clearly demonstrated for anthrax toxin (Puhar and Montecucco, 2007). Considering that specific populations

(domains) of endosomes are composed of different proteins and lipids (Falguieres et al., 2009; Gruenberg and van der Goot, 2006; Sobo et al., 2007), it is conceivable that this difference can be detected by the mutations at position E1-226 which define the efficiency of CHIKV cell entry process.

Further investigations are required to determine whether or not the hypothesis of receptor-mediated targeting of CHIKV to the specific endosomal compartment which favors fusion and cell entry of CHIKV with E1-A226V mutation can fully explain process of CHIKV adaptation to *Ae. albopictus*. In this regard the first and most important advancement should be the development of a cells culture system based on midgut epithelial cells of *Ae. albopictus*, that could accurately reproduce the effect of different mutations on CHIKV infectivity *in vitro*. Such a cell system would allow the investigation and characterization of the cellular proteins and entire endosomal pathways which are necessary for CHIKV cell entry. Also it will be important to determine the identity of the cells surface receptor(s) that is used by CHIKV for entry into endosomal compartments of midgut epithelial cells of *Ae. albopictus.*

More studies also will be necessary to explore the detailed evolutionary mechanisms that control the adaptation of arboviruses to a new host species. Thus, in this dissertation I demonstrated that the smallest genetic changes, such as a single E1-A226V mutation, could be sufficient to significantly influence fitness of arboviruses in the new host, and therefore could be responsible for the viral emergence. However, it remains unknown how these mutations are selected in nature. Phylogenetic studies revealed that RNA-arboviruses evolve about ten times slower than many of their directly-transmitted counterparts (Jenkins et al., 2002; Parvin et al., 1986; Wolfs et al., 1990) presumably because mutations that increase viral fitness in one host usually diminish it in the other. Also, recent studies of VEEV demonstrated that in the condition of alternate replication in vertebrate and invertebrate hosts, virus did not demonstrate detectable fitness gains in either host, however VEEV adaptation to each host was detected when virus replication in alternative host was bypassed (Coffey et al., 2008), indicating that RNA arboviruses may be less likely to change host range than vertebrate-only RNA viruses. Similarly, we did not observe acquisition of the E1-A226V mutation after four rounds of propagation of LR-226A in *Ae. albopictus* - Vero cells cycle, even though the E1-A226V mutation does not attenuate CHIKV in Vero cells (data not shown). This indicates that emergence of the

adaptive mutations in arboviral population is very complex and poorly understood process, that requires futher investigation.

REFERENCES

AbuBakar, S., Sam, I. C., Wong, P. F., MatRahim, N., Hooi, P. S., and Roslan, N. (2007). Reemergence of endemic Chikungunya, Malaysia. Emerg Infect Dis *13*, 147-149.

Adebajo, A. O. (1996). Rheumatic manifestations of tropical diseases. Curr Opin Rheumatol *8*, 85-89.

Adhami, J., and Murati, N. (1987). Pre´sence du moustique Aedes albopictus en Albanie. Revista Mjekesore *1*, 13–16.

Adhami, J., and Reiter, P. (1998). Introduction and establishment of Aedes (Stegomyia) albopictus skuse (Diptera: Culicidae) in Albania. J Am Mosq Control Assoc *14*, 340-343.

Aguilar, P. V., Adams, A. P., Wang, E., Kang, W., Carrara, A. S., Anishchenko, M., Frolov, I., and Weaver, S. C. (2008). Structural and nonstructural protein genome regions of eastern equine encephalitis virus are determinants of interferon sensitivity and murine virulence. J Virol *82*, 4920-4930.

Aguilar, P. V., Weaver, S. C., and Basler, C. F. (2007). Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. J Virol *81*, 3866-3876.

Ahn, A., Schoepp, R. J., Sternberg, D., and Kielian, M. (1999). Growth and stability of a cholesterol-independent Semliki Forest virus mutant in mosquitoes. Virology *262*, 452-456.

Andersson, H., Barth, B. U., Ekstrom, M., and Garoff, H. (1997). Oligomerization-dependent folding of the membrane fusion protein of Semliki Forest virus. J Virol *71*, 9654-9663.

Angelini, P., Macini, P., Finarelli, A. C., Pol, C., Venturelli, C., Bellini, R., and Dottori, M. (2008). Chikungunya epidemic outbreak in Emilia-Romagna (Italy) during summer 2007. Parassitologia *50*, 97-98.

Angelini, R., Finarelli, A. C., Angelini, P., Po, C., Petropulacos, K., Macini, P., Fiorentini, C., Fortuna, C., Venturi, G., Romi, R.*, et al.* (2007a). An outbreak of chikungunya fever in the province of Ravenna, Italy. Euro Surveill *12*, E070906 070901.

Angelini, R., Finarelli, A. C., Angelini, P., Po, C., Petropulacos, K., Silvi, G., Macini, P., Fortuna, C., Venturi, G., Magurano, F.*, et al.* (2007b). Chikungunya in north-eastern Italy: a summing up of the outbreak. Euro Surveill *12*, E071122 071122.

Anishchenko, M., Paessler, S., Greene, I.P., Aguilar, P.V., Carrara, A.S., and Weaver, S.C. (2004). Generation and characterization of closely related epizootic and enzootic infectious cDNA clones for studying interferon sensitivity and emergence mechanisms of Venezuelan equine encephalitis virus. Journal of virology *78*, 1-8.

Anishchenko, M., Bowen, R. A., Paessler, S., Austgen, L., Greene, I. P., and Weaver, S. C. (2006). Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. Proc Natl Acad Sci U S A *103*, 4994-4999.

Aranda, C., Eritja, R., and Roiz, D. (2006). First record and establishment of the mosquito Aedes albopictus in Spain. Med Vet Entomol *20*, 150-152.

Arankalle, V. A., Shrivastava, S., Cherian, S., Gunjikar, R. S., Walimbe, A. M., Jadhav, S. M., Sudeep, A. B., and Mishra, A. C. (2007). Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. J Gen Virol *88*, 1967-1976.

Arcus, Y. M., Houk, E. L., and Hardy, J. L. (1983). Comparative in vitro binding of an arbovirus to midgut microvillar membranes from susceptible and refractory Culex mosquitoes. Fed Proc *42*, 21-41.

Arpino, C., Curatolo, P., and Rezza, G. (2009). Chikungunya and the nervous system: what we do and do not know. Rev Med Virol *19*, 121-129.

Banerjee, K., Mourja, D. T., and Malunjkar, A. S. (1988). Susceptibility & transmissibility of different geographical strains of Aedes aegypti mosquitoes to Chikungunya virus. Indian J Med Res *87*, 134-138.

Barrera, R. (1996). Competition and resistance to starvation in larvae of container-inhabiting Aedes mosquitoes. Ecol Entomol *21*, 117-127.

Beaman, J. R., and Turell, M. J. (1991). Transmission of Venezuelan equine encephalomyelitis virus by strains of Aedes albopictus (Diptera: Culicidae) collected in North and South America. J Med Entomol *28*, 161-164.

Bedekar, S. D., and Pavri, K. M. (1969). Studies with Chikungunya virus. I. Susceptibility of birds and small mammals. Indian J Med Res *57*, 1181-1192.

Benedict, M. Q., Levine, R. S., Hawley, W. A., and Lounibos, L. P. (2007). Spread of the tiger: global risk of invasion by the mosquito Aedes albopictus. Vector Borne Zoonotic Dis *7*, 76-85.

Bernard, K. A., Klimstra, W. B., and Johnston, R. E. (2000). Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology *276*, 93-103.

Binn, L. N., Harrison, V. R., and Randall, R. (1967). Patterns of viremia and antibody observed in rhesus monkeys inoculated with chikungunya and other serologically related group A arboviruses. Am J Trop Med Hyg *16*, 782-785.

Bonilauri, P., Bellini, R., Calzolari, M., Angelini, R., Venturi, L., Fallacara, F., Cordioli, P., Angelini, P., Venturelli, C., Merialdi, G., and Dottori, M. (2008). Chikungunya virus in Aedes albopictus, Italy. Emerg Infect Dis *14*, 852-854.

Boorman, J. P., and Draper, C. C. (1968). Isolations of arboviruses in the Lagos area of Nigeria, and a survey of antibodies to them in man and animals. Trans R Soc Trop Med Hyg *62*, 269-277.

Bordi, L., Carletti, F., Castilletti, C., Chiappini, R., Sambri, V., Cavrini, F., Ippolito, G., Di Caro, A., and Capobianchi, M. R. (2008). Presence of the A226V mutation in autochthonous and imported Italian chikungunya virus strains. Clin Infect Dis *47*, 428-429.

Braks, M. A. H., Honorio, N. A., Lounibos, L. P., Lourenco-de-Oliveira, R., and Juliano, S. A. (2004). Interspecific competition between two invasive species of container mosquitoes, Aedes aegypti and Aedes albopictus (Diptera: Culicidae), in Brazil. Ann Entomol Soc Am *97*, 130-139.

Brault, A. C., Foy, B. D., Myles, K. M., Kelly, C. L., Higgs, S., Weaver, S. C., Olson, K. E., Miller, B. R., and Powers, A. M. (2004a). Infection patterns of o'nyong nyong virus in the malaria-transmitting mosquito, Anopheles gambiae. Insect Mol Biol *13*, 625-635.

Brault, A. C., Powers, A. M., Ortiz, D., Estrada-Franco, J. G., Navarro-Lopez, R., and Weaver, S. C. (2004b). Venezuelan equine encephalitis emergence: enhanced vector infection from a single amino acid substitution in the envelope glycoprotein. Proc Natl Acad Sci U S A *101*, 11344-11349.

Brault, A. C., Powers, A. M., and Weaver, S. C. (2002). Vector infection determinants of Venezuelan equine encephalitis virus reside within the E2 envelope glycoprotein. J Virol *76*, 6387-6392.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljestrom, P., Fazakerley, J., and McInerney, G. M. (2007). Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. J Virol *81*, 8677-8684.

Bredenbeek, P. J., and Rice, C. M. (1992). Animal RNA virus expression systems. Semin Virol *3*, 297-310.

Bres, P., Camicas, J. L., Cornet, M., Robin, Y., and Taufflieb, R. (1969). Considerations sur l`epididemiologe des arbovirus au Senegal. Bull Soc Pathol Exot *62*, 253-9.

Briegel, H., and Timmermann, S. E. (2001). Aedes albopictus (Diptera: Culicidae): physiological aspects of development and reproduction. J Med Entomol *38*, 566-571.

Brighton, S. W. (1984). Chloroquine phosphate treatment of chronic Chikungunya arthritis. An open pilot study. S Afr Med J *66*, 217-218.

Brighton, S. W., Prozesky, O. W., and de la Harpe, A. L. (1983). Chikungunya virus infection. A retrospective study of 107 cases. S Afr Med J *63*, 313-315.

Briolant, S., Garin, D., Scaramozzino, N., Jouan, A., and Crance, J. M. (2004). In vitro inhibition of Chikungunya and Semliki Forest viruses replication by antiviral compounds: synergistic effect of interferon-alpha and ribavirin combination. Antiviral Res *61*, 111-117.

Broche, R. G., and Borja, E. M. (1999). Aedes albopictus in Cuba. J Am Mosq Control Assoc *15*, 569-570.

Bron, R., Wahlberg, J. M., Garoff, H., and Wilschut, J. (1993). Membrane fusion of Semliki Forest virus in a model system: correlation between fusion kinetics and structural changes in the envelope glycoprotein. Embo J *12*, 693-701.

Burness, A. T., Pardoe, I., Faragher, S. G., Vrati, S., and Dalgarno, L. (1988). Genetic stability of Ross River virus during epidemic spread in nonimmune humans. Virology *167*, 639-643.

Byrnes, A. P., and Griffin, D. E. (1998). Binding of Sindbis virus to cell surface heparan sulfate. J Virol *72*, 7349-7356.

Byrnes, A. P., and Griffin, D. E. (2000). Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. J Virol *74*, 644-651.

Cadd, T. L., Skoging, U., and Liljestrom, P. (1997). Budding of enveloped viruses from the plasma membrane. Bioessays *19*, 993-1000.

Calisher, C. H. (1999). Chikungunya, O'nyong nyong and Mayaro viruses (*Togaviridae*), In Encyclopedia of Virology, A. Granoff, and R. G. Webster, eds. (London: Elsevier Science & Technology Books.), pp. 236- 241.

Calisher, C. H., Karabatsos, N., Lazuick, J. S., Monath, T. P., and Wolff, K. L. (1988). Reevaluation of the western equine encephalitis antigenic complex of alphaviruses (family Togaviridae) as determined by neutralization tests. Am J Trop Med Hyg *38*, 447-452.

Calvo, E., Andersen, J., Francischetti, I. M., de, L. C. M., deBianchi, A. G., James, A. A., Ribeiro, J. M., and Marinotti, O. (2004). The transcriptome of adult female Anopheles darlingi salivary glands. Insect Mol Biol *13*, 73-88.

Canavoso, L. E., Jouni, Z. E., Karnas, K. J., Pennington, J. E., and Wells, M. A. (2001). Fat metabolism in insects. Annu Rev Nutr *21*, 23-46.

Carey, D. E. (1971). Chikungunya and dengue: a case of mistaken identity? J Hist Med Allied Sci *26*, 243-262.

Carey, D. E., Myers, R. M., DeRanitz, C. M., Jadhav, M., and Reuben, R. (1969). The 1964 chikungunya epidemic at Vellore, South India, including observations on concurrent dengue. Trans R Soc Trop Med Hyg *63*, 434-445.

Carletti, F., Bordi, L., Chiappini, R., Ippolito, G., Sciarrone, M. R., Capobianchi, M. R., Di Caro, A., and Castilletti, C. (2007). Rapid detection and quantification of chikungunya virus by a one-step reverse transcription polymerase chain reaction real-time assay. Am J Trop Med Hyg *77*, 521-524.

Carrieri, M., Bacch, M., Bellini, R., and Maini, S. (2003). On the competition occurring between Aedes albopictus and Culex pipiens (Diptera: Culicidae) in Italy, l. Environ Entomol *32*, 1313-1321.

CDC. (2007). Update: chikungunya fever diagnosed among international travelers--United States, 2006. MMWR Morb Mortal Wkly Rep *56*, 276-277.

CDC. Information on *Aedes albopictus*. http://wwwcdcgov/ncidod/dvbid/arbor/albopic_newhtm, (retrieved 05/2009).

Chamberlain, R. W., and Sudia, W. D. (1955). The effects of temperature upon the extrinsic incubation of eastern equine encephalitis in mosquitoes. Am J Hyg *62*, 295-305.

Chamberlain, R. W., and Sudia, W. D. (1961). Mechanism of transmission of viruses by mosquitoes. Annu Rev Entomol *6*, 371-390.

Charrel, R. N., de Lamballerie, X., and Raoult, D. (2007a). Chikungunya outbreaks--the globalization of vectorborne diseases. N Engl J Med *356*, 769-771.

Charrel, R. N., de Lamballerie, X., and Raoult, D. (2007b). Chikungunya outbreaks: The authors reply. N Engl J Med *356*, 2651-2652.

Chastel, C. (1964). [Human Infections In Cambodia By The Chikungunya Virus Or A Closely Related Agent. 3. Epidemiology.]. Bull Soc Pathol Exot Filiales *57*, 65-82.

Chastel, C. (2005). [Chikungunya virus: its recent spread to the southern Indian Ocean and Reunion Island (2005-2006)]. Bull Acad Natl Med *189*, 1827-1835.

Chatterjee, P. K., Eng, C. H., and Kielian, M. (2002). Novel mutations that control the sphingolipid and cholesterol dependence of the Semliki Forest virus fusion protein. J Virol *76*, 12712-12722.

Chatterjee, P. K., Vashishtha, M., and Kielian, M. (2000). Biochemical consequences of a mutation that controls the cholesterol dependence of Semliki Forest virus fusion. J Virol *74*, 1623-1631.

Cherian, S. S., Walimbe, A. M., Jadhav, S. M., Gandhe, S. S., Hundekar, S. L., Mishra, A. C., and Arankalle, V. A. (2009). Evolutionary rates and timescale comparison of

Chikungunya viruses inferred from the whole genome/E1 gene with special reference to the 2005-07 outbreak in the Indian subcontinent. Infect Genet Evol *9*, 16-23.

Chevillon, C., Briant, L., Renaud, F., and Devaux, C. (2008). The Chikungunya threat: an ecological and evolutionary perspective. Trends Microbiol *16*, 80-88.

Choi, H. K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M. G., and Wengler, G. (1991). Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. Nature *354*, 37-43.

Christophers, R. (1960). Aedes aegypti. The yellow fever mosquito: its life history, bionomics, and structure.: (Cambridge: Cambridge University Press.), p 739.

Clayton, R. B. (1964). The Utilization Of Sterols By Insects. J Lipid Res *15*, 3-19.

Coffey, L.L., Vasilakis, N., Brault, A.C., Powers, A.M., Tripet, F., and Weaver, S.C. (2008). Arbovirus evolution in vivo is constrained by host alternation. Proceedings of the National Academy of Sciences of the United States of America *105*, 6970-6975.

Colpitts, T. M., Moore, A. C., Kolokoltsov, A. A., and Davey, R. A. (2007). Venezuelan equine encephalitis virus infection of mosquito cells requires acidification as well as mosquito homologs of the endocytic proteins Rab5 and Rab7. Virology *369*, 78-91.
Condreay, L. D., and Brown, D. T. (1986). Exclusion of superinfecting homologous virus by Sindbis virus-infected Aedes albopictus (mosquito) cells. J Virol *58*, 81-86.

Condreay, L. D., and Brown, D. T. (1988). Suppression of RNA synthesis by a specific antiviral activity in Sindbis virus-infected Aedes albopictus cells. J Virol *62*, 346-348.

Consigny, P. H., Lecuit, M., and Lortholary, O. (2006). [Chikungunya virus: a reemerging alphavirus]. Med Sci (Paris) *22*, 444-446.

Cordel, H., Quatresous, I., Paquet, C., and Couturier, E. (2006). Imported cases of chikungunya in metropolitan France, April 2005 - February 2006. Euro Surveill *11*, E060420 060423.

Cornet, M., Robin, Y., Chateau, R., Heme, G., Adam, C., Valade, M., Le Gonidec, G., Jan, C., Renaudet, J., Dieng, P*., et al.* (1979). Isolements d`arbovirus au Senegal oriental a partur de moustiques (1972-1977) et notes sur l`epidemiologic des virus par les *Aedes*, en particulier du virus amaril. Cah ORSTOM Ser Entolol Med Perasitol *17*, 149.

Cortazzo, P., Cervenansky, C., Marin, M., Reiss, C., Ehrlich, R., and Deana, A. (2002). Silent mutations affect in vivo protein folding in Escherichia coli. Biochem Biophys Res Commun *293*, 537-541.

Costanzo, K. S., Kesavaraju, B., and Juliano, S. A. (2005). Condition-specific competition in container mosquitoes: the role of noncompeting life-history stages. Ecology *86*, 3289-3295.

Couderc, T., Chretien, F., Schilte, C., Disson, O., Brigitte, M., Guivel-Benhassine, F., Touret, Y., Barau, G., Cayet, N., Schuffenecker, I.*, et al.* (2008). A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. PLoS Pathog *4*, e29.

Cuellar-Jimenez, M. E., Velasquez-Escobar, O. L., Gonzalez-Obando, R., and Morales-Reichmann, C. A. (2007). [Detection of Aedes albopictus (Skuse) (Diptera: Culicidae) in the city of Cali, Valle del Cauca, Colombia]. Biomedica *27*, 273-279.

Dash, P. K., Parida, M. M., Santhosh, S. R., Verma, S. K., Tripathi, N. K., Ambuj, S., Saxena, P., Gupta, N., Chaudhary, M., Babu, J. P.*, et al.* (2007). East Central South African genotype as the causative agent in reemergence of Chikungunya outbreak in India. Vector Borne Zoonotic Dis *7*, 519-527.

Daugherty, M. P., Alto, B. W., and Juliano, S. A. (2000). Invertebrate carcasses as a resource for competing Aedes albopictus and Aedes aegypti (Diptera: Culicidae). J Med Entomol *37*, 364-372.

Davis, N. C. (1931). The effect of various temperatures in modifying the extrinsic incubation period of the yellow fever virus in Aedes aegypti. Am J Hyg *16*, 163-176.

Davis, N. L., Willis, L. V., Smith, J. F., and Johnston, R. E. (1989). In vitro synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. Virology *171*, 189-204.

de Groot, R. J., Hardy, W. R., Shirako, Y., and Strauss, J. H. (1990). Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. Embo J *9*, 2631-2638.

De, I., Fata-Hartley, C., Sawicki, S. G., and Sawicki, D. L. (2003). Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. J Virol *77*, 13106-13116.

de Lamballerie, X., Leroy, E., Charrel, R. N., Ttsetsarkin, K., Higgs, S., and Gould, E. A. (2008). Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J *5*, 33.

de Lamballerie, X., Ninove, L., and Charrel, R. N. (2009). Antiviral treatment of chikungunya virus infection. Infect Disord Drug Targets *9*, 101-104.

Delatte, H., Paupy, C., Dehecq, J. S., Thiria, J., Failloux, A. B., and Fontenille, D. (2008). [Aedes albopictus, vector of chikungunya and dengue viruses in Reunion Island: biology and control]. Parasite *15*, 3-13.

Deller, J. J., Jr., and Russell, P. K. (1967). An analysis of fevers of unknown origin in American soldiers in Vietnam. Ann Intern Med *66*, 1129-1143.

Depoortere, E., and Coulombier, D. (2006). Chikungunya risk assessment for Europe: recommendations for action. Euro Surveill *11*, E060511 060512.

DeTulleo, L., and Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. Embo J *17*, 4585-4593.

Diallo, M., Thonnon, J., Traore-Lamizana, M., and Fontenille, D. (1999). Vectors of Chikungunya virus in Senegal: current data and transmission cycles. Am J Trop Med Hyg *60*, 281-286.

Doms, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. (1993). Folding and assembly of viral membrane proteins. Virology *193*, 545-562.

Duffus, W. A., Levy-Mintz, P., Klimjack, M. R., and Kielian, M. (1995). Mutations in the putative fusion peptide of Semliki Forest virus affect spike protein oligomerization and virus assembly. J Virol *69*, 2471-2479.

Durbin, R. K., and Stollar, V. (1985). Sindbis virus mutants able to replicate in methionine-deprived Aedes albopictus cells. Virology *144*, 529-533.

Earp, L. J., Delos, S. E., Netter, R. C., Bates, P., and White, J. M. (2003). The avian retrovirus avian sarcoma/leukosis virus subtype A reaches the lipid mixing stage of fusion at neutral pH. J Virol *77*, 3058-3066.

ECDC. (2007). Chikungunya outbreak in Italy: what risk for Europe? http://www.ecdceuint/Health_topics/Chikungunya_Fever/italy/070914_ITA_chickunguya.html, (retrieved 10/2007).

Economopoulou, A., Dominguez, M., Helynck, B., Sissoko, D., Wichmann, O., Quenel, P., Germonneau, P., and Quatresous, I. (2009). Atypical Chikungunya virus infections: clinical manifestations, mortality and risk factors for severe disease during the 2005-2006 outbreak on Reunion. Epidemiol Infect *137*, 534-541.

Edelman, R., Tacket, C. O., Wasserman, S. S., Bodison, S. A., Perry, J. G., and Mangiafico, J. A. (2000). Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. Am J Trop Med Hyg *62*, 681-685.

Enserink, M. (2006). Infectious diseases. Massive outbreak draws fresh attention to little-known virus. Science *311*, 1085.

Enserink, M. (2007). EPIDEMIOLOGY: Tropical Disease Follows Mosquitoes to Europe. Science *317*, 1485a.

Enserink, M. (2008). Entomology. A mosquito goes global. Science *320*, 864-866.

Falguieres, T., Luyet, P. P., and Gruenberg, J. (2009). Molecular assemblies and membrane domains in multivesicular endosome dynamics. Exp Cell Res *315*, 1567-1573.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approachusing the bootstrap. Evolution *39*, 783-791.

Fernandez, Z., Moncayo, A. C., Carrara, A. S., Forattini, O. P., and Weaver, S. C. (2003). Vector competence of rural and urban strains of Aedes (Stegomyia) albopictus (Diptera: Culicidae) from Sao Paulo State, Brazil for IC, ID, and IF subtypes of Venezuelan equine encephalitis virus. J Med Entomol *40*, 522-527.

Forattini, O. P. (1986). Aedes (Stegomyia) albopictus (Skuse) identification in Brazil. Revista de Saude Publics (Sao Paulo) *20*, 244-245.

Frohman, M. A. (1993). Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. Methods Enzymol *218*, 340-356.

Frolova, E. I., Fayzulin, R. Z., Cook, S. H., Griffin, D. E., Rice, C. M., and Frolov, I. (2002). Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. J Virol *76*, 11254-11264.

Fung, K. L., and Gottesman, M. M. (2009). A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function. Biochim Biophys Acta *1794*, 860-871.

Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E., and Frolov, I. (2007a). Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. J Virol *81*, 13552-13565.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., and Frolov, I. (2007b). The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. J Virol *81*, 2472-2484.

Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U., and Liljestrom, P. (1990). The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. J Cell Biol *111*, 867-876.

Garry, R. F., Bishop, J. M., Parker, S., Westbrook, K., Lewis, G., and Waite, M. R. (1979). Na+ and K+ concentrations and the regulation of protein synthesis in Sindbis virus-infected chick cells. Virology *96*, 108-120.

Geigenmuller-Gnirke, U., Nitschko, H., and Schlesinger, S. (1993). Deletion analysis of the capsid protein of Sindbis virus: identification of the RNA binding region. J Virol *67*, 1620-1626.

Gerardin, P., Barau, G., Michault, A., Bintner, M., Randrianaivo, H., Choker, G., Lenglet, Y., Touret, Y., Bouveret, A., Grivard, P*., et al.* (2008a). Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Reunion. PLoS Med *5*, e60.

Gerardin, P., Guernier, V., Perrau, J., Fianu, A., Le Roux, K., Grivard, P., Michault, A., de Lamballerie, X., Flahault, A., and Favier, F. (2008b). Estimating Chikungunya prevalence in La Reunion Island outbreak by serosurveys: two methods for two critical times of the epidemic. BMC Infect Dis *8*, 99.

Gibbons, D. L., Ahn, A., Liao, M., Hammar, L., Cheng, R. H., and Kielian, M. (2004a). Multistep regulation of membrane insertion of the fusion peptide of Semliki Forest virus. J Virol *78*, 3312-3318.

Gibbons, D. L., Erk, I., Reilly, B., Navaza, J., Kielian, M., Rey, F. A., and Lepault, J. (2003). Visualization of the target-membrane-inserted fusion protein of Semliki Forest virus by combined electron microscopy and crystallography. Cell *114*, 573-583.

Gibbons, D. L., Reilly, B., Ahn, A., Vaney, M. C., Vigouroux, A., Rey, F. A., and Kielian, M. (2004b). Purification and crystallization reveal two types of interactions of the fusion protein homotrimer of Semliki Forest virus. J Virol *78*, 3514-3523.

Gilotra, S. K., and Shah, K. V. (1967). Laboratory studies on transmission of Chikungunya virus by mosquitoes. Am J Epidemiol *86*, 379-385.

Girard, Y. A., Klingler, K. A., and Higgs, S. (2004). West Nile virus dissemination and tissue tropisms in orally infected Culex pipiens quinquefasciatus. Vector Borne Zoonotic Dis *4*, 109-122.

Glomb-Reinmund, S., and Kielian, M. (1998a). fus-1, a pH shift mutant of Semliki Forest virus, acts by altering spike subunit interactions via a mutation in the E2 subunit. J Virol *72*, 4281-4287.

Glomb-Reinmund, S., and Kielian, M. (1998b). The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. Virology *248*, 372-381.

Golberg, L., and De Meillon, B. (1948). The nutrition of the larva of Aedes aegypti Linnaeus; lipid requirements. Biochem J *43*, 373-379.

Gomez de Cedron, M., Ehsani, N., Mikkola, M. L., Garcia, J. A., and Kaariainen, L. (1999). RNA helicase activity of Semliki Forest virus replicase protein NSP2. FEBS Lett *448*, 19-22.

Gorchakov, R., Frolova, E., Sawicki, S., Atasheva, S., Sawicki, D., and Frolov, I. (2008). A new role for ns polyprotein cleavage in Sindbis virus replication. J Virol *82*, 6218-6231.

Gratz, N. G. (2004). Critical review of the vector status of Aedes albopictus. Med Vet Entomol *18*, 215-227.

Green, D. W., Rowley, W. A., Wong, Y. W., Brinker, J. P., Dorsey, D. C., and Hausler, W. J., Jr. (1980). The significance of western equine encephalomyelitis viral infections in Aedes trivittatus (Diptera: Culicidae) in Iowa. I. Variation in susceptibility of Aedes

trivittatus to experimental infection with three strains of western equine encephalomyelitis virus. Am J Trop Med Hyg *29*, 118-124.

Gruenberg, J., and van der Goot, F. G. (2006). Mechanisms of pathogen entry through the endosomal compartments. Nat Rev Mol Cell Biol *7*, 495-504.

Gubler, D. J., and Rosen, L. (1976). Variation among geographic strains of Aedes albopictus in susceptibility to infection with dengue viruses. Am J Trop Med Hyg *25*, 318-325.

Hahn, Y. S., Grakoui, A., Rice, C. M., Strauss, E. G., and Strauss, J. H. (1989a). Mapping of RNA- temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. J Virol *63*, 1194-1202.

Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989b). Mapping of RNA- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J Virol *63*, 3142-3150.

Hardy, J. L., Houk, E. J., Kramer, L. D., and Reeves, W. C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu Rev Entomol *28*, 229-262.

Harrison, V. R., Binn, L. N., and Randall, R. (1967a). Comparative immunogenicities of chikungunya vaccines prepared in avian and mammalian tissues. Am J Trop Med Hyg *16*, 786-791.

Harrison, V. R., Eckels, K. H., Bartelloni, P. J., and Hampton, C. (1971). Production and evaluation of a formalin-killed Chikungunya vaccine. J Immunol *107*, 643-647.

Harrison, V. R., Marshall, J. D., and Guilloud, N. B. (1967b). The presence of antibody to Chikungunya and other serologically related viruses in the sera of sub-human primate imports to the United States. J Immunol *98*, 979-981.

Hawley, W. A. (1988). The biology of Aedes albopictus. J Am Mosq Control Assoc Suppl *1*, 1-39.

Heil, M. L., Albee, A., Strauss, J. H., and Kuhn, R. J. (2001). An amino acid substitution in the coding region of the E2 glycoprotein adapts Ross River virus to utilize heparan sulfate as an attachment moiety. J Virol *75*, 6303-6309.

Helenius, A., Kartenbeck, J., Simons, K., and Fries, E. (1980). On the entry of Semliki forest virus into BHK-21 cells. J Cell Biol *84*, 404-420.

Higgs, S. (2004). How do mosquito vectors live with their viruses? In Microbe-Vector Interactions in Vector-Borne Diseases, S. H. Gillespie, G. L. Smith, and A. Osbourn, eds. (Cambridge, UK: Cambridge University Press), pp. 103-137.

Higgs, S. (2006). The 2005-2006 Chikungunya epidemic in the Indian Ocean. Vector Borne Zoonotic Dis *6*, 115-116.

Higgs, S., Olson, K. E., Kamrud, K. I., Powers, A. M., and Beaty, B. J. (1997). Viral expression systems and viral infections in insects., In The Molecular Biology of Disease Vectors: A Methods Manual, J. M. Crampton, C. B. Beard, and C. Louis, eds. (London. UK: Chapman & Hall), pp. 457–483.

Higgs, S., Olson, K. E., Klimowski, L., Powers, A. M., Carlson, J. O., Possee, R. D., and Beaty, B. J. (1995). Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. Insect Mol Biol *4*, 97-103.

Houk, E. J., Arcus, Y. M., Hardy, J. L., and Kramer, L. D. (1990). Binding of western equine encephalomyelitis virus to brush border fragments isolated from mesenteronal epithelial cells of mosquitoes. Virus Res *17*, 105-117.

Hsieh, P., and Robbins, P. W. (1984). Regulation of asparagine-linked oligosaccharide processing. Oligosaccharide processing in Aedes albopictus mosquito cells. J Biol Chem *259*, 2375-2382.

Hulst, M. M., van Gennip, H. G., and Moormann, R. J. (2000). Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E(rns). J Virol *74*, 9553-9561.

Ibanez-Bernal, S., and Martinez-Campos, C. (1994). Aedes albopictus in Mexico. J Am Mosq Control Assoc *10*, 231-232.

Inoue, S., Morita, K., Matias, R. R., Tuplano, J. V., Resuello, R. R., Candelario, J. R., Cruz, D. J., Mapua, C. A., Hasebe, F., Igarashi, A., and Natividad, F. F. (2003). Distribution of three arbovirus antibodies among monkeys (Macaca fascicularis) in the Philippines. J Med Primatol *32*, 89-94.

Jadhav, M., Namboodripad, M., Carman, R. H., Carey, D. E., and Myers, R. M. (1965). Chikungunya disease in infants and children in Vellore: a report of clinical and haematological features of virologically proved cases. Indian J Med Res *53*, 764-776.

James, A. A., and Rossignol, P. A. (1991). Mosquito salivary glands: Parasitological and molecular aspects. Parasitol Today *7*, 267-271.

Jenkins, G.M., Rambaut, A., Pybus, O.G., and Holmes, E.C. (2002). Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. Journal of molecular evolution *54*, 156-165.

Juliano, S. A., Lounibos, L. P., and O'Meara, G. F. (2004). A field test for competitive effects of Aedes albopictus on A. aegypti in South Florida: differences between sites of coexistence and exclusion? Oecologia *139*, 583-593.

Jupp, P. G., and McIntosh, B. M. (1988). Chikungunya virus disease., In The Arboviruses: Epidemiology and Ecology, M. T. P., ed. (Boca Raton, FL: CRC Press), pp. 137-157.

Jupp, P. G., McIntosh, B. M., Dos Santos, I., and DeMoor, P. (1981). Laboratory vector studies on six mosquito and one tick species with chikungunya virus. Trans R Soc Trop Med Hyg *75*, 15-19.

Kamer, G., and Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. Nucleic Acids Res *12*, 7269-7282.

Karabatos, N. (1985). International catalog of arboviruses (San Antonio, TX: American Society of Tropical Medicine and Hygiene).

Kariuki Njenga, M., Nderitu, L., Ledermann, J. P., Ndirangu, A., Logue, C. H., Kelly, C. H., Sang, R., Sergon, K., Breiman, R., and Powers, A. M. (2008). Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. J Gen Virol *89*, 2754-2760.

Karpf, A. R., Blake, J. M., and Brown, D. T. (1997). Characterization of the infection of Aedes albopictus cell clones by Sindbis virus. Virus Res *50*, 1-13.

Kato, N., Mueller, C. R., Fuchs, J. F., McElroy, K., Wessely, V., Higgs, S., and Christensen, B. M. (2008). Evaluation of the function of a type I peritrophic matrix as a physical barrier for midgut epithelium invasion by mosquito-borne pathogens in Aedes aegypti. Vector Borne Zoonotic Dis *8*, 701-712.

Kay, B. H., and Jennings, C. D. (2002). Enhancement or modulation of the vector competence of Ochlerotatus vigilax (Diptera: Culicidae) for ross river virus by temperature. J Med Entomol *39*, 99-105.

Keene, K.M., Foy, B.D., Sanchez-Vargas, I., Beaty, B.J., Blair, C.D., and Olson, K.E. (2004). RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. Proceedings of the National Academy of Sciences of the United States of America *101*, 17240-17245.

Kerr, P. J., Weir, R. C., and Dalgarno, L. (1993). Ross River virus variants selected during passage in chick embryo fibroblasts: serological, genetic, and biological changes. Virology *193*, 446-449.

Khan, A. H., Morita, K., Parquet Md Mdel, C., Hasebe, F., Mathenge, E. G., and Igarashi, A. (2002). Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. J Gen Virol *83*, 3075-3084.

Kielian, M. (2006). Class II virus membrane fusion proteins. Virology *344*, 38-47.

Kielian, M., Chatterjee, P. K., Gibbons, D. L., and Lu, Y. E. (2000). Specific roles for lipids in virus fusion and exit. Examples from the alphaviruses. Subcell Biochem *34*, 409-455.

Kielian, M., and Helenius, A. (1985). pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. J Cell Biol *101*, 2284-2291.

Kielian, M., and Jungerwirth, S. (1990). Mechanisms of enveloped virus entry into cells. Mol Biol Med *7*, 17-31.

Kielian, M., Klimjack, M. R., Ghosh, S., and Duffus, W. A. (1996). Mechanisms of mutations inhibiting fusion and infection by Semliki Forest virus. J Cell Biol *134*, 863-872.

Kielian, M., and Rey, F. A. (2006). Virus membrane-fusion proteins: more than one way to make a hairpin. Nat Rev Microbiol *4*, 67-76.

Kielian, M. C., and Helenius, A. (1984). Role of cholesterol in fusion of Semliki Forest virus with membranes. J Virol *52*, 281-283.

Kielian, M. C., Marsh, M., and Helenius, A. (1986). Kinetics of endosome acidification detected by mutant and wild-type Semliki Forest virus. Embo J *5*, 3103-3109.

Kimchi-Sarfaty, C., Oh, J. M., Kim, I. W., Sauna, Z. E., Calcagno, A. M., Ambudkar, S. V., and Gottesman, M. M. (2007). A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science *315*, 525-528.

Kirkham, M., Fujita, A., Chadda, R., Nixon, S. J., Kurzchalia, T. V., Sharma, D. K., Pagano, R. E., Hancock, J. F., Mayor, S., and Parton, R. G. (2005). Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol *168*, 465-476.

Klimjack, M. R., Jeffrey, S., and Kielian, M. (1994). Membrane and protein interactions of a soluble form of the Semliki Forest virus fusion protein. J Virol *68*, 6940-6946.

Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D., and Ryman, K. D. (2003). DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol *77*, 12022-12032.

Klimstra, W. B., Ryman, K. D., and Johnston, R. E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. J Virol *72*, 7357-7366.

Klobucar, A., Merdic, E., Benic, N., Baklaic, Z., and Krcmar, S. (2006). First record of Aedes albopictus in Croatia. J Am Mosq Control Assoc *22*, 147-148.

Knight, K. L., and Stone, A. (1977). A catalogue of the mosquitoes of the world., Vol VI, 2-nd ed. (Lenham, MD: The Thomas Say Foundation) p. 156.

Knudsen, A. B., Romi, R., and Majori, G. (1996). Occurrence and spread in Italy of Aedes albopictus, with implications for its introduction into other parts of Europe. J Am Mosq Control Assoc *12*, 177-183.

Kolokoltsov, A. A., Fleming, E. H., and Davey, R. A. (2006). Venezuelan equine encephalitis virus entry mechanism requires late endosome formation and resists cell membrane cholesterol depletion. Virology *347*, 333-342.

Kondratieff, B. C., and Black, W. C. t. (2005). The Arthropods, In Biology of Disease Vectors, W. C. Marquardt, Black W. C. 4th, S. Higgs, J. E. Freier, A. A. James, H. H.

Hagedorn, B. Kondratieff, J. Hemingway, and C. G. Moore, eds. (New York: Elsevier Academic Press), pp. 3-8.

Koonin, E. V., and Dolja, V. V. (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol *28*, 375-430.

Kramer, L. D., Hardy, J. L., Presser, S. B., and Houk, E. J. (1981). Dissemination barriers for western equine encephalomyelitis virus in Culex tarsalis infected after ingestion of low viral doses. Am J Trop Med Hyg *30*, 190-197.

Kramer, L. D., and Scherer, W. F. (1976). Vector competence of mosquitoes as a marker to distinguish Central American and Mexican epizootic from enzootic strains of Venezuelan encephalitis virus. Am J Trop Med Hyg *25*, 336-346.

Krastinova, E., Quatresous, I., and Tarantola, A. (2006). Imported cases of chikungunya in metropolitan France: update to June 2006. Euro Surveill *11*, E060824 060821.

Kuhn, R. J., Griffin, D. E., Zhang, H., Niesters, H. G., and Strauss, J. H. (1992). Attenuation of Sindbis virus neurovirulence by using defined mutations in nontranslated regions of the genome RNA. J Virol *66*, 7121-7127.

Kuhn, R. J., Hong, Z., and Strauss, J. H. (1990). Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. J Virol *64*, 1465-1476.

Kuhn, R. J., Niesters, H. G., Hong, Z., and Strauss, J. H. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. Virology *182*, 430-441.

Kumar, N. P., Joseph, R., Kamaraj, T., and Jambulingam, P. (2008). A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India. J Gen Virol *89*, 1945-1948.

La Linn, M., Eble, J. A., Lubken, C., Slade, R. W., Heino, J., Davies, J., and Suhrbier, A. (2005). An arthritogenic alphavirus uses the alpha1beta1 integrin collagen receptor. Virology *336*, 229-239.

Lakadamyali, M., Rust, M. J., Babcock, H. P., and Zhuang, X. (2003). Visualizing infection of individual influenza viruses. Proc Natl Acad Sci U S A *100*, 9280-9285.

Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006). Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell *124*, 997-1009.

Lanciotti, R. S., Kosoy, O., Lavenir, J. J., Panella, O. J., Velez, J. O., Lambert, A. J., and Campbell, G. L. (2007). Chikungunya Virus in US Travelers Returning from India, 2006. Emerging Infectious Diseases *13*, 764-767.

Laras, K., Sukri, N. C., Larasati, R. P., Bangs, M. J., Kosim, R., Djauzi, Wandra, T., Master, J., Kosasih, H., Hartati, S.*, et al.* (2005). Tracking the re-emergence of epidemic chikungunya virus in Indonesia. Trans R Soc Trop Med Hyg *99*, 128-141.

Larsen, J. R., and Ashley, R. F. (1971). Demonstration of Venezuelan equine encephalomyelitis virus in tissues of Aedes Aegypti. Am J Trop Med Hyg *20*, 754-760.

Lastarza, M. W., Grakoui, A., and Rice, C. M. (1994a). Deletion and duplication mutations in the C-terminal nonconserved region of Sindbis virus nsP3: effects on phosphorylation and on virus replication in vertebrate and invertebrate cells. Virology *202*, 224-232.

LaStarza, M. W., Lemm, J. A., and Rice, C. M. (1994b). Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. J Virol *68*, 5781-5791.

Le Blanc, I., Luyet, P. P., Pons, V., Ferguson, C., Emans, N., Petiot, A., Mayran, N., Demaurex, N., Faure, J., Sadoul, R.*, et al.* (2005). Endosome-to-cytosol transport of viral nucleocapsids. Nat Cell Biol *7*, 653-664.

Lemm, J. A., Durbin, R. K., Stollar, V., and Rice, C. M. (1990). Mutations which alter the level or structure of nsP4 can affect the efficiency of Sindbis virus replication in a host-dependent manner. J Virol *64*, 3001-3011.

Lemm, J. A., and Rice, C. M. (1993). Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. J Virol *67*, 1916-1926.

Leroy, E. M., Nkoghe, D., Ollomo, B., Nze-Nkogue, C., Becquart, P., Grard, G., Pourrut, X., Charrel, R., Moureau, G., Ndjoyi-Mbiguino, A., and De-Lamballerie, X. (2009). Concurrent chikungunya and dengue virus infections during simultaneous outbreaks, Gabon, 2007. Emerg Infect Dis *15*, 591-593.

Lescar, J., Roussel, A., Wien, M. W., Navaza, J., Fuller, S. D., Wengler, G., Wengler, G., and Rey, F. A. (2001). The Fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell *105*, 137-148.

Levine, B., and Griffin, D. E. (1993). Molecular analysis of neurovirulent strains of Sindbis virus that evolve during persistent infection of scid mice. J Virol *67*, 6872-6875.

Levine, B., Huang, Q., Isaacs, J. T., Reed, J. C., Griffin, D. E., and Hardwick, J. M. (1993). Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature *361*, 739-742.

Li, G. P., La Starza, M. W., Hardy, W. R., Strauss, J. H., and Rice, C. M. (1990). Phosphorylation of Sindbis virus nsP3 in vivo and in vitro. Virology *179*, 416-427.

Ligon, B. L. (2006). Reemergence of an unusual disease: the chikungunya epidemic. Semin Pediatr Infect Dis *17*, 99-104.

Liljestrom, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J Virol *65*, 4107-4113.

Liu, S., Leung, H. J., and Leppla, S. H. (2007). Characterization of the interaction between anthrax toxin and its cellular receptors. Cell Microbiol *9*, 977-987.

Loewy, A., Smyth, J., von Bonsdorff, C. H., Liljestrom, P., and Schlesinger, M. J. (1995). The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. J Virol *69*, 469-475.

Lounibos, L. P., G.F., O. M., Nishimura, M., Escher, R. L., Nelson, T., Campos, E. G., and Juliano, S. A. (2001). Testing predictions of displacement of native Aedes by the invasive Asian Tiger Mosquito Aedes albopictus in Florida, USA. Biol Invasions *3*, 151-166.

Lu, Y. E., Cassese, T., and Kielian, M. (1999). The cholesterol requirement for sindbis virus entry and exit and characterization of a spike protein region involved in cholesterol dependence. J Virol *73*, 4272-4278.

Ludwig, G. V., Christensen, B. M., Yuill, T. M., and Schultz, K. T. (1989). Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus-vector infection model. Virology *171*, 108-113.

Ludwig, G. V., Kondig, J. P., and Smith, J. F. (1996). A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. J Virol *70*, 5592-5599.

Lugo Edel, C., Moreno, G., Zachariah, M. A., Lopez, M. M., Lopez, J. D., Delgado, M. A., Valle, S. I., Espinoza, P. M., Salgado, M. J., Perez, R.*, et al.* (2005). Identification of Aedes albopictus in urban Nicaragua. J Am Mosq Control Assoc *21*, 325-327.

Lumsden, W. H. (1955). An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. II. General description and epidemiology. Trans R Soc Trop Med Hyg *49*, 33-57.

Luo, T., and Brown, D. T. (1993). Purification and characterization of a Sindbis virus-induced peptide which stimulates its own production and blocks virus RNA synthesis. Virology *194*, 44-49.

Luo, T., and Brown, D. T. (1994). A 55-kDa protein induced in Aedes albopictus (mosquito) cells by antiviral protein. Virology *200*, 200-206.

Mandl, C. W., Kroschewski, H., Allison, S. L., Kofler, R., Holzmann, H., Meixner, T., and Heinz, F. X. (2001). Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. J Virol *75*, 5627-5637.

Mangiafico, J. A. (1971). Chikungunya virus infection and transmission in five species of mosquito. Am J Trop Med Hyg *20*, 642-645.

Marquardt, M. T., Phalen, T., and Kielian, M. (1993). Cholesterol is required in the exit pathway of Semliki Forest virus. J Cell Biol *123*, 57-65.

Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. Cell *124*, 729-740.

Marsh, M., Kielian, M. C., and Helenius, A. (1984). Semliki forest virus entry and the endocytic pathway. Biochem Soc Trans *12*, 981-983.

Mason, P. J., and Haddow, A. J. (1957). An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53; an additional note on Chikungunya virus isolations and serum antibodies. Trans R Soc Trop Med Hyg *51*, 238-240.

Mayne, J. T., Bell, J. R., Strauss, E. G., and Strauss, J. H. (1985). Pattern of glycosylation of Sindbis virus envelope proteins synthesized in hamster and chicken cells. Virology *142*, 121-133.

McCrae, A. W., Henderson, B. E., Kirya, B. G., and Sempala, S. D. (1971). Chikungunya virus in the Entebbe area of Uganda: isolations and epidemiology. Trans R Soc Trop Med Hyg *65*, 152-168.

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L., and Higgs, S. (2006). Role of the yellow fever virus structural protein genes in viral dissemination from the Aedes aegypti mosquito midgut. J Gen Virol *87*, 2993-3001.

McGill, P. E. (1995). Viral infections: alpha-viral arthropathy. Baillieres Clin Rheumatol *9*, 145-150.

McIntosh, B. M. (1961). Susceptibility of some African wild rodents to infection with various arthropod-borne viruses. Trans R Soc Trop Med Hyg *55*, 63-68.

McIntosh, B. M. (1970). Antibody against Chikungunya virus in wild primates in Southern Africa. S Afr J Med Sci *35*, 65-74.

McIntosh, B. M., and Jupp, P. G. (1970). Attempts to transmit Chikungunya virus with six species of mosquito. J Med Entomol *7*, 615-618.

McIntosh, B. M., Jupp, P. G., and Dos Santos, I. (1977). Rural epidemic of chikungunya in South Africa with involvement of *Aedes (Diceromyia*) *furcifer* (Edwards) and baboons. South African Journal of Science *73*, 267-269.

McIntosh, B. M., Paterson, H. E., Donaldson, J. M., and de Sousa, J. (1963). Chikungunya virus: viral susceptibility and transmission studies with some vertebrates. S Afr J Med Sci *28*, 45.

McIntosh, B. M., Paterson, H. E., McGillivray, G., and Desousa, J. (1964). Further Studies On The Chikungunya Outbreak In Southern Rhodesia In 1962. I. Mosquitoes, Wild Primates And Birds In Relation To The Epidemic. Ann Trop Med Parasitol *58*, 45-51.

McKnight, K. L., Simpson, D. A., Lin, S. C., Knott, T. A., Polo, J. M., Pence, D. F., Johannsen, D. B., Heidner, H. W., Davis, N. L., and Johnston, R. E. (1996). Deduced consensus sequence of Sindbis virus strain AR339: mutations contained in laboratory strains which affect cell culture and in vivo phenotypes. J Virol *70*, 1981-1989.

Melton, J. V., Ewart, G. D., Weir, R. C., Board, P. G., Lee, E., and Gage, P. W. (2002). Alphavirus 6K proteins form ion channels. J Biol Chem *277*, 46923-46931.

Mi, S., and Stollar, V. (1990). Both amino acid changes in nsP1 of Sindbis virusLM21 contribute to and are required for efficient expression of the mutant phenotype. Virology *178*, 429-434.

Mi, S., and Stollar, V. (1991). Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli. Virology *184*, 423-427.

Miller, B., and Mitchell, C. (1991a). Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito Aedes aegypti. AmJ Trop Med Hyg *45*, 399-407.

Miller, B. R., and Mitchell, C. J. (1991b). Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito Aedes aegypti. Am J Trop Med Hyg *45*, 399-407.

Mitchell, C. J., and Gubler, D. J. (1987). Vector competence of geographic strains of Aedes albopictus and Aedes polynesiensis and certain other Aedes (Stegomyia) mosquitoes for Ross River virus. J Am Mosq Control Assoc *3*, 142-147.

Mitchell, C. J., Miller, B. R., and Gubler, D. J. (1987). Vector competence of Aedes albopictus from Houston, Texas, for dengue serotypes 1 to 4, yellow fever and Ross River viruses. J Am Mosq Control Assoc *3*, 460-465.

Mitchell, C. J., Niebylski, M. L., Smith, G. C., Karabatsos, N., Martin, D., Mutebi, J. P., Craig, G. B., Jr., and Mahler, M. J. (1992). Isolation of eastern equine encephalitis virus from Aedes albopictus in Florida. Science *257*, 526-527.

Moehring, J. M., Inocencio, N. M., Robertson, B. J., and Moehring, T. J. (1993). Expression of mouse furin in a Chinese hamster cell resistant to Pseudomonas exotoxin A and viruses complements the genetic lesion. J Biol Chem *268*, 2590-2594.

Molina-Cruz, A., Gupta, L., Richardson, J., Bennett, K., Black, W. t., and Barillas-Mury, C. (2005). Effect of mosquito midgut trypsin activity on dengue-2 virus infection and dissemination in Aedes aegypti. Am J Trop Med Hyg *72*, 631-637.

Molinari, M., and Helenius, A. (1999). Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. Nature *402*, 90-93.

Monath, T. P. (1988). Yellow fever., In The arboviruses: epidemiology and ecology, T. P. Monath, ed. (Boca Raton, FL.: CRC Press), pp. 139–241.

Morrison, J. G. (1979). Chikungunya fever. Int J Dermatol *18*, 628-629.

Mourya, D. T., Gokhale, M. D., Malunjkar, A. S., Bhat, H. R., and Banerjee, K. (1994). Inheritance of oral susceptibility of Aedes aegypti to Chikungunya virus. Am J Trop Med Hyg *51*, 295-300.

Mourya, D. T., Malunjkar, A. S., and Banerjee, K. (1987). Susceptibility & transmissibility of Aedes aegypti to four strains of chikungunya virus. Indian J Med Res *86*, 185-190.

Mourya, D. T., Ranadive, S. N., Gokhale, M. D., Barde, P. V., Padbidri, V. S., and Banerjee, K. (1998). Putative chikungunya virus-specific receptor proteins on the midgut brush border membrane of Aedes aegypti mosquito. Indian J Med Res *107*, 10-14.

Moutailler, S., Krida, G., Schaffner, F., Vazeille, M., and Failloux, A. B. (2008). Potential vectors of Rift Valley fever virus in the Mediterranean region. Vector Borne Zoonotic Dis *8*, 749-753.

Muyembe-Tamfum, J. J., Peyrefitte, C. N., Yogolelo, R., Mathina Basisya, E., Koyange, D., Pukuta, E., Mashako, M., Tolou, H., and Durand, J. P. (2003). [Epidemic of Chikungunya virus in 1999 and 200 in the Democratic Republic of the Congo]. Med Trop (Mars) *63*, 637-638.

Myles, K. M., Kelly, C. L., Ledermann, J. P., and Powers, A. M. (2006). Effects of an opal termination codon preceding the nsP4 gene sequence in the O'Nyong-Nyong virus genome on Anopheles gambiae infectivity. J Virol *80*, 4992-4997.

Myles, K. M., Pierro, D. J., and Olson, K. E. (2003). Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti. J Virol *77*, 8872-8881.

Nakao, E., and Hotta, S. (1973). Immunogenicity of purified, inactivated chikungunya virus in monkeys. Bull World Health Organ *48*, 559-562.

National Informatics Centre, (2008). Health Bulletin. Dengue fever http://india.gov.in/citizen/health/archive_index.php. (retrieved 12/08). In Health Bulletin. Dengue fever.

Navaratnarajah, C. K., and Kuhn, R. J. (2007). Functional characterization of the Sindbis virus E2 glycoprotein by transposon linker-insertion mutagenesis. Virology *363*, 134-147.

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P. W., Blystone, S. D., Brown, E. J., and Baxt, B. (1998). Foot-and-mouth disease virus virulent for cattle utilizes the integrin alpha(v)beta3 as its receptor. J Virol *72*, 3587-3594.

Nieva, J. L., Bron, R., Corver, J., and Wilschut, J. (1994). Membrane fusion of Semliki Forest virus requires sphingolipids in the target membrane. Embo J *13*, 2797-2804.

Noridah, O., Paranthaman, V., Nayar, S. K., Masliza, M., Ranjit, K., Norizah, I., Chem, Y. K., Mustafa, B., Kumarasamy, V., and Chua, K. B. (2007). Outbreak of chikungunya due to virus of Central/East African genotype in Malaysia. Med J Malaysia *62*, 323-328.

NVBDCP (2008). Dengue/DHF. New Delhi: National Vector Borne Disease Control Programme http://www.nvbdcp.gov.in/Dengueupdate.html.(retrieved 12/08).

Oberste, M. S., Parker, M. D., and Smith, J. F. (1996). Complete sequence of Venezuelan equine encephalitis virus subtype IE reveals conserved and hypervariable domains within the C terminus of nsP3. Virology *219*, 314-320.

Ogata, K., and Lopez Samayoa, A. (1996). Discovery of Aedes albopictus in Guatemala. J Am Mosq Control Assoc *12*, 503-506.

Osterrieth, P., Deleplanque-Liegeois, P., and Renoirte, R. (1960). [Research on the Chikungunya virus in the Belgian Congo. II. Serological investigation.]. Ann Soc Belg Med Trop *40*, 205-213.

Ozden, S., Huerre, M., Riviere, J. P., Coffey, L. L., Afonso, P. V., Mouly, V., de Monredon, J., Roger, J. C., El Amrani, M., Yvin, J. L.*, et al.* (2007). Human muscle satellite cells as targets of Chikungunya virus infection. PLoS ONE *2*, e527.

Paganin, F., Borgherini, G., Staikowsky, F., Arvin-Berod, C., and Poubeau, P. (2006). [Chikungunya on Reunion Island: chronicle of an epidemic foretold]. Presse Med *35*, 641-646.

Pages, F., Peyrefitte, C. N., Mve, M. T., Jarjaval, F., Brisse, S., Iteman, I., Gravier, P., Nkoghe, D., and Grandadam, M. (2009). Aedes albopictus mosquito: the main vector of the 2007 Chikungunya outbreak in Gabon. PLoS ONE *4*, e4691.

Paredes, A., Alwell-Warda, K., Weaver, S. C., Chiu, W., and Watowich, S. J. (2001). Venezuelan equine encephalomyelitis virus structure and its divergence from old world alphaviruses. J Virol *75*, 9532-9537.

Parola, P., de Lamballerie, X., Jourdan, J., Rovery, C., Vaillant, V., Minodier, P., Brouqui, P., Flahault, A., Raoult, D., and Charrel, R. N. (2006). Novel chikungunya virus variant in travelers returning from Indian Ocean islands. Emerg Infect Dis *12*, 1493-1499.

Parvin, J.D., Moscona, A., Pan, W.T., Leider, J.M., and Palese, P. (1986). Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. Journal of virology *59*, 377-383.

Pastorino, B., Muyembe-Tamfum, J. J., Bessaud, M., Tock, F., Tolou, H., Durand, J. P., and Peyrefitte, C. N. (2004). Epidemic resurgence of Chikungunya virus in democratic Republic of the Congo: identification of a new central African strain. J Med Virol *74*, 277-282.

Paterson, H. E., and McIntosh, B. M. (1964). Further Studies On The Chikungunya Outbreak In Southern Rhodesia In 1962. Ii. Transmission Experiments With The *Aedes* Furcifer-Taylori Group Of Mosquitoes And With A Member Of The Anopheles Gambiae Complex. Ann Trop Med Parasitol *58*, 52-55.

Paul, S. D., and Singh, K. R. (1968). Experimental infection of *Macaca radiata* with Chikungunya virus and transmission of virus by mosquitoes. Indian J Med Res *56*, 802-811.

Paupy, C., Delatte, H., Bagny, L., Corbel, V., and Fontenille, D. (2009). Aedes albopictus, an arbovirus vector: From the darkness to the light. Microbes Infect.

Peiris, J. S., Dittus, W. P., and Ratnayake, C. B. (1993). Seroepidemiology of dengue and other arboviruses in a natural population of toque macaques (Macaca sinica) at Polonnaruwa, Sri Lanka. J Med Primatol *22*, 240-245.

Pennington, J. E., and Wells, M. A. (2005). The adult midgut: structure and function., In In Biology of Disease Vectors, W. C. Marquardt, Black W. C. 4th, S. Higgs, J. E. Freier, A. A. James, H. H. Hagedorn, B. Kondratieff, J. Hemingway, and C. G. Moore, eds. (New York, NY: Elsevier Academic Press.), pp. 289-296.

Peranen, J. (1991). Localization and phosphorylation of Semliki Forest virus non-structural protein nsP3 expressed in COS cells from a cloned cDNA. J Gen Virol *72 (Pt 1)*, 195-199.

Peranen, J., and Kaariainen, L. (1991). Biogenesis of type I cytopathic vacuoles in Semliki Forest virus-infected BHK cells. J Virol *65*, 1623-1627.

Peranen, J., Rikkonen, M., Liljestrom, P., and Kaariainen, L. (1990). Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. J Virol *64*, 1888-1896.

Peranen, J., Takkinen, K., Kalkkinen, N., and Kaariainen, L. (1988). Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. J Gen Virol *69 (Pt 9)*, 2165-2178.

Peyrefitte, C. N., Bessaud, M., Pastorino, B. A., Gravier, P., Plumet, S., Merle, O. L., Moltini, I., Coppin, E., Tock, F., Daries, W.*, et al.* (2008). Circulation of Chikungunya virus in Gabon, 2006-2007. J Med Virol *80*, 430-433.

Peyrefitte, C. N., Rousset, D., Pastorino, B. A., Pouillot, R., Bessaud, M., Tock, F., Mansaray, H., Merle, O. L., Pascual, A. M., Paupy, C.*, et al.* (2007). Chikungunya virus, Cameroon, 2006. Emerg Infect Dis *13*, 768-771.

Pfeiffer, J. K., and Kirkegaard, K. (2005). Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. PLoS Pathog *1*, e11.

Phalen, T., and Kielian, M. (1991). Cholesterol is required for infection by Semliki Forest virus. J Cell Biol *112*, 615-623.

Pialoux, G., Gauzere, B. A., Jaureguiberry, S., and Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. Lancet Infect Dis *7*, 319-327.

Pierro, D. J., Myles, K. M., Foy, B. D., Beaty, B. J., and Olson, K. E. (2003). Development of an orally infectious Sindbis virus transducing system that efficiently disseminates and expresses green fluorescent protein in Aedes aegypti. Insect Mol Biol *12*, 107-116.

Pierro, D. J., Powers, E. L., and Olson, K. E. (2007). Genetic determinants of Sindbis virus strain TR339 affecting midgut infection in the mosquito Aedes aegypti. J Gen Virol *88*, 1545-1554.

Pierro, D. J., Powers, E. L., and Olson, K. E. (2008). Genetic determinants of Sindbis virus mosquito infection are associated with a highly conserved alphavirus and flavivirus envelope sequence. J Virol *82*, 2966-2974.

Pilitt, D. R., and Jones, J. C. (1972). A qualitative method for estimating the degree of engorgement of Aedes aegypti adults. J Med Entomol *9*, 334-337.

Pons, V., Luyet, P. P., Morel, E., Abrami, L., van der Goot, F. G., Parton, R. G., and Gruenberg, J. (2008). Hrs and SNX3 functions in sorting and membrane invagination within multivesicular bodies. PLoS Biol *6*, e214.

Powers, A. M., Brault, A. C., Tesh, R. B., and Weaver, S. C. (2000). Re-emergence of Chikungunya and O'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. J Gen Virol *81*, 471-479.

Powers, A. M., and Logue, C. H. (2007). Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol *88*, 2363-2377.

Pugachev, K. V., Mason, P. W., Shope, R. E., and Frey, T. K. (1995). Double-subgenomic Sindbis virus recombinants expressing immunogenic proteins of Japanese encephalitis virus induce significant protection in mice against lethal JEV infection. Virology *212*, 587-594.

Puhar, A., and Montecucco, C. (2007). Where and how do anthrax toxins exit endosomes to intoxicate host cells? Trends Microbiol *15*, 477-482.

Rainey, G. J., Wigelsworth, D. J., Ryan, P. L., Scobie, H. M., Collier, R. J., and Young, J. A. (2005). Receptor-specific requirements for anthrax toxin delivery into cells. Proc Natl Acad Sci U S A *102*, 13278-13283.

Ramful, D., Carbonnier, M., Pasquet, M., Bouhmani, B., Ghazouani, J., Noormahomed, T., Beullier, G., Attali, T., Samperiz, S., Fourmaintraux, A., and Alessandri, J. L. (2007). Mother-to-child transmission of Chikungunya virus infection. Pediatr Infect Dis J *26*, 811-815.

Rampal, Sharda, M., and Meena, H. (2007). Neurological complications in Chikungunya fever. J Assoc Physicians India *55*, 765-769.

Ravi, V. (2006). Re-emergence of chikungunya virus in India. Indian J Med Microbiol *24*, 83-84.

Reisen, W. K., Fang, Y., and Martinez, V. M. (2006). Effects of temperature on the transmission of west nile virus by Culex tarsalis (Diptera: Culicidae). J Med Entomol *43*, 309-317.

Reiter, P., Fontenille, D., and Paupy, C. (2006). Aedes albopictus as an epidemic vector of chikungunya virus: another emerging problem? Lancet Infect Dis *6*, 463-464.

Renault, P., Solet, J. L., Sissoko, D., Balleydier, E., Larrieu, S., Filleul, L., Lassalle, C., Thiria, J., Rachou, E., de Valk, H.*, et al.* (2007). A major epidemic of chikungunya virus infection on Reunion Island, France, 2005-2006. Am J Trop Med Hyg *77*, 727-731.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F.*, et al.* (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet *370*, 1840-1846.

Rice, C. M., Levis, R., Strauss, J. H., and Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J Virol *61*, 3809-3819.

Rikkonen, M., Peranen, J., and Kaariainen, L. (1994). ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. J Virol *68*, 5804-5810.

Robin, S., Ramful, D., Le Seach, F., Jaffar-Bandjee, M. C., Rigou, G., and Alessandri, J. L. (2008). Neurologic manifestations of pediatric chikungunya infection. J Child Neurol *23*, 1028-1035.

Robinson, M. C. (1955). An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features. Trans R Soc Trop Med Hyg *49*, 28-32.

Rodhain, F. (1996). [Problems posed by the spread of Aedes albopictus]. Bull Soc Pathol Exot *89*, 137-140; discussion 140-131.

Rodriguez Tovar, M. L., and Ortega Martinez, M. G. (1994). Aedes albopictus in Muzquiz city, Coahuila, Mexico. J Am Mosq Control Assoc *10*, 587.

Roiz, D., Eritja, R., Escosa, R., Lucientes, J., Marques, E., Melero-Alcibar, R., Ruiz, S., and Molina, R. (2007). A survey of mosquitoes breeding in used tires in Spain for the detection of imported potential vector species. J Vector Ecol *32*, 10-15.

Roiz, D., Eritja, R., Molina, R., Melero-Alcibar, R., and Lucientes, J. (2008). Initial distribution assessment of Aedes albopictus (Diptera: Culicidae) in the Barcelona, Spain, area. J Med Entomol *45*, 347-352.

Romi, R. (2001). [Aedes albopictus in Italy: an underestimated health problem]. Ann Ist Super Sanita *37*, 241-247.

Romi, R., Severini, F., and Toma, L. (2006). Cold acclimation and overwintering of female Aedes albopictus in Roma. J Am Mosq Control Assoc *22*, 149-151.

Romoser, W. S., Faran, M. E., Bailey, C. L., and Lerdthusnee, K. (1992). An immunocytochemical study of the distribution of Rift Valley fever virus in the mosquito Culex pipiens. Am J Trop Med Hyg *46*, 489-501.

Ross, R. W. (1956a). A laboratory technique for studying the insect transmission of animal viruses, employing a bat-wing membrane, demonstrated with two African viruses. J Hyg (Lond) *54*, 192-200.

Ross, R. W. (1956b). The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. J Hyg (Lond) *54*, 177-191.

Rossi, G. C., Pascual, N. T., and Krsticevic, F. J. (1999). First record of Aedes albopictus (Skuse) from Argentina. J Am Mosq Control Assoc *15*, 422.

Roussel, A., Lescar, J., Vaney, M. C., Wengler, G., Wengler, G., and Rey, F. A. (2006). Structure and interactions at the viral surface of the envelope protein E1 of Semliki Forest virus. Structure *14*, 75-86.

Russo, A. T., White, M. A., and Watowich, S. J. (2006). The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. Structure *14*, 1449-1458.

Ryman, K. D., and Klimstra, W. B. (2008). Host responses to alphavirus infection. Immunol Rev *225*, 27-45.

Sabatini, A., Raineri, V., Trovato, G., and Coluzzi, M. (1990). [Aedes albopictus in Italy and possible diffusion of the species into the Mediterranean area]. Parassitologia *32*, 301-304.

Salvan, M., and Mouchet, J. (1994). Aedes albopictus et Aedes aegypti a` l'Ile de La Re´union. Ann Soc Belge Me´d Trop *74*, 323-326.

Salvatella, R., and Rosa, R. (2003). Culícidos y salud humana en el Uruguay. Entomol Vect *10*, 431-436.

Sambri, V., Cavrini, F., Rossini, G., Pierro, A., and Landini, M. P. (2008). The 2007 epidemic outbreak of Chikungunya virus infection in the Romagna region of Italy: a new perspective for the possible diffusion of tropical diseases in temperate areas? New Microbiol *31*, 303-304.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual, 2nd edn (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Sanchez-Vargas, I., Travanty, E.A., Keene, K.M., Franz, A.W., Beaty, B.J., Blair, C.D., and Olson, K.E. (2004). RNA interference, arthropod-borne viruses, and mosquitoes. Virus research *102*, 65-74.

Sang, R. C., Ahmed, O., Faye, O., Kelly, C. L., Yahaya, A. A., Mmadi, I., Toilibou, A., Sergon, K., Brown, J., Agata, N.*, et al.* (2008). Entomologic investigations of a chikungunya virus epidemic in the Union of the Comoros, 2005. Am J Trop Med Hyg *78*, 77-82.

Sardelis, M. R., Turell, M. J., Dohm, D. J., and O'Guinn, M. L. (2001). Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. Emerg Infect Dis *7*, 1018-1022.

Sardelis, M. R., Turell, M. J., O'Guinn, M. L., Andre, R. G., and Roberts, D. R. (2002). Vector competence of three North American strains of Aedes albopictus for West Nile virus. J Am Mosq Control Assoc *18*, 284-289.

Sariola, M., Saraste, J., and Kuismanen, E. (1995). Communication of post-Golgi elements with early endocytic pathway: regulation of endoproteolytic cleavage of Semliki Forest virus p62 precursor. J Cell Sci *108 (Pt 6)*, 2465-2475.

Sarkar, J. K., Chatterjee, S. N., Chakravarti, S. K., and Mitra, A. C. (1965). Chikungunya virus infection with haemorrhagic manifestations. Indian J Med Res *53*, 921-925.

Sawicki, D. L., Perri, S., Polo, J. M., and Sawicki, S. G. (2006). Role for nsP2 Proteins in the Cessation of Alphavirus Minus-Strand Synthesis by Host Cells. J Virol *80*, 360-371.

Sawicki, D. L., and Sawicki, S. G. (1993). A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. J Virol *67*, 3605-3610.

Sawicki, D. L., Sawicki, S. G., Keranen, S., and Kaariainen, L. (1981). Specific Sindbis virus-coded function for minus-strand RNA synthesis. J Virol *39*, 348-358.

Saxena, S. K., Singh, M., Mishra, N., and Lakshmi, V. (2006). Resurgence of chikungunya virus in India: an emerging threat. Euro Surveill *11*, E060810 060812.

Schaffner, F., Bouletreau, B., Guillet, P., and Karch, S. (2001). Aedes albopictus (Skuse, 1894) established in metropolitan France. European Mosquito Bulletin *9*, 1-2.

Schaffner, F., and Karch, S. (2000). [First report of Aedes albopictus (Skuse, 1984) in metropolitan France]. C R Acad Sci III *323*, 373-375.

Schaffner, F., Van Bortel, W., and Coosemans, M. (2004). First record of Aedes (Stegomyia) albopictus in Belgium. J Am Mosq Control Assoc *20*, 201-203.

Schlesinger, S. (2001). Alphavirus vectors: development and potential therapeutic applications. Expert Opin Biol Ther *1*, 177-191.

Schlesinger, S., and Schlesinger, M. J. (2001a). *Togaviridae*: The viruses and their replication, In Fields' Virology, Fourth Edition, D. M. Knipe, and P. M. Howley, eds. (New York: Lippincott, Williams and Wilkins), pp. 895-916.

Schlesinger, S., and Schlesinger, M. J. (2001b). *Togaviridae*: The viruses and their replication., In Fields' Virology, D. M. Knipe, and P. M. Howley, eds. (New York: Lippincott, Williams and Wilkins), pp. 895-916.

Schmidt, M. F., Bracha, M., and Schlesinger, M. J. (1979). Evidence for covalent attachment of fatty acids to Sindbis virus glycoproteins. Proc Natl Acad Sci U S A *76*, 1687-1691.

Scholte, E., and Schaffner, F. (2007). Waiting for the tiger: establishment and spread of the Asian tiger mosquito in Europe., In Emerging pests and vector-borne diseases in Europe., Takken W, and Knols B, eds. (Wageningen, Netherlands: Wageningen Academic Publishers), pp. 241-2607.

Scholte, E. J., Dijkstra, E., Blok, H., De Vries, A., Takken, W., Hofhuis, A., Koopmans, M., De Boer, A., and Reusken, C. B. (2008). Accidental importation of the mosquito Aedes albopictus into the Netherlands: a survey of mosquito distribution and the presence of dengue virus. Med Vet Entomol *22*, 352-358.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M. C., Lavenir, R., Pardigon, N., Reynes, J. M., Pettinelli, F*., et al.* (2006). Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med *3*, e263.

Scobie, H. M., Thomas, D., Marlett, J. M., Destito, G., Wigelsworth, D. J., Collier, R. J., Young, J. A., and Manchester, M. (2005). A soluble receptor decoy protects rats against anthrax lethal toxin challenge. J Infect Dis *192*, 1047-1051.

Scott, T. W., and Burrage, T. G. (1984). Rapid infection of salivary glands in Culiseta melanura with eastern equine encephalitis virus: an electron microscopic study. Am J Trop Med Hyg *33*, 961-964.

Scott, T. W., Lorenz, L. H., and Weaver, S. C. (1990). Susceptibility of Aedes albopictus to infection with eastern equine encephalomyelitis virus. J Am Mosq Control Assoc *6*, 274-278.

Seabaugh, R. C., Olson, K. E., Higgs, S., Carlson, J. O., and Beaty, B. J. (1998). Development of a chimeric sindbis virus with enhanced per Os infection of Aedes aegypti. Virology *243*, 99-112.

Sefton, B. M. (1977). Immediate glycosylation of Sindbis virus membrane proteins. Cell *10*, 659-668.

Sempala, S. D., and Kirya, B. G. (1973). Laboratory transmission of Chikungunya virus by *Aedes* (*Stegomyia*) *apicoargenteus* Theobald (Diptera, Culicidae). Am J Trop Med Hyg *22*, 263-266.

Seneviratne, S. L., and Perera, J. (2006). Fever epidemic moves into Sri Lanka. Bmj *333*, 1220-1221.

Sergon, K., Njuguna, C., Kalani, R., Ofula, V., Onyango, C., Konongoi, L. S., Bedno, S., Burke, H., Dumilla, A. M., Konde, J*., et al.* (2008). Seroprevalence of Chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. Am J Trop Med Hyg *78*, 333-337.

Sergon, K., Yahaya, A. A., Brown, J., Bedja, S. A., Mlindasse, M., Agata, N., Allaranger, Y., Ball, M. D., Powers, A. M., Ofula, V.*, et al.* (2007). Seroprevalence of Chikungunya virus infection on Grande Comore Island, union of the Comoros, 2005. Am J Trop Med Hyg *76*, 1189-1193.

Service, M. (2007). Chikungunya Risk of transmission in the USA. Wing Beats *18*.

Shirako, Y., and Strauss, J. H. (1994). Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. J Virol *68*, 1874-1885.

Sieczkarski, S. B., and Whittaker, G. R. (2002). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J Virol *76*, 10455-10464.

Sieczkarski, S. B., and Whittaker, G. R. (2003). Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. Traffic *4*, 333-343.

Silberkang, M., Havel, C. M., Friend, D. S., McCarthy, B. J., and Watson, J. A. (1983). Isoprene synthesis in isolated embryonic Drosophila cells. I. Sterol-deficient eukaryotic cells. J Biol Chem *258*, 8503-8511.

Simard, F., Nchoutpouen, E., Toto, J. C., and Fontenille, D. (2005). Geographic distribution and breeding site preference of Aedes albopictus and Aedes aegypti (Diptera: culicidae) in Cameroon, Central Africa. J Med Entomol *42*, 726-731.

Simon, F., Parola, P., Grandadam, M., Fourcade, S., Oliver, M., Brouqui, P., Hance, P., Kraemer, P., Mohamed, A. A., de Lamballerie, X.*, et al.* (2007). Chikungunya Infection: An Emerging Rheumatism Among Travelers Returned From Indian Ocean Islands. Report of 47 Cases. Medicine (Baltimore) *86*, 123-137.

Simon, F., Savini, H., and Parola, P. (2008). Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. Med Clin North Am *92*, 1323-1343, ix.

Simpson, D. I., Williams, M. C., O'Sullivan, J. P., Cunningham, J. C., and Mutere, F. A. (1968). Studies on arboviruses and bats (Chiroptera) in East Africa. II. Isolation and haemagglutination-inhibition studies on bats collected in Kenya and throughout Uganda. Ann Trop Med Parasitol *62*, 432-440.

Singh, K. R., and Pavri, K. M. (1967). Experimental studies with chikungunya virus in Aedes aegypti and Aedes albopictus. Acta Virol *11*, 517-526.

Sjoberg, M., and Garoff, H. (2003). Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. J Virol *77*, 3441-3450.

Skoging, U., Vihinen, M., Nilsson, L., and Liljestrom, P. (1996). Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid. Structure *4*, 519-529.

Smit, J. M., Bittman, R., and Wilschut, J. (1999). Low-pH-dependent fusion of Sindbis virus with receptor-free cholesterol- and sphingolipid-containing liposomes. J Virol *73*, 8476-8484.

Smit, J. M., Klimstra, W. B., Ryman, K. D., Bittman, R., Johnston, R. E., and Wilschut, J. (2001). PE2 cleavage mutants of Sindbis virus: correlation between viral infectivity and pH-dependent membrane fusion activation of the spike heterodimer. J Virol *75*, 11196-11204.

Smit, J. M., Waarts, B. L., Kimata, K., Klimstra, W. B., Bittman, R., and Wilschut, J. (2002). Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. J Virol *76*, 10128-10137.

Smith, D. R., Adams, A. P., Kenney, J. L., Wang, E., and Weaver, S. C. (2008). Venezuelan equine encephalitis virus in the mosquito vector Aedes taeniorhynchus: infection initiated by a small number of susceptible epithelial cells and a population bottleneck. Virology *372*, 176-186.

Smith, G. C., and Francy, D. B. (1991). Laboratory studies of a Brazilian strain of Aedes albopictus as a potential vector of Mayaro and Oropouche viruses. J Am Mosq Control Assoc *7*, 89-93.

Smith, T. J., Cheng, R. H., Olson, N. H., Peterson, P., Chase, E., Kuhn, R. J., and Baker, T. S. (1995). Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. Proc Natl Acad Sci U S A *92*, 10648-10652.

Sobo, K., Chevallier, J., Parton, R. G., Gruenberg, J., and van der Goot, F. G. (2007). Diversity of raft-like domains in late endosomes. PLoS One *2*, e391.

Sourisseau, M., Schilte, C., Casartelli, N., Trouillet, C., Guivel-Benhassine, F., Rudnicka, D., Sol-Foulon, N., Le Roux, K., Prevost, M. C., Fsihi, H., *et al.* (2007). Characterization of reemerging chikungunya virus. PLoS Pathog *3*, e89.

Spence, L. P., and Thomas, L. (1959). Application of haemagglutination and complementfixation techniques to the identification and serological classification of arthropod-borne viruses; studies on Chikungunya and Makonde viruses. Trans R Soc Trop Med Hyg *53*, 248-255.

Sprenger, D., and Wuithiranyagool, T. (1986). The discovery and distribution of Aedes albopictus in Harris County, Texas. J Am Mosq Control Assoc *2*, 217-219.

Strauss, E. G., Stec, D. S., Schmaljohn, A. L., and Strauss, J. H. (1991). Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. J Virol *65*, 4654-4664.

Strauss, J. H., and Strauss, E. G. (1990). Alphavirus proteinases. Semin Virol *1*, 347-356.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. Microbiol Rev *58*, 491-562.

Strauss, J. H., and Strauss, E. G. (2001). Virus evolution: how does an enveloped virus make a regular structure? Cell *105*, 5-8.

Strauss, J. H., and Strauss, E. G. (2002). Family Togaviridae, In Viruses and human disease (London, UK: Academic Press), pp. 76-85.

Suopanki, J., Sawicki, D. L., Sawicki, S. G., and Kaariainen, L. (1998). Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. J Gen Virol *79 (Pt 2)*, 309-319.

Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, (MA: Sinauer Associates.).

Tesh, R. B., Gubler, D. J., and Rosen, L. (1976). Variation among goegraphic strains of Aedes albopictus in susceptibility to infection with chikungunya virus. Am J Trop Med Hyg *25*, 326-335.

Thiruvengadam, K. V., Kalyanasundaram, V., and Rajgopal, J. (1965). Clinical and pathological studies on chikungunya fever in Madras city. Indian J Med Res *53*, 729-744.

Toma, L., Severini, F., Di Luca, M., Bella, A., and Romi, R. (2003). Seasonal patterns of oviposition and egg hatching rate of Aedes albopictus in Rome. J Am Mosq Control Assoc *19*, 19-22.

Tong, L., Wengler, G., and Rossmann, M. G. (1993). Refined structure of Sindbis virus core protein and comparison with other chymotrypsin-like serine proteinase structures. J Mol Biol *230*, 228-247.

Tsetsarkin, K., Higgs, S., McGee, C. E., De Lamballerie, X., Charrel, R. N., and Vanlandingham, D. L. (2006). Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. Vector Borne Zoonotic Dis *6*, 325-337.

Tsetsarkin, K. A., Vanlandingham, D. L., McGee, C. E., and Higgs, S. (2007). A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. PLoS Pathog *3*, e201.

Turell, M. J., Barth, J., and Coleman, R. E. (1999). Potential for Central American mosquitoes to transmit epizootic and enzootic strains of Venezuelan equine encephalitis virus. J Am Mosq Control Assoc *15*, 295-298.

Turell, M. J., and Beaman, J. R. (1992). Experimental transmission of Venezuelan equine encephalomyelitis virus by a strain of Aedes albopictus (Diptera: Culicidae) from New Orleans, Louisiana. J Med Entomol *29*, 802-805.

Turell, M. J., Beaman, J. R., and Tammariello, R. F. (1992). Susceptibility of selected strains of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) to chikungunya virus. J Med Entomol *29*, 49-53.

Turell, M. J., and Malinoski, F. J. (1992). Limited potential for mosquito transmission of a live, attenuated chikungunya virus vaccine. Am J Trop Med Hyg *47*, 98-103.

Turell, M. J., O'Guinn, M. L., Dohm, D. J., and Jones, J. W. (2001). Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol *38*, 130-134.

Turell, M. J., O'Guinn, M. L., Navarro, R., Romero, G., and Estrada-Franco, J. G. (2003). Vector competence of Mexican and Honduran mosquitoes (Diptera: Culicidae) for

enzootic (IE) and epizootic (IC) strains of Venezuelan equine encephalomyelitis virus. J Med Entomol *40*, 306-310.

Turell, M. J., Rossi, C. A., and Bailey, C. L. (1985). Effect of extrinsic incubation temperature on the ability of Aedes taeniorhynchus and Culex pipiens to transmit Rift Valley fever virus. Am J Trop Med Hyg *34*, 1211-1218.

Ubol, S., and Griffin, D. E. (1991). Identification of a putative alphavirus receptor on mouse neural cells. J Virol *65*, 6913-6921.

Ubol, S., Tucker, P. C., Griffin, D. E., and Hardwick, J. M. (1994). Neurovirulent strains of Alphavirus induce apoptosis in bcl-2-expressing cells: role of a single amino acid change in the E2 glycoprotein. Proc Natl Acad Sci U S A *91*, 5202-5206.

Ulug, E. T., Garry, R. F., and Bose, H. R., Jr. (1989). The role of monovalent cation transport in Sindbis virus maturation and release. Virology *172*, 42-50.

Ulug, E. T., Garry, R. F., Waite, M. R., and Bose, H. R., Jr. (1984). Alterations in monovalent cation transport in Sindbis virus-infected chick cells. Virology *132*, 118-130.

Umashankar, M., Sanchez-San Martin, C., Liao, M., Reilly, B., Guo, A., Taylor, G., and Kielian, M. (2008). Differential cholesterol binding by class II fusion proteins determines membrane fusion properties. J Virol *82*, 9245-9253.

Vanlandingham, D. L., Hong, C., Klingler, K., Tsetsarkin, K., McElroy, K. L., Powers, A. M., Lehane, M. J., and Higgs, S. (2005a). Differential infectivities of o'nyong-nyong and chikungunya virus isolates in Anopheles gambiae and Aedes aegypti mosquitoes. Am J Trop Med Hyg *72*, 616-621.

Vanlandingham, D. L., Tsetsarkin, K., Hong, C., Klingler, K., McElroy, K. L., Lehane, M. J., and Higgs, S. (2005b). Development and characterization of a double subgenomic chikungunya virus infectious clone to express heterologous genes in Aedes aegypti mosquitoes. Insect Biochem Mol Biol *35*, 1162-1170.

Vanlandingham, D. L., Tsetsarkin, K., Klingler, K. A., Hong, C., McElroy, K. L., Lehane, M. J., and Higgs, S. (2006). Determinants of vector specificity of o'nyong nyong and chikungunya viruses in Anopheles and Aedes mosquitoes. Am J Trop Med Hyg *74*, 663-669.

Vashishtha, M., Phalen, T., Marquardt, M. T., Ryu, J. S., Ng, A. C., and Kielian, M. (1998). A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit. J Cell Biol *140*, 91-99.

Vasiljeva, L., Merits, A., Auvinen, P., and Kaariainen, L. (2000). Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. J Biol Chem *275*, 17281-17287.

Vasiljeva, L., Merits, A., Golubtsov, A., Sizemskaja, V., Kaariainen, L., and Ahola, T. (2003). Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. J Biol Chem *278*, 41636-41645.

Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., Thiria, J., Dehecq, J. S., Fontenille, D., Schuffenecker, I*., et al.* (2007). Two Chikungunya Isolates from the Outbreak of La Reunion (Indian Ocean) Exhibit Different Patterns of Infection in the Mosquito, Aedes albopictus. PLoS ONE *2*, e1168.

Vazeille, M., Moutailler, S., Pages, F., Jarjaval, F., and Failloux, A. B. (2008). Introduction of Aedes albopictus in Gabon: what consequences for dengue and chikungunya transmission? Trop Med Int Health *13*, 1176-1179.

Vihinen, H., Ahola, T., Tuittila, M., Merits, A., and Kaariainen, L. (2001). Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. J Biol Chem *276*, 5745-5752.

Vihinen, H., and Saarinen, J. (2000). Phosphorylation site analysis of Semliki forest virus nonstructural protein 3. J Biol Chem *275*, 27775-27783.

Volkova, E., Frolova, E., Darwin, J. R., Forrester, N. L., Weaver, S. C., and Frolov, I. (2008). IRES-dependent replication of Venezuelan equine encephalitis virus makes it highly attenuated and incapable of replicating in mosquito cells. Virology *377*, 160-169.

Waarts, B. L., Bittman, R., and Wilschut, J. (2002). Sphingolipid and cholesterol dependence of alphavirus membrane fusion. Lack of correlation with lipid raft formation in target liposomes. J Biol Chem *277*, 38141-38147.

Wang, E., Volkova, E., Adams, A. P., Forrester, N., Xiao, S. Y., Frolov, I., and Weaver, S. C. (2008). Chimeric alphavirus vaccine candidates for chikungunya. Vaccine *26*, 5030-5039.

Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S., and Strauss, J. H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol *66*, 4992-5001.

Wang, K. S., Schmaljohn, A. L., Kuhn, R. J., and Strauss, J. H. (1991a). Antiidiotypic antibodies as probes for the Sindbis virus receptor. Virology *181*, 694-702.

Wang, K. S., and Strauss, J. H. (1991). Use of a lambda gt11 expression library to localize a neutralizing antibody-binding site in glycoprotein E2 of Sindbis virus. J Virol *65*, 7037-7040.

Wang, Y. F., Sawicki, S. G., and Sawicki, D. L. (1991b). Sindbis virus nsP1 functions in negative-strand RNA synthesis. J Virol *65*, 985-988.

Wang, Y. F., Sawicki, S. G., and Sawicki, D. L. (1994). Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. J Virol *68*, 6466-6475.

Warrier, R., Linger, B. R., Golden, B. L., and Kuhn, R. J. (2008). Role of sindbis virus capsid protein region II in nucleocapsid core assembly and encapsidation of genomic RNA. J Virol *82*, 4461-4470.

Watts, D. M., Burke, D. S., Harrison, B. A., Whitmire, R. E., and Nisalak, A. (1987). Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. Am J Trop Med Hyg *36*, 143-152.

Weaver, S., Dalgarno, L., Frey, T. K., Huang, H. V., Kinney, R. M., Rice, C. M., Roehrig, J. T., Shope, R., and Strauss, E. G. (2000). Family Togaviridae., In Virus taxonomy. Classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses., M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeogh, C. R. Pringle, and R. B. e. Wickner, eds. (San Diego,TX: Academic Press), pp. 879-889.

Weaver, S. C. (1986). Electron microscopic analysis of infection patterns for Venezuelan equine encephalomyelitis virus in the vector mosquito, Culex (Melanoconion) taeniopus. Am J Trop Med Hyg *35*, 624-631.

Weaver, S. C., and Barrett, A. D. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol *2*, 789-801.

Weaver, S. C., Lorenz, L. H., and Scott, T. W. (1992). Pathologic changes in the midgut of Culex tarsalis following infection with Western equine encephalomyelitis virus. Am J Trop Med Hyg *47*, 691-701.

Weaver, S. C., Scherer, W. F., Cupp, E. W., and Castello, D. A. (1984). Barriers to dissemination of Venezuelan encephalitis viruses in the Middle American enzootic vector mosquito, Culex (Melanoconion) taeniopus. Am J Trop Med Hyg *33*, 953-960.

Weaver, S. C., Scott, T. W., and Lorenz, L. H. (1990). Patterns of eastern equine encephalomyelitis virus infection in Culiseta melanura (Diptera: Culicidae). J Med Entomol *27*, 878-891.

Weaver, S. C., Scott, T. W., Lorenz, L. H., Lerdthusnee, K., and Romoser, W. S. (1988). Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. J Virol *62*, 2083-2090.

Weaver, S. C., Scott, T. W., Lorenz, L. H., and Repik, P. M. (1991). Detection of eastern equine encephalomyelitis virus deposition in Culiseta melanura following ingestion of radiolabeled virus in blood meals. Am J Trop Med Hyg *44*, 250-259.

Weinbren, M. P., Haddow, A. J., and Williams, M. C. (1958). The occurrence of Chikungunya virus in Uganda. I. Isolation from mosquitoes. Trans R Soc Trop Med Hyg *52*, 253-257.

Weinstein, D. B. (1979). A single-step adsorption method for removal of lipoproteins and preparation of cholesterol-free serum. Circulation *60*, 204.

Wendell, M. D., Wilson, T. G., Higgs, S., and Black, W. C. (2000). Chemical and gamma-ray mutagenesis of the white gene in Aedes aegypti. Insect Mol Biol *9*, 119-125.

Weng, M. H., Lien, J. C., Wang, Y. M., Wu, H. L., and Chin, C. (1997). Susceptibility of three laboratory strains of Aedes albopictus (Diptera: Culicidae) to Japanese encephalitis virus from Taiwan. J Med Entomol *34*, 745-747.

Wengler, G., Wurkner, D., and Wengler, G. (1992). Identification of a sequence element in the alphavirus core protein which mediates interaction of cores with ribosomes and the disassembly of cores. Virology *191*, 880-888.

White, A., Berman, S., and Lowenthal, J. P. (1972). Comparative immunogenicities of Chikungunya vaccines propagated in monkey kidney monolayers and chick embryo suspension cultures. Appl Microbiol *23*, 951-952.

White, J., and Helenius, A. (1980). pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc Natl Acad Sci U S A *77*, 3273-3277.

Wigelsworth, D. J., Krantz, B. A., Christensen, K. A., Lacy, D. B., Juris, S. J., and Collier, R. J. (2004). Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen. J Biol Chem *279*, 23349-23356.

Wolfe, N. D., Dunavan, C. P., and Diamond, J. (2007). Origins of major human infectious diseases. Nature *447*, 279-283.

Wolfe, N. D., Kilbourn, A. M., Karesh, W. B., Rahman, H. A., Bosi, E. J., Cropp, B. C., Andau, M., Spielman, A., and Gubler, D. J. (2001). Sylvatic transmission of arboviruses among Bornean orangutans. Am J Trop Med Hyg *64*, 310-316.

Wolfs, T.F., de Jong, J.J., Van den Berg, H., Tijnagel, J.M., Krone, W.J., and Goudsmit, J. (1990). Evolution of sequences encoding the principal neutralization epitope of human immunodeficiency virus 1 is host dependent, rapid, and continuous. Proceedings of the National Academy of Sciences of the United States of America *87*, 9938-9942.

Woodall, J. (1995). Aedes albopictus - Honduras, http://www.promedmail.org. Archive Number 19951214.1251 (retrieved 05/2009).

Woodward, T. M., Miller, B. R., Beaty, B. J., Trent, D. W., and Roehrig, J. T. (1991). A single amino acid change in the E2 glycoprotein of Venezuelan equine encephalitis virus affects replication and dissemination in Aedes aegypti mosquitoes. J Gen Virol *72 (Pt 10)*, 2431-2435.

Yergolkar, P. N., Tandale, B. V., Arankalle, V. A., Sathe, P. S., Sudeep, A. B., Gandhe, S. S., Gokhle, M. D., Jacob, G. P., Hundekar, S. L., and Mishra, A. C. (2006). Chikungunya outbreaks caused by African genotype, India. Emerg Infect Dis *12*, 1580-1583.

Zhang, W., Heil, M., Kuhn, R. J., and Baker, T. S. (2005). Heparin binding sites on Ross River virus revealed by electron cryo-microscopy. Virology *332*, 511-518.

Ziegler, S. A., Lu, L., da Rosa, A. P., Xiao, S. Y., and Tesh, R. B. (2008). An animal model for studying the pathogenesis of chikungunya virus infection. Am J Trop Med Hyg *79*, 133-139.

VITA

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PRESENT POSTITION AND ADDRESS

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BIOGRAPHICAL

Date of Birth:	September 17, 1979
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Current Address:	112 Tarpon Ave. Galveston, Texas 77550 USA

EDUCATION

2005-2009 Ph.D. (in progress)	Experimental Pathology, The University of Texas Medical Branch Galveston, Texas Cumulative GPA 3.94
1997-2002	B.S. and M.S. (Biological Sciences), Novosibirsk State University and Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk, Russia.

RESEARCH ACTIVITIES

7/05-present	Graduate Research Assistant/Graduate Student Laboratory of Stephen Higgs, Ph.D Experimental Pathology University of Texas Medical Branch Galveston, TX
2003-2005	Rearch Assistant I Laboratory of Stephen Higgs, Ph.D Experimental Pathology University of Texas Medical Branch Galveston, TX, USA

HONORS

2009	Poster session Pathology Trainee Day award.
2009	McLaughlin endowment travel award.
2008-2009	McLaughlin endowment Predoctoral Fellowship award.
2008	Center for Tropical Diseases Graduate Student Award
2008	1st place in poster session Pathology Trainee Day.
2008	"Who`s Who Among Students in American universities and Colleges"
2007	Arthur Zimang Award.

TEACHING RESPONSIBILITIES (UTMB)

Graduate School

Fall 2006	Assistant Mentor to senior Graduate Student (Bradley Schneider) Basic molecular biology techniques: SDS-PAGE electrophoresis, Western blotting
Spring-Summer 2007	Assistant Mentor to senior Graduate Student (Charles McGee) Basic molecular biology techniques.
Fall 2007	Assistant Mentor to Post-Doctoral Fellow (Naomi Forrester). Advanced molecular biology techniques. Construction of infectious clone of Mayaro virus
Winter 2008	Assistant Mentor to first year Graduate Student (Mary Miller) Site directed mutagenesis of the E2 protein of CHIKV strains Ag41855 and La Reunion.
Spring 2008	Assistant Mentor to first year Graduate Student (Katie Taylor). Rescuing and characterization of pseudo-revertants of CHIKV with lethal mutation in position 226 of E1 protein.

Medical School

Spring 2006 — Assistant Instructor in 4 labs with Department of Pathology faculty.

Topics:
- Humoral immunity and cytokines.
- Chills, fevers, aches and pains
- Neoplasia
- Pediatric and developmental pathology

(Partial fulfillment of Department of Pathology course requirements)

School of Allied Health Sciences
Clinical Laboratories Sciences

Summer 2006 Co-Instructor: Special Topics Module
Clinical Virology Acute vs. Chronic Viral Disease
Infectious Arthropod-borne Disease: The Flaviviruses
(Partial fulfillment of Department of Pathology course requirements)

MEMBERSHIP IN SCIENTIFIC SOCIETIES

2007-present American Society for Virology
2005-present American Society for Tropical Medicine and Hygiene

PUBLICATIONS

A. ARTICLES IN PEER-REVIEWED JOURNALS

1. McGee CE, Shustov AV, **Tsetsarkin K**, Frolov IV, Mason PW, Vanlandingham DL, Higgs S, 2009. Infection, Dissemination, and Transmission of a West Nile Virus Green Fluorescent Protein Infectious Clone by Culex pipiens quinquefasciatus Mosquitoes. Vector Borne Zoonotic Dis.
2. McElroy KL, Girard YA, McGee CE, **Tsetsarkin KA**, Vanlandingham DL, Higgs S, 2008. Characterization of the Antigen Distribution and Tissue Tropisms of Three Phenotypically Distinct Yellow Fever Virus Variants in Orally Infected Aedes aegypti Mosquitoes. Vector Borne Zoonotic Dis.
3. de Lamballerie X, Leroy E, Charrel RN, **Tsetsarkin K**, Higgs S, Gould EA, 2008. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J 5: 33.
4. McGee CE, Lewis MG, Claire MS, Wagner W, Lang J, Guy B, **Tsetsarkin K**, Higgs S, Decelle T, 2008. Recombinant Chimeric Virus with Wild-Type Dengue 4 Virus Premembrane and Envelope and Virulent Yellow Fever Virus Asibi Backbone Sequences Is Dramatically Attenuated in Nonhuman Primates. J Infect Dis.
5. McGee CE, **Tsetsarkin K**, Vanlandingham DL, McElroy KL, Lang J, Guy B, Decelle T, Higgs S, 2008. Substitution of Wild-Type Yellow Fever Asibi Sequences for 17D Vaccine Sequencesin ChimeriVax-Dengue 4 Does Not Enhance Infection of Aedes aegypti Mosquitoes. J Infect Dis.
6. **Tsetsarkin KA**, Vanlandingham DL, McGee CE, Higgs S, 2007. A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. PLoS Pathog 3: e201.

7. Sim C, Hong YS, **Tsetsarkin KA**, Vanlandingham DL, Higgs S, Collins FH, 2007. Anopheles gambiae heat shock protein cognate 70B impedes onyong-nyong virus replication. BMC Genomics 8: 231.
8. Vanlandingham DL, **Tsetsarkin K**, Klingler KA, Hong C, McElroy KL, Lehane MJ, Higgs S, 2006. Determinants of vector specificity of o'nyong nyong and chikungunya viruses in Anopheles and Aedes mosquitoes. Am J Trop Med Hyg 74: 663-9.
9. McElroy KL, **Tsetsarkin KA**, Vanlandingham DL, Higgs S, 2006. Role of the yellow fever virus structural protein genes in viral dissemination from the Aedes aegypti mosquito midgut. J Gen Virol 87: 2993-3001.
10. McElroy KL, **Tsetsarkin KA**, Vanlandingham DL, Higgs S, 2006. Manipulation of the yellow fever virus non-structural genes 2A and 4B and the 3'non-coding region to evaluate genetic determinants of viral dissemination from the Aedes aegypti midgut. Am J Trop Med Hyg 75: 1158-64.
11. **Tsetsarkin K**, Higgs S, McGee CE, De Lamballerie X, Charrel RN, Vanlandingham DL, 2006. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. Vector Borne Zoonotic Dis 6: 325-37.
12. Vanlandingham DL, Hong C, Klingler K, **Tsetsarkin K**, McElroy KL, Powers AM, Lehane MJ, Higgs S, 2005. Differential infectivities of o'nyong-nyong and chikungunya virus isolates in Anopheles gambiae and Aedes aegypti mosquitoes. Am J Trop Med Hyg 72: 616-21.
13. McElroy KL, **Tsetsarkin KA**, Vanlandingham DL, Higgs S, 2005. Characterization of an infectious clone of the wild-type yellow fever virus Asibi strain that is able to infect and disseminate in mosquitoes. J Gen Virol 86: 1747-51.
14. Vanlandingham DL, **Tsetsarkin K**, Hong C, Klingler K, McElroy KL, Lehane MJ, Higgs S, 2005. Development and characterization of a double subgenomic chikungunya virus infectious clone to express heterologous genes in Aedes aegypti mosquitoes. Insect Biochem Mol Biol 35: 1162-70.

B. SUBMITTED

1. **Tsetsarkin K**, McGee CE, Volk S, Vanlandingham DL, Weaver S, Higgs S, 2009. EPISTATIC ROLES OF E2 GLYCOPROTEIN MUTATIONS IN ADAPTATION OF CHIKUNGUNYA VIRUS TO AEDES ALBOPICTUS AND AE. AEGYPTI MOSQUITOES. PLOS One.
2. Volk S, Garcia T, Sall A, Schuh A, **Tsetsarkin K,** Adams P, Holmes E, Higgs S, Maharaj P, Brault A, Weaver S, 2009. Chikungunya Virus Evolutionary Rates Vary According to Transmission Cycle. PLoS Pathog.

C. ABSTRACTS/PRESENTATION

C. E. McGee, **K. Tsetsarkin**, J. Lang, B. Guy, T. Decelle, M. G. L., M. St. Claire, W. Wagner, D. L. Vanlandingham, K. L. McElroy, and St. Higgs. Recombinant chimeric virus with wild type dengue 4 premembrane and envelope and wild type yellow fever Asibi backbone sequences is dramatically attenuated in Aedes aegypti mosquitoes and Cynomolgus macaques. Poster. American Society for Virology 27th annual meeting. July 12-16, 2008. Cornell University, Ithaca, NY. Abstract #22-9.

K. A. Tsetsarkin, C. E. McGee, D. L. Vanlandingham, S. Higgs. Chikungunya virus – mechanism of adaptation to *Ae.albopictus* mosquito. Poster. American Society for Virology 27th annual meeting. July 12-16, 2008. Cornell University, Ithaca, NY. Abstract #34-1.

C. E. McGee, **K. Tsetsarkin**, J. Lang, B. Guy, T. Decelle, M. G. Lewis, M. St. Claire, W. Wagner, D. L. Vanlandingham, K. L. McElroy, and S. Higgs. Recombinant chimeric virus with wild type dengue 4 premembrane and envelope and wild type yellow fever Asibi backbone sequences is dramatically attenuated in *Aedes aegypti* mosquitoes and *Cynomolgus macaques*. Poster. Department of Pathology 14th Annual Research Day. May 6, 2008. Galveston TX. Abstract #7.

K. Tsetsarkin, Dana L. Vanlandingham, Charles E. McGee, and Stephen Higgs. A single mutation in chikungunya virus affects vector specificity and epidemic potential. Poster. Department of Pathology 14th Annual Research Day. May 6, 2008. Galveston TX. Abstract #11.

C. E. McGee, **K. Tsetsarkin**, J. Lang, B. Guy, T. Decelle, M. G. Lewis, M. St. Claire, W. Wagner, D. L. Vanlandingham, K. L. McElroy, and S. Higgs Recombinant chimeric virus with wild type Dengue 4 prM-E and wild type yellow fever Asibi backbone sequences is dramatically attenuated in non-human primates and Aedes aegypti mosquitoes. Poster. Seventh McLaughlin Symposium in Infection and Immunity. November 15-17, 2007. Galveston, TX. Abstract #2.

C. E. McGee, A. V. Shustov, **K. Tsetsarkin**, I. V. Frolov, P. W. Mason, D. L. Vanlandingham, and S. Higgs. Infection, Dissemination, and Transmission of a West Nile virus Green Fluorescent Protein Infectious Clone by *Culex pipiens quinquefasciatus* Mosquitoes. Oral Presentation American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #658.

C. E. McGee, **K. Tsetsarkin**, J. Lang, B. Guy, T. Decelle, M. G. Lewis, M. S. Claire, W. Wagner, D. L. Vanlandingham, K. L. McElroy, and S. Higgs. Recombinant chimeric virus with wild type Dengue 4 prM-E and wild type yellow fever Asibi backbone sequences is dramatically attenuated in *Aedes aegypti* mosquitoes and non-human primates. Poster. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #2696.

K. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, X. De Lamballerie, R.N. Charrel, and Stephen Higgs. Infectious Clones of Chikungunya Virus (La Reunion Isolate) for Vector Competence Studies. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. Poster. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #613.

K. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, X. De Lamballerie, R.N. Charrel, and S. Higgs. Infectious Clones of Chikungunya Virus (La Reunion Isolate) for Vector Competence Studies. Poster. American Society for Virology 26th Annual Meeting. July 14-18, 2007. Corvallis, Oregon. Abstract #413

C. E. McGee., A. V. Shustov, **K. Tsetsarkin**, I. V. Frolov, P. W. Mason, D. L. Vanlandingham, and S. Higgs. Infection, Dissemination, and Transmission of a West Nile virus Green Fluorescent Protein Infectious Clone by *Culex pipiens quinquefasciatus* Mosquitoes. Poster. 18th Annual Course on Biology of Disease Vectors. June 3-16, 2007. Manaus, Amazonas-Brazil.

C. E. McGee, A. V. Shustov, **K. Tsetsarkin,** I. V. Frolov, P. W. Mason, D. L. Vanlandingham, and S. Higgs. Infection, Dissemination, and Transmission of a West Nile virus Green Fluorescent Protein Infectious Clone by *Culex pipiens quinquefasciatus* Mosquitoes. Poster. Department of Pathology 13th Annual Research Day. May 2, 2007. Galveston, TX. Abstract #16

K. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, X. De Lamballerie, R.N. Charrel, and S. Higgs. Infectious Clones of Chikungunya Virus (La Reunion Isolate) for Vector Competence Studies. Poster. Department of Pathology 13th Annual Research Day. May 2, 2007. Galveston, TX. Abstract #18

K. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, X. De Lamballerie, R.N. Charrel, and S. Higgs. American Society of Tropical Medicine and Hygiene 55th Annual Meeting. November 12-16, 2006. Atlanta, GA. Abstract #2604

K L. McElroy, Y A. Girard*, C E. McGee*, **K A. Tsetsarkin**, D L. Vanlandingham, and S Higgs. Distribution and tissue tropisms of three phenotypically distinct yellow fever virus strains in *Aedes aegypti.* Poster. American Society of Tropical Medicine and Hygiene 55th Annual Meeting. November 12-16, 2006. Atlanta, GA. Abstract #809

K. Tsetsarkin, D. L. Vanlandingham, C. E. McGee, X. De Lamballerie, R.N. Charrel, and S. Higgs. Infectious Clones of La Reunion Isolate of Chikungunya Virus for Vector Studies. Poster. Experimental Pathology BBSC Program Orientation Presentation and Poster Session. October 25, 2006. Galveston, TX.

K L. McElroy, **K A. Tsetsarkin,** Y A. Girard, C E. McGee, D L. Vanlandingham, S Higgs. Genetic and Biological Determinants of Viral Dissemination from the Mosquito Midgut: Yellow fever virus in *Aedes aegypti*. Oral Presentation by Kate L. McElroy. American Society of Tropical Medicine and Hygiene 54th Annual Meeting. December 10-15, 2005. Washington, D.C. Abstract #1074.