DISSERTATION

APPLICATION OF GENETIC TOOLS TO IDENTIFY THE DETERMINANTS OF ARBOVIRUS INFECTION OF THE *AEDES AEGYPTI* MIDGUT

Submitted by:

Dennis John Pierro

Department of Microbiology, Immunology and Pathology

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DENNIS JOHN PIERRO ENTITLED APPLICATION OF GENETIC TOOLS TO IDENTIFY THE DETERMINANTS OF ARBOVIRUS INFECTION OF THE *AEDES AEGYPTI* MIDGUT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Barry Beaty

Ann M. Powers

R A Brown

Adviser

Carol D Blair

Department Head (and Committee Member)

ABSTRACT OF DISSERTATION

APPLICATION OF GENETIC TOOLS TO IDENTIFY THE DETERMINANTS OF ARBOVIRUS INFECTION OF THE *AEDES AEGYPTI* MIDGUT

Aedes aegypti is one of the most important mosquito species in medical entomology today. It is a known vector of dengue (DENV) and yellow fever viruses as well as being susceptible to a number of other arboviruses capable of causing disease in humans and domestic animals. It has very rapidly achieved an expansive distribution throughout most of the tropical and subtropical regions on the world. *Ae. aegypti* has also evolved into a "domesticated" species by living and breeding within the human communities increasing its contact frequency with people.

Arbovirus infection of *Ae. aegypti* begins with the acquisition of an infectious bloodmeal into the mosquito's midgut. Midgut epithelial cells then become infected eventually leading to midgut escape and the potential to retransmit the arbovirus via an infected salivary gland. However, the midgut epithelial cells are known to be selectively susceptible to arbovirus infection and constitute a barrier to arbovirus infection. This barrier is thought to be a result of the genetics of the mosquito and the arbovirus.

The arboviral genetic determinants of midgut infection are very poorly understood despite the extreme importance of this organ in arboviral transmission cycles. This dissertation intends to investigate the viral genetic determinants of midgut infection through the use of infectious clone (ic) technology. Four arboviral genomes were investigated for their ability to differentially infect *Ae. aegypti* midguts: Sindbis virus (SINV) strains TE/5'2J, TR339, MRE16 and DENV-2 strain 1409.

Through the use of the SINV TE/5'2J ic as a backbone, the TR339 genetic determinants of *Ae. aegypti* midgut infection were associated with the E2 glycoprotein, specifically at amino acid (aa) positions E2-55 and E2-70. These sites were identified to affect the midgut infection rate (MIR) independently and in combination with each other. Structurally, theses sites were found in predicted loop regions of the protein. Phenotypically, I observed that mammalian derived viruses more efficiently established an infection of midgut cells than mosquito derived viruses independently of the TR339 genetic determinants.

Also through the use of the SINV TE/5'2J ic, the MRE16 genetic determinants of *Ae. aegypti* midgut infection were associated with the E2 glycoprotein as well, specifically at aa positions E2-95-6 and E2-116-119. These sites were also in loop regions of E2 working collectively to enhance the MIR. Interestingly, a conserved aa motif (i.e. PPF/.GDS) as well as a common structural configuration was identified among the envelope proteins of the alpha- and flaviviruses. Moreover, the specific genetic determinants of MRE16 and TR339 led to the hypothesis that envelope proteins with protruding loop regions, which have variable aa sequences, was a common structure involved in alpha- and flavivirus infections of midguts.

The arbovirus DENV-2 strain 1409 is proposed to be an early isolate of the newly established American/Asian DENV-2 genotype that has caused significant number of disease outbreaks in the tropical Americas. The 1409 isolate has been well characterized in the laboratory for its infection potential in *Ae. aegypti* midguts and represents an ideal candidate for ic construction and future genetic determinant assays. This dissertation describes the technical hurdles overcome to successfully generate and characterize a full-length infectious clone of DENV-2 1409.

The work and data described in this dissertation adds to the knowledge of mosquito midgut infections by presenting insights into the specific residues responsible for midgut infections. It also provides a new tool for further investigations into infection determinants of arboviruses. Moreover, future research projects can be designed from the information in this dissertation to better identify the determinants of mosquito midgut infections with the ultimate goal of applying such information for the control and elimination of arboviral diseases.

Dennis John Pierro
Department of Microbiology, Immunology and Pathology
Colorado State University
Fort Collins, CO 80523
Summer 2007

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Dennis J. Pierro
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Literature Review

Introduction: Public Health impact of arthropod-borne viruses.

Infectious and parasitic diseases, including HIV/AIDS, tuberculosis and measles, account for approximately 19.5% of all deaths worldwide (WHO 2003). Arthropod-borne infectious diseases, including malaria, dengue and leishmania, account for roughly 12.5% of these deaths or 2.3% of all deaths worldwide. A subset of the arthropod-borne diseases is the arthropod-borne viruses (arboviruses) that can cause a broad spectrum of diseases in humans and domestic animals. Arboviruses, such as dengue (mosquito-borne), sandfly fever (sandfly-borne) and Kyasanar forest disease (tick-borne) viruses, are reported to cumulatively kill over 33,000 people worldwide every year (WHO 2003). These reported values are widely believed to be large underestimations of the global mortality rate for these diseases. The underestimation is thought to be a result of inaccessible clinical sites, poor diagnosis and/or inadequate reporting mechanisms. In fact, it is estimated that 2.5 billion people (two-fifths of the world population) are at risk for dengue virus infections alone (WHO 1997). The disease burden of arboviruses in hyperendemic countries is substantial, with up to 292 per 1000 children infected each year, and up to 8 per 1000 children hospitalized per year (Deen, Harris et al. 2006). In addition, arbovirus-infected domestic animals can lead to severe morbidity or mortality (e.g. Venezuelan equine encephalitis infected horses) with an economic loss in the form of decreased agricultural production or inefficient animal husbandry (Bram, George et al. 2002; Gould, Higgs et al. 2006).

The public health impact of arthropod-borne diseases has been recognized for some time and various prevention programs (as well as the hurdles to prevention) have

been proposed (Maroli and Majori 1991; 1995; Gubler 1998; Beaty 2005; Hemingway, Beaty et al. 2006). Gubler *et al.* suggest that effective arboviral disease prevention programs must have several integrated components, including active laboratory-based surveillance, emergency response, education of the medical community to ensure effective case management, community-based integrated mosquito control, and effective use of vaccines when they become available (Gubler 1998). In addition, sustained financial and political support for effective programs locally, regionally and globally are needed to aid in arbovirus disease control because patchy prevention programs can become ineffectual (Gubler and Clark 1996; Guzman, Alvarez et al. 1999; Mudur 2006; Ooi, Goh et al. 2006).

Complicating the public health efforts of endemic arbovirus disease control is the issue of epidemic disease. For example, the emergence of West Nile virus for the first time in North America in 1999 has resulted in over 16,700 new arboviral disease cases, more than 665 deaths and it is expected to be a public health concern throughout the Americas for many years to come (Hayes and Gubler 2006). The emergence of Chikungunya virus in India and islands in the Indian Ocean in 2006 may be the consequence of a variant virus that is predicted to result in approximately 2 million cases of disease in 2006 alone (Mudur 2006; Parola, de Lamballerie et al. 2006; Charrel, de Lamballerie et al. 2007). Moreover, the reemergence of arthropod-borne diseases in quiescent areas is a problematic common occurrence (Guzman, Alvarez et al. 1999; Hubalek and Halouzka 1999). In fact, the wide-spread use of an effective and long lasting vaccine against the mosquito-borne yellow fever virus has not yet stopped the recurrence of tens of thousands of yellow fever disease cases every year (Gubler 2004;

Barrett and Higgs 2007).

A multifaceted and long-term approach to arboviral disease control is necessary and needs to take into account all of the above facts. The control of arboviruses will also benefit from the development of new technologies capable of reducing disease (e.g. genetic modification of mosquitoes). Understanding the issues surrounding the dynamic relationship between the virus, the arthropod and the vertebrate host will also impact the efficiency and rate at which arboviruses are controlled and possibly eradicated. Other issues include understanding the the impact of globalization of the human population on arthropod-borne diseases, the correct use of chemicals for vector control, and the hurdles to the development of new antiviral drugs and vaccines. Moreover, since many of the world's poorest citizens are at the highest risk for disease, strategies for overcoming the socioeconomic barriers to disease prevention need to be developed and implemented.

A significant amount of *in vitro* research has recently gone into understanding the molecular mechanisms of arbovirus infections. Such data give insights into some of the parameters for virus infection, but only provide limited insights into the details of arboviral transmission in the wild. Interlinking questions about host susceptibility requirements, neuroinvasion mechanisms, vector competence requisites, determinants of viral infection, innate defense modulation, etc., are still far from being answered. Disease control measures will be greatly enhanced by examining the details of natural arboviral transmission.

Arbovirus-vector-host cycles.

Arboviruses are characterized by using a blood-sucking arthropod to transmit a

virus between vertebrate hosts, which excludes the vector-borne plant viruses and the invertebrate pathogenic viruses (WHO 1985). They are classically maintained in natural cycles by hematophagous arthropods such as Diptera (mosquitoes and biting flies), Hemiptera (bloodfeeding bugs) and Acari (ticks) that transmit the arbovirus to vertebrates during a bloodfeeding event sometime in their life cycle (Woodring JL 1996). In turn, an infected vertebrate can amplify the virus and act as a reservoir for infections of numerous naïve arthropods during feedings. This vector-host (i.e. arthropod-vertebrate) cyclic interaction is a defining characteristic in the long term survival of most arboviruses.

Of the 534 arboviruses registered in the International Catalog of Arboviruses, 214 are known or probable arboviruses, 287 are considered probably arboviruses and 33 are listed as probably or definitely not arboviruses (Karabatsos 1985. Updated 2001.; Gubler 2002). Approximately 135 of the arboviruses are known to cause illness in humans. These arboviruses are taxonomically diverse, consisting of seven families and numerous genera. Six of the seven families are RNA viruses: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Rhabdoviridae*, *Reoviridae*, *and Orthomyxoviridae*. The first three families constitute arboviruses with the largest impact on world public health (Gubler 2002). There is only one known DNA arbovirus, African swine fever virus (family *Asfarviridae*) (Brown 1986; Dixon KL 1999).

Both the arachnids and the insects are involved in biological arbovirus transmission. There are approximately 25,800 known arachnids capable of bloodsucking behavior and there are over 17,000 species of blood-sucking insects, which is equal to about 1.5% of all recognized insects (Black 2005; Kuno and Chang

2005). Of these insects, approximately 3,600 mosquitoes (Order Diptera, family Culicidae, subfamilies *Anophelinae* and *Culicinae*) are capable of bloodfeeding behavior. However, the ability of a vector to become infected and then transmit an arbovirus to a host (also called vector competence) is unique to each arthropod. Infections of mosquito vectors by arboviruses and the establishment of a transmission cycle are dependent on a variety of physiological, environmental and genetic factors. Such factors include the infection determinants of the virus, the susceptibility and permissiveness of different mosquito cell types, viral dose, environmental conditions (e.g. temperature, light cycles), feeding behaviors, host selection and others. It is interesting to note that even within a mosquito species (e.g. *Culex pipiens*), subspecies can have dramatically different vector competence (Lee, Rowley et al. 2000; Bennett, Olson et al. 2002; Fonseca, Keyghobadi et al. 2004).

The vast majority of arboviral diseases are zoonotic, having primary enzootic transmission cycles involving arthropods with wild animals as well as involving arthropods with humans and domestic animals that often represent tangential or dead-end hosts (Weaver and Barrett 2004; Weaver 2006). A typical mosquito-borne arbovirus enzootic transmission cycle involves avian, rodent, or nonhuman primates as a reservoir and/or amplification host and mosquitoes (figure 1.1, page 7, top center loop). The simplest mechanism for human infection from this enzootic cycle is where "spillover" infections occur. This can happen when a natural enzootic cycle is in close proximity to humans or to domestic animals, allowing mosquitoes to easily find these potential bloodmeal sources. Alternatively, spillover can occur when amplification of the enzootic virus results in higher numbers of circulating infected mosquitoes that in

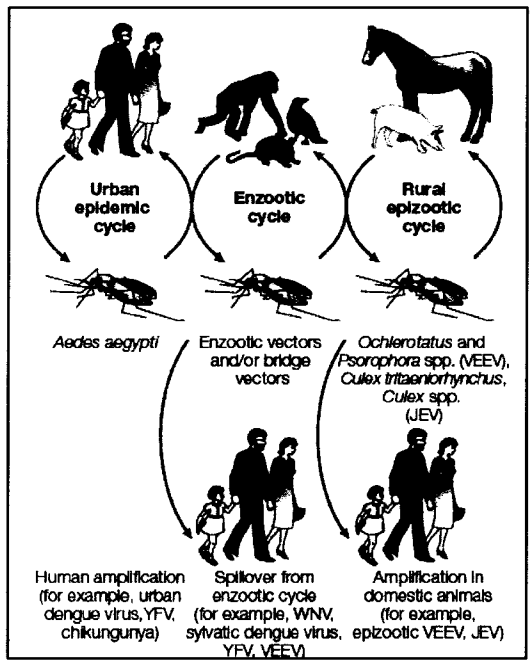


Figure 1.1. Representative arbovirus transmission cycles involving mosquitoes (Weaver and Barrett 2004)

turn infect humans. Spillover from an enzootic cycle can also occur as a result of bridge vectors, which can have broader host preferences. Secondary amplification, or a rural epizootic cycle (figure 1.1, top right loop), is another mechanism for arbovirus transmission to humans whereby local domesticated animals become infected and act as amplifiers to ultimately increase the frequency of infected mosquitoes in human habitats and increase human encounters. A third mechanism is the urban cycle (figure 1.1, top left) that utilizes humans for virus amplification, resulting in self sustaining epidemic cycles and the potential for large outbreaks of disease (Weaver and Barrett 2004).

Mosquito-borne virus infection of a vertebrate.

The means of arbovirus transmission from a mosquito vector to a vertebrate host is by delivery of virus-containing saliva during bloodfeeding, after which, the virus

infects host cells and a viremia develops. However, the requirements for virus delivery and establishment in a vertebrate are quite complicated and start well before the introduction of a virus into the host. Numerous events must accurately occur in order to achieve a successful transmission cycle.

The ability of an arbovirus to infect a host is initially dictated by the mosquito's host selection skills and feeding behavior. Host selection is the preference of a mosquito to feed on a specific vertebrate species. Mosquitoes that have a host selection preference that configures to a particular transmission cycle (figure 1.1, page 7) are likely to promote disease transmission. For example, opportunistic feeders such as *Culex quinquefasciatus, Cx. fatigan* and *Cx. tarsalis* are more likely to promote a "spillover" of an enzootic cycle or a transmission from a rural epizootic cycle to a human because of their ability to selectively feed on wild animals and humans (Reisen and Boreham 1979; Wekesa, Yuval et al. 1997; Elizondo-Quiroga, Flores-Suarez et al. 2006). In addition, the presence and activity of a bridge vector (i.e. an arthropod that facilitates virus transmission to humans but is not a significant component of the zoonotic cycle), such as *Cx. salinarius* for West Nile virus, can also significantly influence disease transmission from both of these cycles (Medlock, Snow et al. 2005; Molaei, Andreadis et al. 2006). Alternatively, the population density of anthrophilic (i.e. attracted to humans) mosquitoes, such as *Aedes aegypti* and *Ae. albopictus,* in urban epidemic transmission cycles (figure 1.1, left side) can greatly affect disease transmission rates between humans (Gratz 2004; Moncayo, Fernandez et al. 2004; Ponlawat and Harrington 2005). Host seeking behavior relies on a variety of cues, such as kairomones (chemical produced by the host), visual patterns, temperature, etc.,

to attract a mosquito to a host (Lothrop and Reisen 2001). Moreover, the feeding behavior of an infected mosquito, such as circadian activity (i.e. feeding time), repetitive feeding rates, probing times, mosquito survival rates, etc., can also influence disease transmission (Reisen, Meyer et al. 1993; Anderson and Brust 1997; Reisen, Lothrop et al. 1997; Ribeiro 2000). In summary, the combined events that lead up to the delivery of mosquito saliva into a host are a varied array of extremely critical events in the arboviral transmission process.

Following the delivery of an arbovirus to a vertebrate, the virus must evade early host defenses, such as complement and cell mediated immunity (e.g. interferon induction), to achieve a primary infection. This evasion can be aided by components in the saliva that can modulate the immune system such as the suppression IFNγ or the up regulation of IL-4, among others (reviewed by Titus) (Zeidner, Higgs et al. 1999; Schneider, Soong et al. 2004; Titus, Bishop et al. 2006). Following evasion, a primary infection can be established in localized resident dendritic cells, such as Langerhans cells; in monocytes/macrophages; or the virus can bypass localized cells and primarily infect cells of the draining lymph nodes (Johnston, Halliday et al. 2000; MacDonald and Johnston 2000; Wu, Grouard-Vogel et al. 2000; Barratt-Boyes, Zimmer et al. 2002). After the initial infection is established, replication of the virus results in a secondary and more-systemic infection leading to viremia. Pathogenesis, when observed, is often correlated with viremia; however, arbovirus disease in humans is often sub-clinical, acute and self-limiting.

Arbovirus acquisition by a mosquito.

Transmission of an arbovirus from a vertebrate to a mosquito is initiated by the uptake of virus-containing blood during ingestion of a viremic bloodmeal. As in the case of vertebrate host infections, the requirements for mosquito infection and establishment of a transmission cycle are complicated and initially rely on an interplay of events. Some of these events occur before the mosquito even ingests a bloodmeal, while others are intrinsic to the mosquito. For example, the type, abundance and contact frequency of vertebrate hosts available to a particular mosquito are important early, external factors in the capacity of a mosquito to become infected and then transmit a virus (Favier, Chalvet-Monfray et al. 2006; Lord, Rutledge et al. 2006). Mosquito host selection and feeding behavior are also important factors in virus transmission to the mosquito (Day 2005). In addition, environmental conditions such as the daily temperature, recent rain patterns (which impact mosquito regeneration rates), and the abundance of appropriate habitat, (which can increase mosquito densities) are also external factors important in the likelihood that a mosquito will become infected (Kay and Jennings 2002; Favier, Chalvet-Monfray et al. 2006; Munga, Minakawa et al. 2006; Reisen, Fang et al. 2006).

After mosquito host selection, probing and imbing occur, the infected blood passes along the mosquito's alimentary tract and is delivered to the midgut. The arbovirus then comes into contact with some of the mosquito's innate defenses such as secreted digestive enzymes and the development of a peritrophic matrix. Digestive enzymes secreted into the lumen of the mosquito midgut are intended to break down the components of the bloodmeal and are traditionally thought to have a negative impact on virus infectivity due to their multi-targeted approach (Billingsley 1990). However, it has

been observed that some arboviruses (e.g. bunya- and orbiviruses) may require proteolytic processing prior to midgut infection (Ludwig, Christensen et al. 1989; Ludwig, Israel et al. 1991; Mertens, Burroughs et al. 1996; Xu, Wilson et al. 1997). It is interesting to note that this requirement for proteolytic processing may be specific to insect cell infection and not mammalian. The peritrophic matrix defense mechanism is a chitinous gel-like structure that originates as a secretion from the midgut cells themselves in response to blood accumulation. It eventually surrounds the bloodmeal to physically separate the blood bolus from the epithelial cells (Shao, Devenport et al. 2001). Accordingly, to enhance the likelihood of midgut infection, arboviruses must appropriately negotiate the proteolytic processing of the digestive enzymes and rapidly infect epithelial cells prior to the development of the peritrophic matrix structure.

Following the successful bypass of the defenses of the midgut-lumen, arboviruses must adhere to a susceptible midgut epithelial cell to enter. The two main mechanisms proposed for arbovirus cell recognition and entry are receptor mediated endocytosis and direct cell surface fusion. Both of these lead to cytosolic internalization of the nucleocapsid and delivery of the genomic RNA into the cytosol, where replication and packaging occurs (Saito and Gale 2007). No specific cell surface receptor(s) for arbovirus infection has yet been definitively identified, although high-affinity laminin receptors, heparin sulfate receptors as well as non specific binding proteins have been implicated as attachment proteins (Wang, Kuhn et al. 1992; Byrnes and Griffin 1998; Klimstra, Ryman et al. 1998; Mourya, Ranadive et al. 1998; Mercado-Curiel, Esquinca-Aviles et al. 2006). Infection of the midgut appears to be a barrier to infection for many arboviruses due to its restricted cell susceptibility.

Release of infectious arbovirus particles from the midgut epithelium has been observed to be a rate limiting transmission step and is known as the midgut escape barrier (Kramer, Hardy et al. 1981; Turell, Gargan et al. 1984; Weaver, Scherer et al. 1984; McElroy, Tsetsarkin et al. 2006). Determinants for midgut escape have been linked to the arboviral genomes (Beaty, Miller et al. 1982). Successful virus dissemination into the haemoceol from the midgut then can lead to secondary infection of other mosquito tissues and organs including the mosquito salivary gland. Infection of and escape from the salivary gland is the final major mosquitoe barrier to virus transmission (Kramer, Hardy et al. 1981; Paulson, Grimstad et al. 1989). Once infected, the salivary gland acts as the portal for virus transmission to a new vertebrate via the saliva. The time from mosquito ingestion of an arbovirus, to the infection of additional mosquito organs, to transmission to a new host via saliva is called the extrinsic incubation period (Hardy 1988).

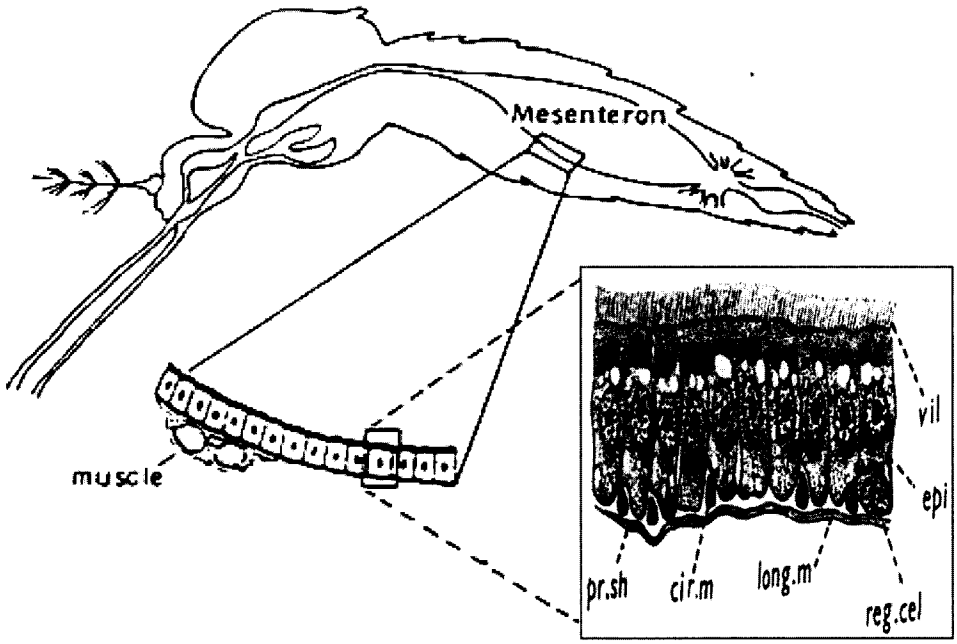


Figure 1.2. Cross section of a mosquito and its midgut (in box). vil, microvilli; epi, columnar epithelial cells; reg.cel, regenerative cells; lon.m, longitudinal musculature cell; cir.m, circular musculature cell; pr.sh, basal lamina (Jobling and Lewis 1987. Anatomical drawings of biting flies. British Museum of Natural History, London, UK)

Arbovirus infection of the mosquito midgut.

As stated above, the infection of the mosquito midgut is a limiting step in an arbovirus transmission cycle. Mosquitoes that do not support arbovirus infection of the midgut are considered to have a midgut infection barrier (MIB). This barrier can be a result of the loss of functions in receptor binding, uncoating, transcription, or translation events (Black, Bennett et al. 2002). The arbovirus MIB is thought to be a result of genetic factors of both the virus and vector (Bennett, Olson et al. 2002; Black, Bennett et al. 2002; Brault, Powers et al. 2002).

The midgut wall is composed of a single layer of polarized, tight-junction columnar epithelial cells surrounded externally by a lattice of muscular cells and a

porous basal lamina (figure 1.2) (Hardy 1988). On the lumen side, the midgut cells are populated with protruding microvilli as well as being innervated and permeated by respiratory tracheals and nerve fibers (Bertram and Bird 1961; Hecker, Freyvogel et al. 1971; Hecker, Freyvogel et al. 1971; Hecker 1977; Brown, Raikhel et al. 1985; Billingsley 1990; Zieler, Garon et al. 2000). Other cells of the midgut are the endocrine cells, which tend to be smaller than the columnar cells and are positioned more basally and have narrower, if any, apical extensions (Hecker, Freyvogel et al. 1971; Hecker 1977; Brown, Raikhel et al. 1985; Brown 1989; Billingsley 1990; Weaver and Scott 1990; Zieler, Garon et al. 2000). Small, basally located cells observed in midguts shortly after emergence have been postulated to be regenerative cells (Hecker, Freyvogel et al. 1971; Hecker 1977; Brown, Raikhel et al. 1985; Brown 1989; Billingsley 1990; Weaver and Scott 1990; Zieler, Garon et al. 2000). In addition, it has been observed that the posterior end of the midgut has a slightly different endothelial cell morphology. (Shahabuddin and Pimenta 1998; Cociancich, Park et al. 1999; Zieler, Garon et al. 2000).

The mosquito midgut has been known to be the site for primary infection of arboviruses for more than 50 years (McLean 1955; Chamberlain, Sudia et al. 1959; LaMotte 1960). Doi used fluorescent antibody assay in 1970 to first observe arbovirus infection of midguts 4 days post oral infection (Doi 1970). Larsen and Ashley used electron microscopy in 1971 to identify an arbovirus association with the malpighian tubules of the midgut (Larsen and Ashley 1971). Additionally, Whitfield *et. al.* used electron microscopy in 1973 to track an arbovirus infection in a midgut (Whitfield, Murphy et al. 1973). Similar studies were subsequently performed for additional

observations of arboviruses-midgut associations (Scott, Hildreth et al. 1984; Weaver 1986; Weaver, Scott et al. 1988; Weaver, Lorenz et al. 1992).

Determinants of arboviral infection of the midgut.

An early determinant of midgut infection is the arboviral concentration required to overcome a "threshold of infection" (Chamberlain and Sudia 1961; Hardy 1988). The importance of this threshold has been established for a number of arboviruses for some time (Barnett 1956; Hurlbut 1956; Gresser, Hardy et al. 1958; Altma 1963; Jupp and McIntosh 1970; Jupp and McIntosh 1970; Hayles, McLintock et al. 1972; Jupp, McIntosh et al. 1972; Howard and Wallis 1974; Freier and Beier 1984). This dose dependency effect on midgut infection is interesting in that seemingly high titers of cell culture derived virus, in the range of 10^{5-7} PFU/ml, are generally required for arbovirus infection (Weaver, Scott et al. 1988; Bennett, Olson et al. 2002; Tiawsirisup, Platt et al. 2004; Mahmood, Chiles et al. 2006). Considering that mosquitoes generally ingest an artificial bloodmeal volume of approximately 2.5 µl during feedings, viral titers of between 250-25,000 virus particles can be necessary for efficient infection of the midgut (Ogunrinade 1980). However, this threshold appears to be lower in live viremic hosts as the bloodmeal source than artificial ones for unknown reasons (Kramer, Hardy et al. 1981; Lord, Rutledge et al. 2006).

The genetics of the mosquito also play a very important role in midgut infections (Miller and Mitchell 1991; Bosio, Beaty et al. 1998; Bennett, Beaty et al. 2005). Using *Ae. aegypti* crosses, mosquitoes have been identified as having genetic determinants for high and low dengue virus susceptibility phenotypes (Gubler and Rosen 1976; Gubler,

Nalim et al. 1979). It is interesting to note that mosquitoes showing genetic relatedness also demonstrate similarity in oral infection rates with yellow fever virus (Tabachnick, Wallis et al. 1985). Bosio *et. al.* have identified two quantitative trait loci for a midgut infection barrier to dengue virus type 2 and mapped them on chromosomes *II* and *III* of the genome (Bosio, Fulton et al. 2000). Midgut escape barriers have also been identified with one being on multiple chromosomes (Bennett, Flick et al. 2005) Clearly mosquito genetics play a role in midgut infections, but to date no specific genes have been implicated.

The influence of arboviral genetic determinants on midgut infection and dissemination rates has been reported (Beaty, Miller et al. 1982; Weaver, Scott et al. 1988; Seabaugh, Olson et al. 1998; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; McElroy, Tsetsarkin et al. 2006; Vanlandingham, Tsetsarkin et al. 2006). Efficient arbovirus infections in mosquito cells have been associated with both the structural and nonstructural genes of arboviruses (Johnson, Chambers et al. 2002; Vanlandingham, Tsetsarkin et al. 2006). It has been shown that the La Crosse virus (genus *Bunyavirus*, family *Bunyaviridae*) has determinants in its G1 glycoprotein that condition midgut infection (Sudin, Beaty et al. 1987). Alphavirus studies have shown that a number of the virus genetic determinants that specifically affect midgut infection are associated with the viral envelope 2 (E2) glycoprotein (Woodward, Miller et al. 1991; Turell, Ludwig et al. 1999; Brault, Powers et al. 2002; Brault, Powers et al. 2002; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; Weaver, Anishchenko et al. 2004).

The alphavirus E2 glycoprotein and its genetic determinants of midgut infection

Several unique determinants for midgut infection have been identified in the E2 protein of alphaviruses. Specifically, a deletion of amino acid residues 200-229 in a Sindbis virus (SINV) resulted in significantly reduced midgut infection rate in *Ae. aegypti* mosquitoes (Myles, Pierro et al. 2003). In VEEV, a single isoleucine-to-phenylalanine change at position E2-207 significantly impacted midgut infection rates in *Ae. aegypti* midguts (Woodward, Miller et al. 1991). Also in VEEV, a serine-to-asparagine change at amino acid position 218 was shown to be a significant determinant of midgut infection rates in *Ae. taeniorhynchus* mosquitoes (Brault, Powers et al. 2004). This 218 site is located in the E2 putative cell-receptor binding domain (CRBD) spanning amino acids 170 to 220 and studies characterizing this domain predict that the CRBD is exposed on the viral surface (Stec, Waddell et al. 1986; Davis, Pence et al. 1987; Strauss, Stec et al. 1991; Smith, Cheng et al. 1995).

***Aedes aegypti* ancestry**

The *Ae. aegypti* mosquito is thought to have originated in Africa and is considered one of the most medically important mosquito species worldwide (Failloux, Vazeille et al. 2002). There are two main sub-species of *Ae. aegypti* involved in disease transmission: 1) *Ae. aegypti aegypti*, a light colored mosquito that is widespread in the tropics and subtropics, and is now considered a "domesticated" species since it breeds primarily in domestic environments; and 2) *Ae. aegypti formaosus*, a dark colored mosquito that is found mainly in tropical African forests, and that breeds in tree- and rock-holes (Failloux, Vazeille et al. 2002). The worldwide distribution of *Ae. ae.*

aegypti is thought to have started as a result of a tropical African tree dwelling species spreading into North Africa where it took advantage of human water storage containers for breeding (Tabachnick and Powell 1979). Human trading and trafficking introduced this species to the Mediterranean, West Africa, East Africa and on to the rest of the world (Failloux, Vazeille et al. 2002). This species was likely introduced into the New World from West Africa as a result of the 15th to 19th century African slave trade. Advances in the shipping industry in the 18th and 19th century likely led to its introduction from East and/or North Africa into Asia (Smith 1956). Military shipping during World War II likely resulted in introductions into the Pacific Islands (Monath 1994; Failloux, Vazeille et al. 2002). *Ae. ae. formaosus* has not achieved global distribution patterns and is still considered a tree-dwelling species of West Africa (Gabon and Ivory Coast) and some islands of the Indian Ocean (e.g. Europa Island and La Rĕunion) (Failloux, Vazeille et al. 2002).

Ae. aegypti, primarily *Ae. ae. aegypti,* is a major vector in the epidemic disease cycles of dengue and yellow fever viruses as well as being a competent vector for a number of other arboviruses including chickungunya virus and SINV. For the sake of simplicity, *Ae. ae. aegypti* will be represented as *Ae. aegypti* throughout the remaining chapters of this dissertation.

Model system for identifying virus genetic determinants of midgut infection.

SINV is a widely investigated virus and is considered the prototype alphavirus. Use of SINVs benefits the investigator by being categorized as a biosafety level 2 pathogen, which reduces the risk and deleterious effects of a laboratory acquired

infection as compared with other alphaviruses. Moreover, its genome structure, replication strategy and infection pattern have been extensively studied. Full-length cDNA infectious clones of SINVs have been generated, as have trans-acting replicon packaging systems. In addition, SINV transducing systems have been developed for the expression of heterologous genes as well as the delivery of RNA molecules (i.e. for the stimulation of the RNAi pathway) concurrently with a SINV infection. SINV is known to infect a variety of mosquitoes but its ability to infect and be transmitted by *Ae. aegypti* mosquitoes, which is a vector for a variety of medically important arboviruses such as dengue, yellow fever and chikungunya viruses, makes SINV a very powerful tool in medical entomology studies. In addition, the *Ae. aegypti* mosquito has been colonized in the laboratory and is easily manipulated for experimental use. The combination of the *Ae. aegypti* mosquito together with the SINV infectious clone technology is a very useful system in arboviral disease research.

SINV background

SINV (family *Togaviridae*, genus *Alphavirus*) is an arbovirus that is maintained in nature in a transmission cycle involving mosquito species, such as *Culex spp., Culiseta spp.* and *Aedes spp.*, and vertebrates (Doherty, Carley et al. 1977). SINV has a broad vertebrate host range; however, the primary host in nature is avian (Karabatsos 1985. Updated 2001.). The avian host is suggested to have widely distributed the virus, with isolations having been made in Europe, Africa, Asia and Australia (Calisher 1988; Strauss and Strauss 1994). Genetic studies have identified three major genotypes of SINV; the Paleoarctic-Ethiopian (P/E) genotype, which is found in Europe and Africa,

the Oriental-Australian (O/A) genotype, which is found in Asia and Australasia, and the Southwest (SW) genotype, which is found in the southwestern region of Western Australia (Rentier-Delrue and Young 1980; Olson and Trent 1985; Sammels, Lindsay et al. 1999; Saleh, Poidinger et al. 2003). In addition, numerous SINV-like genotypes have been identified (Liang, Li et al. 2000; Powers, Brault et al. 2001; Saleh, Poidinger et al. 2004).

There are numerous wild type and laboratory derived SINV stains in research use today. Currently, two major wild-type strains are being investigated for their genetic determinants of mosquito midgut infections: AR339 and MRE16. The prototype SINV, strain AR339, was originally isolated from a pool of *Cx. pipiens* and *Cx. univittatus* mosquitoes collected in the Sindbis health district of Egypt, and represents a P/E genotype (Taylor, Hurlbut et al. 1955). A full-length cDNA infectious clone (ic), termed TR339, that represents the putative AR339 consensus sequence, has been generated (McKnight, Simpson et al. 1996; Klimstra, Ryman et al. 1998). The SINV strain MRE16 was isolated between 1966 and 1969 from a pool of *Cx. tritaeniorhynchus* mosquitoes in Malaysia and represents a O/A genotype (Pudney 1979). A full-length infectious clone of MRE16 has also been generated (Myles, Pierro et al. 2003).

SINV molecular biology and virus structure

The SINV genome is a positive-sense, single stranded, nonsegmented RNA of approximately 11,400 nucleotides (nt) (Strauss and Strauss 1994; Schlesinger 2001). The genome contains a 5' 7-methyl-guanosine cap with a 3' poly(A) tail and is directly translated into a polyprotein that is co- and post-translationally processed into the four

nonstructural proteins that make up the viral replication complex. This complex produces a negative sense RNA molecule that is used as a template for the production of genome-length as well as subgenomic RNA strands. The subgenomic RNA encodes a polyprotein that is co- and post-translationally processed into the five structural proteins, which are (in genome order) the capsid, envelope (E) 3, E2, 6K and E1.

The SINV outer protein shell structure is an icosahedron having a three fold axis of symmetry composed of E1-E2 heterotrimers arranged in a T=4 lattice. The outer shell surface can be sectioned into an inner base (skirt) region composed mostly of laterally lying E1 and an outer region that is thought to be populated by E2 homotrimers laced with E1 (Anthony and Brown 1991; Pletnev, Zhang et al. 2001; Zhang, Mukhopadhyay et al. 2002; Paredes, Ferreira et al. 2004). The surface glycosylated E1 and E2 are anchored to the viral membrane and the innermembrane portion of E2 is known to associate with the nucleocapsid structure (Strauss and Strauss 1994). The viral nucleocapsid is composed of 240 copies of a virus encoded capsid protein arranged in an icosahedral lattice. Inside the SINV nucleocapsid is a single copy of the positive sense RNA genome (Strauss and Strauss 1994).

The alphavirus E1 glycoprotein is a class II membrane fusion protein consisting mostly of β sheets, two glycosylation sites and contains an internal fusion peptide region (Lescar, Roussel et al. 2001; Pletnev, Zhang et al. 2001). The atomic structure of the SINV E1 has been generated (Zhang, Mukhopadhyay et al. 2002). The E1 protein lies parallel to the viral membrane in a homotrimer formation that forms an E1-E2 heterotrimer lattice on the virus surface (Zhang, Mukhopadhyay et al. 2002). During alphavirus penetration into target cells, E1 undergoes a conformational change, in

response to low pH, from a network of E1-E2 heterotrimers at the viral surface to form a cell-membrane-inserted E1 homotrimer leading to viral and cellular membrane fusion and the cytoplasmic insertion of the viral genome (Wahlberg, Bron et al. 1992; Wahlberg and Garoff 1992; Klimjack, Jeffrey et al. 1994; Gibbons, Vaney et al. 2004).

The atomic structure of E2 has not been determined, although a number of studies have provided insights into its configuration (Smith, Cheng et al. 1995; Pletnev, Zhang et al. 2001; Mukhopadhyay, Zhang et al. 2006). The SINV E2 glycoprotein has two glycosylation sites, is approximately 51kDa and rises nearly 50 Å above the skirt region as a heterotrimer mixture with E1 (Pletnev, Zhang et al. 2001; Paredes, Ferreira et al. 2004). The E2 monomer stands spike-like and perpendicular to the viral membrane as a homotrimer. The E2 of many alphaviruses including SINV, Venezuelan equine encephalitis virus (VEEV) and Ross River virus (RRV) are sensitive to neutralizing antibodies (Johnson, Brubaker et al. 1990; Pence, Davis et al. 1990; Strauss, Stec et al. 1991; Ubol and Griffin 1991; Agapov, Razumov et al. 1994; Pereboev, Razumov et al. 1996; Vrati, Kerr et al. 1996). Escape mutants to neutralizing antibodies have indicated that the putative CRBD is located on the viral surface. Further analysis shows that these E2 spike tips revolve 180° away from the center of the trimer (Mukhopadhyay, Zhang et al. 2006).

Cellular virogenesis

As previously stated, the proposed mechanisms for cellular entry of alphaviruses are receptor mediated endocytosis and direct plasma membrane fusion/penetration. Numerous studies have provided support for SINV use of both of these mechanisms.

Direct penetration by SINV has been shown in mammalian cells as a means to bypass receptor mediated endocytosis (Wengler, Koschinski et al. 2003; Paredes, Ferreira et al. 2004); however, it is unclear if receptors are required for this mechanism. For receptor mediated endocytosis in vertebrate cells, murine and human histocompatability antigens may serve as alphavirus receptors (Helenius, Morein et al. 1978). Ubol and Griffin identified two proteins that may serve the same function on mouse neuronal cells (Ubol and Griffin 1991). Wang et al. suggested that a 67-kDa high-affinity laminin receptor functions as a common receptor for SIN virus infection of BHK cells (Wang, Schmaljohn et al. 1991). Maassen and Terhorst showed that a lymphoblastic cell-surface protein with a molecular weight of 90,000, which is associated with the human histocompatibility antigens, was involved in SINV binding (Maassen and Terhorst 1981). Klimstra et. al. proposed that lectin molecules, such as DC-SIGN and L-SIGN, may represent common attachment receptor molecules for arthropod-borne viruses (Klimstra, Nangle et al. 2003). Moreover, the widely expressed glycosaminoglycan heparan sulfates can participate in the binding of SINV to cells (Byrnes and Griffin 1998; Byrnes and Griffin 2000; Ryman, Gardner et al. 2007). Ryman et. al. have shown that SINV with an enhanced affinity for heparan sulfate can lead to adult mouse virulence with a specificity for neural cells (Ryman, Gardner et al. 2007). When this information is analyzed together, it is hypothesized that there are multiple mammalian receptors for SINV attachment, some of which are highly conserved throughout the vertebrates (Strauss, Wang et al. 1994).

For receptor mediated endocytosis in insect cells, Ludwig et. al. have identified a 32-kDa polypeptide on mosquito cells with affinity to Venezuelan equine encephalitis

virus and to laminin (Ludwig, Kondig et al. 1996). Interestingly, this group determined that the 32-kDa polypeptide from C6/36 cells and the 67-kDa polypeptide from BHK cells had a structural relationship and that some monoclonal antibodies could cross-react with these two polypeptides. This type of receptor conservation can help explain the broad host range of SINV.

Following the virus binding to a cell surface receptor, a clathrin coated vesicle develops and internalizes the virus. A drop in pH within this endosome induces membrane fusion of the viral and vesicle membranes via the fusion domain of E1. Membrane fusion allows for entry of the nucleocapsid structure into the cytoplasm of the cell where it then uncoats and releases the viral genome. The naked RNA is directly translated by the host cell's translation machinery, giving rise to the SINV non-structural proteins (nsP) that make up the virus' replication complex. This complex, in association with internal organelles, produces full-length negative strand and subsequently more full-length positive strand RNA as well as a subgenomic RNA molecule, called 26S, which is translated into the SINV structural proteins. It has been shown that nsP2 of SINV down regulates host cell transcription in an effort to avoid the host cell's defense mechanisms (Garmashova, Gorchakov et al. 2007). For reassembly, the replicated RNA genome and capsid subunits self-assemble in the cytoplasm to form the nuecleocapsid that is then transported to the cell's plasma membrane. The viral envelope proteins develop in the endoplasmic reticulum and golgi apparatus of the cell and are also transported to the cell surface membrane. The nucleocapsid buds through the plasma membrane where it acquires a lipid membrane coat embedded with the viral surface glycoproteins E1 and E2 to produce a fully developed infectious virus (Griffin 2001) .

Manipulation of the SINV genome.

Arboviral research has benefited greatly from the development of positive-strand RNA infectious clone technology. SINV genomes can now be assembled as full-length cDNA copies contained within a replicative plasmid for manipulation using bacterial systems. Rice et. al. first developed a full-length SINV infectious clone in 1987 (Rice, Levis et al. 1987). Manipulation of SINV genomes can take the form of site-directed mutations in the genome, the production of chimeric SINV infectious clones, the development of transducing systems for the transcription and/or expression of heterologous gene sequences (i.e. reporter genes such as GFP), and the engineering of replicon systems for single round infections of the SINV genome (Rice, Levis et al. 1987; Polo, Davis et al. 1988; Grakoui, Levis et al. 1989; Hahn, Strauss et al. 1989; Li and Rice 1989; Frolov, Frolova et al. 1997). Identifying the genetic determinants of SINV that affect the mosquito midgut infection rate generally involves the manipulation of the SINV genome at specific locations (either as a site-directed change or a chimeric construct) followed by virus generation and analysis (Olson, Myles et al. 2000; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; Foy, Myles et al. 2004). Chapters 3 and 4 of this manuscript describe the manipulation of the SINV genome to identify the viral genetic determinants of *Ae. aegypti* midgut infections.

Flavivirus as models for midgut infections.

The members of the Flavivirus genus (family *Flaviviridae*) are known to have a significant impact on world public health. The flavivirus genus contains both tick-borne as well as mosquito-borne members; the latter of which are sub-classified antigenically

into 3 serocomplexes: the dengue, Japanese encephalitis and yellow fever virus serocomplexes. Interestingly, the *Ae. aegypti* mosquito has been found to be experimentally susceptible to members of all three of these serocomplexes, indicating a possible conserved infection determinant (Rosen, Roseboom et al. 1985). Moreover, the structure of the flavivirus E protein and the E1 of SINV, also infectious to *Ae. aegypti*, have been shown to be very similar and both harbor an internal fusion peptide (Zhang, Mukhopadhyay et al. 2002). From this, it may be speculated that the flavivirus and alphavirus genera share functional and structural components of midgut infection in *Ae. aegypti* mosquitoes. The development of an infectious clone of a representative flavivirus with known *Ae. aegypti* midgut infection characteristics would greatly facilitate research on the genetic determinants of flavivirus infection of the midgut.

One such flavivirus is the DENV-2 strain 1409 (an American/Asian genotype), which has undergone a number of investigations concerning midgut infections. In particular, the 1409 strain has been shown to have significant differences in the midgut infection rate (MIR) of geographically distinct strains of *Ae. aegypti* (Bennett, Olson et al. 2002). It is also thought that bloodmeal digestion, and possibly 1409 proteolytic processing, are mediated by midgut trypsins that influence the rate of DENV-2 infection in *Ae. aegypti* mosquitoes (Molina-Cruz, Gupta et al. 2005). Bosio et al have identified two quantitative trait loci that condition DENV-2 1409 midgut infection which were located on chromosomes II and III of the *Ae. aegypti* genome (Bosio, Beaty et al. 1998; Bosio, Fulton et al. 2000). Virogenesis of DENV-2 1409 in the midguts of *Ae. aegypti* begins with an expanding focus of infection that eventually resolves itself starting at 10 dpi (Salazar, Richardson et al. 2007). Moreover, the recently identified DENV-2

American/Asian genotype, which includes the 1409 isolate, is an Asian genotype DENV-2 introduced into the Americas, with subsequent proliferation and circulation of the virus in this region (Uzcategui, Camacho et al. 2001; Twiddy, Farrar et al. 2002; Lorono-Pino, Farfan-Ale et al. 2004). Introduction of viruses from this genotype, of which 1409 is speculated to be an early isolate, was linked to a significant increase in the number of DHF cases across Latin America (Uzcategui, Camacho et al. 2001). DENV-2 from the Asian and American/Asian genotypes more readily infect North American *Ae. aegypti* mosquitoes than do the DENV-2 American genotypes (Armstrong and Rico-Hesse 2001). The American/Asian genotype viruses are also believed to have supplanted the American genotype in many areas, leaving a more virulent and readily transmitted DENV-2 in its place. From this information, the DENV-2 strain 1409 would be an ideal candidate for infectious clone development, which can ultimately be used as a tool in midgut infection assays. Chapter 4 of this manuscript describes its construction and characterization.

Summary and goals

The global impact of arboviruses on public health is considerable and merits robust, long term disease control strategies to reduce mortality, morbidity and loss of economic opportunity. These strategies need to take into account the many dynamics of arbovirus disease. The virus, the host and the vector each have unique characteristics adjoined to each other within a transmission cycle. Unfortunately, many of the transmission control stategies used to date have proven unsustainable or unsuccessful and resulted in the resurgence of a variety of arboviruses worldwide. Clearly, continued

study into the components of arbovirus disease transmission cycles may foster additional and/or more effective control measures.

Little is understood about the infection of mosquitoes by arboviruses. It is known that the arboviral infection of a mosquito midgut is a limiting step in disease transmission for many arboviruses. This MIB is clearly an important component of a disease cycle, but the mechanisms controlling this barrier are very poorly understood. Previous investigations into the MIB have shown that both the mosquito and the arbovirus have determinants that affect the infection of the midgut. However, much more information is needed to identify the specific determinants and assess their impact on the MIB of mosquitoes. With such information, the mosquito midgut infection barrier might be exploited for arbovirus disease control.

One of the prototypic arboviruses is SINV, which is known to infect C*ulex* and *Aedes spp* mosquitoes. There are two SINV strains that have been used in experimental mosquito MIB assays; TR339 and MRE16. These two viruses have the capacity to efficiently infect the midgut of *Ae. aegypti* mosquitoes, their genome sequences are known, and infectious clones of each have been developed. In addition, the medically important *Ae. aegypti* mosquitoes have been colonized in the laboratory for experimental infection assays. Using these materials, this work presented here applied infectious clone technology to 1) identify the genetic determinants of TR339 that influence *Ae. aegypti* midgut infections, to 2) identify the genetic determinants of MRE16 that influence *Ae. aegypti* midgut infections, and to 3) develop a new DENV-2 infectious clone for use in assaying the flavivirus genetic determinants of *Ae. aegypti* midgut infection.

Chapter 2

Genetic determinants of Sindbis virus strain TR339 affecting midgut infection in the mosquito, *Aedes aegypti.*

Abstract

Mosquito midgut epithelial cells (MEC) play a major role in determining whether an arbovirus can successfully infect and be transmitted by mosquitoes. The Sindbis virus (SINV) strain TR339 efficiently infects *Ae. aegypti* MEC, but the SINV strain TE/5'2J poorly infects MEC. SINV determinants for MEC infection have been localized to the E2 glycoprotein. The E2 amino acid sequence of TR339 and TE/5'2J differ at two sites, E2-55 and E2-70. The TE/5'2J virus genome was altered by site-directed mutagenesis to contain the two TR339 residues, E2-55 H→Q (histidine to glutamine) and E2-70 K→E (lysine to glutamic acid). This chapter describes the growth patterns of derived viruses in cell culture and the determination of their midgut infection rate (MIR) in *Ae. aegypti* mosquitoes. These results clearly show that the E2-55 H→Q and the E2-70 K→E mutations in the TE/5'2J virus increase MIR both independently and in combination with each other. TE/5'2J virus containing both TR339 E2 residues had MIRs similar to the parental TR339 virus.

Introduction

As previously mentioned, SINV is an arbovirus that is maintained in nature in a transmission cycle involving mosquito species; such as *Culex spp., Culiseta spp.* and *Aedes spp.*, and wild birds (Doherty, Carley et al. 1977). *Ae. aegypti* mosquitoes, the primary vector for Yellow Fever and Dengue viruses, are susceptible to several SINV strains in the laboratory; however an *Ae. aegypti* MIB to SINV is clearly evident.

Several VEEV and SINV studies have shown that the alphavirus genetic determinants of midgut infection are associated with the E2 glycoprotein (Woodward, Miller et al. 1991; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; Weaver, Anishchenko et al. 2004). In addition, the E2 of many alphaviruses, including SINV, VEEV and RRV, is sensitive to neutralizing antibodies (Johnson, Brubaker et al. 1990; Pence, Davis et al. 1990; Strauss, Stec et al. 1991; Ubol and Griffin 1991; Agapov, Razumov et al. 1994; Pereboev, Razumov et al. 1996; Vrati, Kerr et al. 1996). Moreover, a number of sites involved in virus neutralization have been found between amino acids 170 to 220 of E2 (SINV positions), which may be a putative cell receptor binding site for susceptible cells (Strauss, Stec et al. 1991).

The prototype SINV strain (AR339) was originally isolated from a pool of *Cx. pipiens* and *Cx. univittatus* mosquitoes collected in Egypt (Taylor, Hurlbut et al. 1955). A full-length cDNA infectious clone (ic), termed TR339, that represents the putative AR339 consensus sequence has been generated (McKnight, Simpson et al. 1996; Klimstra, Ryman et al. 1998). Previous characterization of the TR339 virus (propagated in C6/36 cells) for infection rate and viral distribution in the midgut of *Ae. aegypti* mosquitoes found a greater than 90% midgut infection rate (MIR) 7 days post infection (dpi) (Myles, Pierro et al. 2004). In contrast, the recombinant SINV strain TE/5'2J, a double subgenomic SINV often used to deliver and express genes of interest in vector species, was constructed from a chimeric mouse neurovirulent variant of AR339 called TE12 (Lustig, Jackson et al. 1988), but it infects less than 15% of mosquitoes when analyzed similarly (Pierro, Myles et al. 2003). Sequence comparison between the SINV TE12 and TR339 genomes predicts two amino acid differences

(histidine to glutamine [H→Q] and lysine to glutamic acid [K→E]) in the E2 glycoprotein at amino acid positions 55 and 70 (E2-H55Q and E2-K70E, respectively). *Ae. aegypti*, together with these SINVs, provide a unique model system to more specifically investigate the arboviral genetic determinants of midgut infection. In this chapter I hypothesize that the genetic determinants of TR339 for enhanced midgut infection reside in the E2 gene, either at E2-55 or E2-70 position(s).

Material and methods

Cell culture. The African green monkey kidney Vero (ATCC CCL-81 P140-170), baby hamster kidney BHK-21 (ATCC CL-10 P60-80), *Ae. albopictus* C6/36 (ATCC CRL-1660 P130-150) and *Ae. aegypti* Aag2 (provided by Dr. A. Raihkel, University of California at Riverside) cells were individually grown in minimal essential medium Eagle (MEM) supplemented with 8% fetal bovine serum (heat inactivated), 2mM L-glutamine, 1x non-essential amino acids for MEM, 100 U/ml penicillin, and 100 ug/ml streptomycin. Cell culture was performed at 5% atmospheric CO_2 in a 37 ^{o}C (Vero and BHK) or 28 ^{o}C (C6/36 and Aag2) chamber.

Infectious clones. The cDNA ic pTE/5'2J was generously provided by Dr. Charles Rice (Rockefeller University, NY, NY) and its use has been previously described (Hahn, Hahn et al. 1992; Pierro, Myles et al. 2003). The cDNA ic pTR339 was provided by Dr. R. Johnston (University of North Carolina, Chapel Hill, NC). The derived consensus sequence of TR339 and its use has been described (McKnight, Simpson et al. 1996; Klimstra, Ryman et al. 1998; Myles, Pierro et al. 2004).

Following sequencing of the E2 gene of TE/5'2J, primers were engineered for site-directed mutagenesis of pTE/5'2J at nucleotides representing E2-55 and E2-70 amino acid residues. Using the entire pTE/5'2J plasmid as the template, the primers sets: 5'-CGCCCAGTTTGGATACGACCAAAGCGGAGCCGCAAGCGC-3', 5'-GCGCTTGCTGCTCCGCTTTGGTCGTATCCAAACTGGGCG-3' and 5'-GT ACCGCTACATGTCGCTTGAGCAGGATCACACCG-3', 5'-CGGTGTGATCC TGCTCAAGCGACATGTAGCGGTAC-3' were used to mutate the pTE/5'2J cDNA to contain either glutamine at E2-55 (pTE/E2-H55Q), glutamic acid at E2-70 (pTE/E2-K70E) or both (pTE/E2-55Q/70E), respectively. The site-directed mutagenesis reaction (sPCR) used the Quick Change PCR Mutagenesis Kit (Stratagene, La Jolla, CA). Mutation insertion was verified by sequence analysis of the plasmid DNA as well as the virus derived RNA genome via RT/PCR. TE/E2-55Q/70E-GFP was created by insertion of enhanced green fluorescence protein (GFP) into the second subgenomic region of TE/E2-55Q/70E as previously described (Pierro, Myles et al. 2003). The amino acid alignments in figure 2.1 (page 37) used the Pfam database and the color codes used for clusters are from Jalview (Finn, Mistry et al. 2006). The predictions for 2° structure and solvent access in figure 2.1 were performed by PredictProtein (Rost, Yachdav et al. 2004).

Generation and characterization of SINV particles. The production of infectious viral particles from cDNA of pTR339, pTE/5'2J or the sPCR mutant clones was as previously described (Pierro, Myles et al. 2003; Myles, Pierro et al. 2004). Virus was generated from the infections clone (i.e. P=0) and then passaged once (P-1) for use in bloodmeals. RNA from P1 virus was isolated, the E2 gene fully sequenced

and no adaptive mutations identified. Plaque titrations of SINV were performed by infecting confluent monolayers of Vero cells as described previously (Miller and Mitchell 1986). Plaque sizes of P-0 were analyzed on Vero cells 5 days post infection. Growth curve analysis was performed using confluent monolayer of cells infected in triplicate with P-0 virus at a multiplicity of infection (MOI) of 0.01. Indirect immunofluoresence assays (IFA) were performed on tissue culture cells, mosquito bodies and midgut tissues using anti-SINV-E1 monoclonal (30.11a) as the primary antibody (1:200) and biotinylated sheep anti-mouse antibody (1:200; Amersham Corp., Arlington Heights, IL) as the secondary antibody. The E1 protein was detected using a fluorescein isothiocyanate-conjugated streptavidin detector (1:200; Amersham). Mosquito body tissue and midguts were examined for SINV infections by IFA using the same antibodies as above and as previously described (Myles, Pierro et al. 2003).

Mosquitoes and oral infections. The use of the mosquito *Ae. aegypti* strain Rexville D (RexD; Rexville D, Puerto Rico) and the bloodmeal delivery mechanism was as previously described (Myles, Pierro et al. 2004). In summary, the propagation of virus used in bloodmeals started with confluent monolayers of either Vero or C6/36 cells that were infected with P-0 virus at a MOI of approximately 0.01 in 8% FBS/MEM and cultured in CO_2 incubators for two days at 37°C (Vero) or three days at 28 °C (C6/36). After incubation, freshly harvested P-1 cell culture supernatant (i.e. non-frozen) containing infectious virions was, when applicable, diluted in uninfected tissue culture supernatant (final volume of 0.8 ml) to a predicted viral titer using the standardized growth curve data as a guide (figure 2.2 on page 39). The virus alliquot was then mixed 1:1 with 0.8 ml of defibrinated sheep blood (Colorado Serum Co, Boulder, CO), warmed

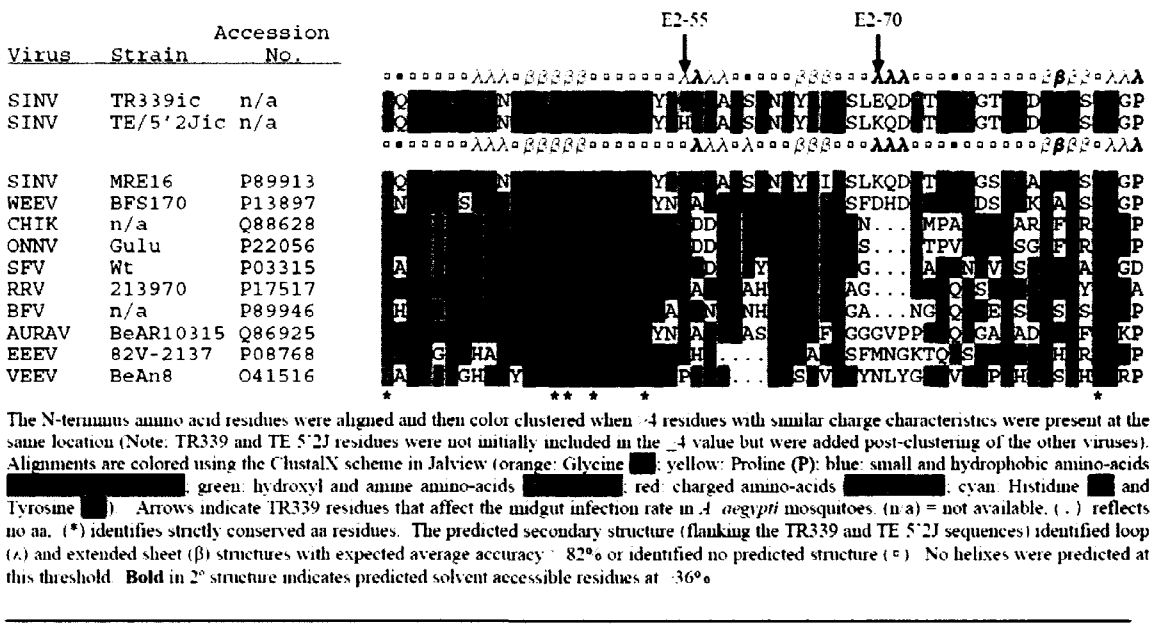
to 37°C and placed in a water-jacket heated (37 °C) glass membrane feeder. Mosquitoes were allowed to probe and feed through a stretched sheet of parafilm on the feeder for no more than 30 min. Fully engorged mosquitoes were collected and maintained in the insectary with ample food and water until assayed. The viral bloodmeal was frozen and later quantified by plaque titration, and the titer was considered acceptable at +/-0.3 log_{10}PFU/ml of the predicted viral titer. Post-bloodmeal viral titers were found to be reduced by only ~10% when compared with pre-bloodmeal titers (data not shown).

Statistical analysis. The MIR was calculated by the number of total IFA positive mosquito midguts divided by the total number of mosquitoes, expressed as a percent, in each replicate. The midgut dissemination rate (MDR) was calculated by the sum of IFA positive mosquito head tissues divided by the total number of IFA positive mosquito midguts, expressed as a percent, for each analyzed replicate. All (geometric) mean MIRs and MDRs were reported as an average of two replicates for each virus with the (geometric) standard error of the mean (SEM) as error bars. The n value for mosquito organs in each analyzed replicate was 21 +/-3 (total n for two replicates = 42 +/-6). Comparison of the MIR and the MDR for statistical significance was performed by combining primary data of replicates and analyzing by Chi Square Test with an alpha cut off of 0.05 using the SAS System© statistical software (Cary, NC). Differences between slope of the MIR were analyzed by a logistic regression model fitting responses (1,0) over dose and virus and dose*virus with a binomial distribution and logistic link using SAS System© statistical software (Cary, NC). The influence of infectious dose can be evaluated by comparing the midgut infectious dose-50 (MID_{50}) of each virus, which can be estimated from figure 2.4 (shaded horizontal bar in MIR. The MID_{50} is the

titer at which 50% of mosquitoes fed an infectious bloodmeal will have SINV antigen in the midgut 9dpi.

Results

Sequence analysis of SINV envelope glycoprotein 2. Nucleotide sequencing of the E2 region of the TR339 and TE/5'2J plasmids confirmed only two coding nucleotide differences resulting in two amino acid differences between the clones. The differences were at genome nucleotide positions 8795 and 8838 (using genome positions from the SINV AR339 HRsp strain published sequence; accession number J02363.1). The nucleotide at position 8795 in TR339 was an adenosine whereas the TE/5'2J sequence had a thymidine. This resulted in a glutamine (Q) residue at amino acid (aa) position E2-55 for TR339 and a histidine (H) aa residue for TE/5'2J. The second variation was a guanosine at nucleotide position 8838 in the TR339 genome compared to an adenosine in TE/5'2J. This resulted in a glutamic acid (E) residue at E2-70 in TR339 and a lysine (K) in TE5'2J.



The N-terminus amino acid residues were aligned and then color clustered when ≥4 residues with similar charge characteristics were present at the same location (Note: TR339 and TE 5'2J residues were not initially included in the ≥4 value but were added post-clustering of the other viruses). Alignments are colored using the ClustalX scheme in Jalview (orange: Glycine; yellow: Proline (P); blue: small and hydrophobic amino-acids; green: hydroxyl and amine amino-acids; red: charged amino-acids; cyan: Histidine and Tyrosine). Arrows indicate TR339 residues that affect the midgut infection rate in *A. aegypti* mosquitoes. (n/a) = not available. (.) reflects no aa. (*) identifies strictly conserved aa residues. The predicted secondary structure (flanking the TR339 and TE 5'2J sequences) identified loop (λ) and extended sheet (β) structures with expected average accuracy > 82% or identified no predicted structure (○). No helixes were predicted at this threshold. **Bold** in 2° structure indicates predicted solvent accessible residues at ≥36%.

Figure 2.1. Cluster alignment of alphavirus E2 residues 32-90.

The TR339 and TE/5'2J N-terminal E2 residues 32-90 were aligned against a panel of alphaviruses and the residues were clustered based on residue characters (figure 2.1 above). The TR339 E2-55 Q residue clustered with the hydroxyl and amine groups while the TE/5'2J H residue did not. Interestingly, none of the E2-70 residues from the panel of alphaviruses formed clusteres; however, a 3-amino acid deletion (E2 70-72) was observed in several other alpphaviruses. Secondary structure prediction analysis identified potential loop regions (identified as λ in figure 2.1) spanning both the E2-55 and the E2-70 residues, which were separated by a beta-sheet (β). The TE/5'2J E2-55 H residue appeared to loose the predicted loop structure (from that predicted in TR339) and added a loop to the serine (S) residue at E2-60, which was not seen in the TR339 E2-60 S residue (figure 2.1) . Predicted solvent accessible residues (bold-type in 2°

structure in figure 2.1) were identified next to E2-55 and at E2-70 for both TR339 and for TE/5'2J. Moreover, the predicted solvent accessibility of the S at E2-60 appears to be affected by the E2-55 H of TE/5'2J.

Generation and characterization of SINV mutants in tissue culture. Primers were designed to mutate E2-55 and E2-70 of TE/5'2J to represent the residues of TR339, resulting in the ic plasmids: SINV TE/E2-H55Q, TE/E2-K70E and TE/E2-55Q/70E. Virus replication rates were analyzed by growth curve analysis in both invertebrate (C6/36 and Aag2) and vertebrate cell lines (Vero and BHK; figure 2.2 on page 39). Maximum virus titers were achieved at 60 and 36 hours post infection for viruses propagated in C6/36 and Aag2 cells, respectively, and 24-36 hours for propagation in either Vero or BHK cells. The maximum virus titers (log_{10} PFU/ml) ranged from 9.1-9.9, 7.8-8.9, 7.9-8.6 and 7.6-8.4 in C6/36, Aag2, Vero and BHK cells, respectively. Interestingly, propagation of TE/5'2J in Aag2 cells achieved the lowest maximal titer of all five viruses and also failed to maintain a persistent high titer over time. We also observed that plaque sizes were larger for viruses that had an E at E2-70 (data not shown) as has been previously reported (McKnight, Simpson et al. 1996).

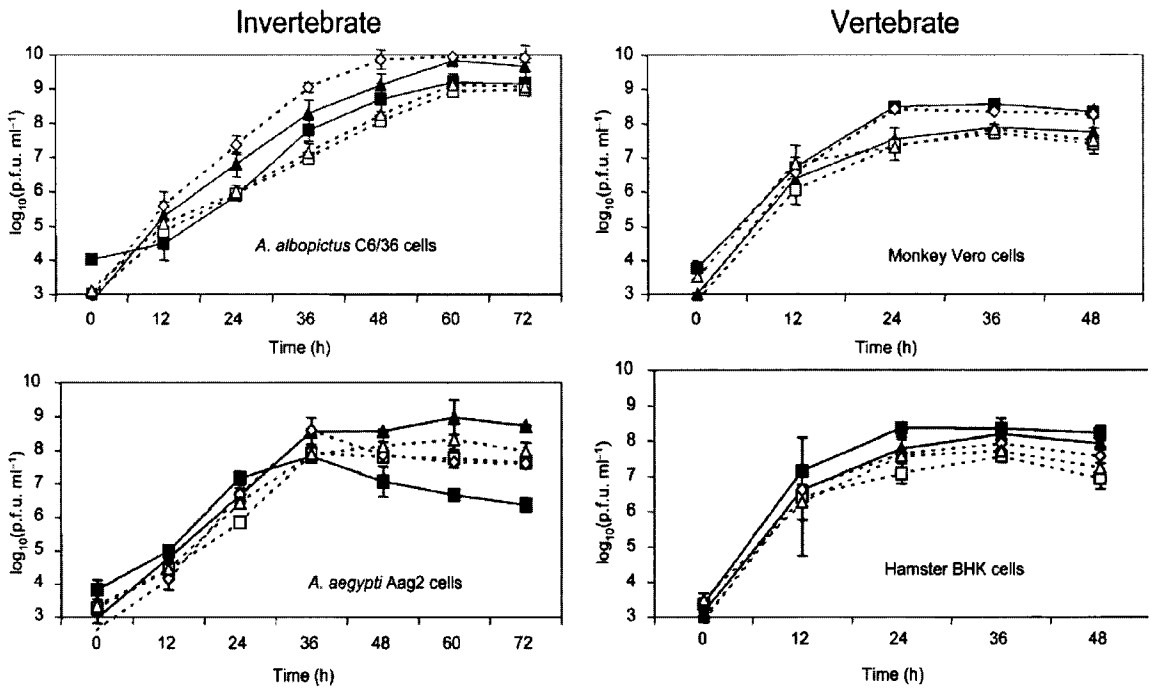


Figure 2.2. Growth curves of SINV in invertebrate and vertebrate cell lines. Triplicate flasks of confluent cell monolayers were initially inoculated at an m.o.i. of 0.01 and incubated at 28 °C (invertebrate) or 37 °C (vertebrate) in a 5 % CO_2 chamber. Viral titer was determined by plaque assay on Vero cells. Error bars, SEM; ▲, TR339; ■, TE/5'2J; □, TE/E2-K70/E; ◊, TE/E2-H55Q; Δ, TE/E2-H55Q/70E.

Pattern of SINV infection of midguts. All of the viruses initiated an infection of the midgut with a discreet focus of infection that spread to adjacent MECs, resulting in an expanding focus (figure 2.3 on page 40). One to four foci of infection were observed in SINV-infected RexD midguts 8-9 dpi regardless of virus used. Neither virus nor cell type had had an influence on the average number of foci per midgut (data not shown). Moreover, the spatial and temporal midgut infection pattern for mosquitoes having a disseminated infection appeared similar to those with only a midgut infection, but no dissemination.

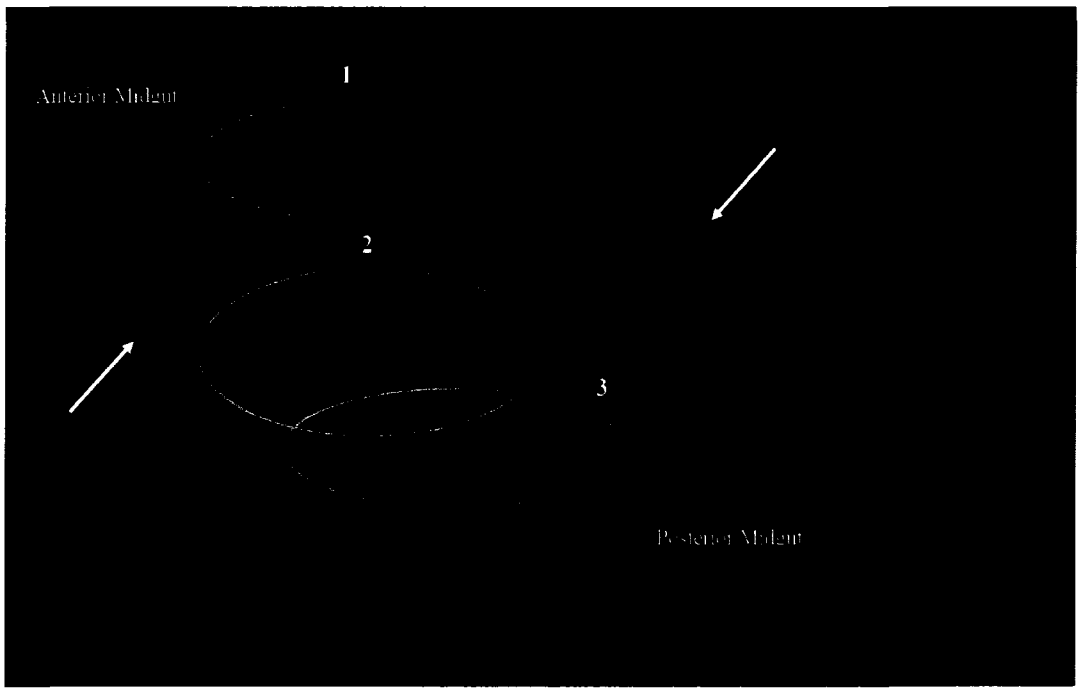


Figure 2.3. SINV foci of infection. IFA staining and fluorescence of three SINV foci of infection (ovals 1–3) in *Ae. aegypti* mosquito midguts 9 days p.i. with SINV TE-55Q/70K. Arrows indicate examples of infected musculature.

Effect of SINV dose on midgut infection rates. RexD mosquitoes were orally fed infectious bloodmeals with various viral titers. After eight to nine dpi, mosquito midguts were dissected (n=21 +/-3 in duplicate) and screened for SINV E1 antigen by IFA to determine the MIR for each virus. The MIR in *Ae. aegypti* mosquitoes was observed to be dose dependent in all viruses analyzed (figure 2.4 on page 41). The influence of infectious dose can be evaluated by comparing the midgut infectious dose-50 (MID_{50}) of each virus, which can be estimated from figure 2.4 (shaded horizontal bar in MIR) The data are presented in table 2.1 (page 42). The MID_{50} is the titer at which 50% of mosquitoes fed an infectious bloodmeal will have SINV antigen in the midgut 9dpi. A lower MID_{50} indicates enhanced efficiency midgut cell infection. The MID_{50}

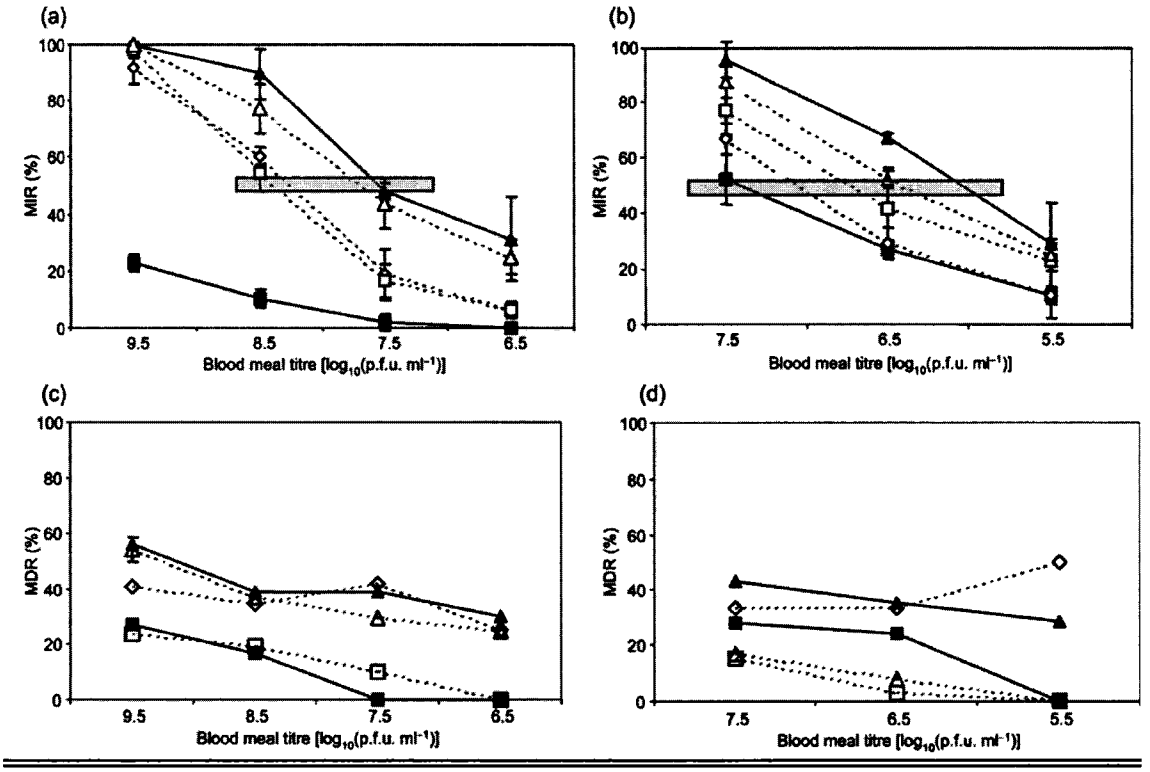


Figure 2.4. MIR (a and b) and MDR (c and d) for viruses propagated in either C6/36 (a and c) or Vero (b and d) cells. MIR is the mean of IFA-positive midguts over n (n=21±3) from two replicates 9 days p.i. MDR is the combined number of IFA-positive heads over the number of positive midguts in two replicates. Shaded horizontal bar is the estimated MID50. Error bars, SEM; ▲, TR339; ■, TE/592J; □, TE/E2-K70/E; ◊, TE/E2-H55Q; Δ, TE/E2-H55Q/70E.

was analyzed for viruses propagated in C6/36 cells and in Vero cells. TR339 virus had the lowest MID_{50} (7.5 and 6 log_{10}, respectively) and TE/5'2J had the highest MID_{50} (>9.5 and 7.5, respectively). The remaining mutants had intermediate MID_{50}'s.

To further assess the virus midgut epithelial cell (MEC) infection potential, the slope of the MIRs (table 2.1 on page 42) for each virus was calculated using the MIR data from figure 2.4 (above). Logistic regression modeling revealed that there was no significant difference between the slopes (i.e. virus*dose) of any pair of viruses for either Vero ($p>0.28$) or C6/36 cell derived viruses ($p>0.2$). Thus, host cell origin and E2 amino acid sequence of the virus had no effect on the MIR slopes.

Table 2.1. Comparison of *Ae. aegypti* midgut infectious dose 50

Virus	Primary Residue E2-55	Primary Residue E270	*Ae. aegypti* MID_{50}[†] C6/36	*Ae. aegypti* MID_{50}[†] Vero	*Ae. aegypti* MID_{50}[†] Δ	MIR Slope[†] C6/36	MIR Slope[†] Vero
TR339	Gln	Glu	7.5	6	1.5	-1.37	-1.6
TE/5'2J	His	Lys	>9.5*	7.5	>2	-1.17	-1.09
TE/E2-H55Q	His	Glu	8.25	7	1.25	-1.67	-1.45
TE/E2-K70E	Gln	Lys	8.25	6.75	1.5	-1.54	-1.28
TE/E2-55Q/70K	Gln	Glu	7.75	6.5	1.25	-1.16	-1.49

[†] from Figure 1, Δ = C6/36 MID50 minus Vero MID50, * MID50 could not be accurately estimated because of maximum titer limits for TE/5'2J virus propagated in C6/36 cells (see Figure 1).

Effect of propagation cell type on MIR. The relative effect of cell type used for virus propagation on MIR was analyzed by standardizing bloodmeal titers for viruses propagated in C6/36 and Vero cells (Figure 2.5 on page 43). A bloodmeal titer of 7.5 $\log_{10}$ PFU/ml, each of the viruses generated in C6/36 cells had a significantly lower mean MIR when compared with the same viruses generated in Vero cells ($p<0.001$). Moreover, the difference between the MID_{50} of virus propagated in Vero and C6/36 cells (represented under Δ in table 2.1) indicated the impact of the propagation cell type on the MIR. From table 2.1, TE/5'2J had the largest Δ MID_{50} (>2 Logs) indicating that the cell type used in propagating this virus had at least a 2 log impact on MIR. The Δ MID_{50} for the remaining viruses ranged from 1.25-1.75 $\log_{10}$. These data indicated that at least 1.0 $\log_{10}$ PFU/ml higher titer of virus in the bloodmeal was required for viruses propagated in C6/36 cells than in Vero cells to achieve an equivalent MID_{50}.

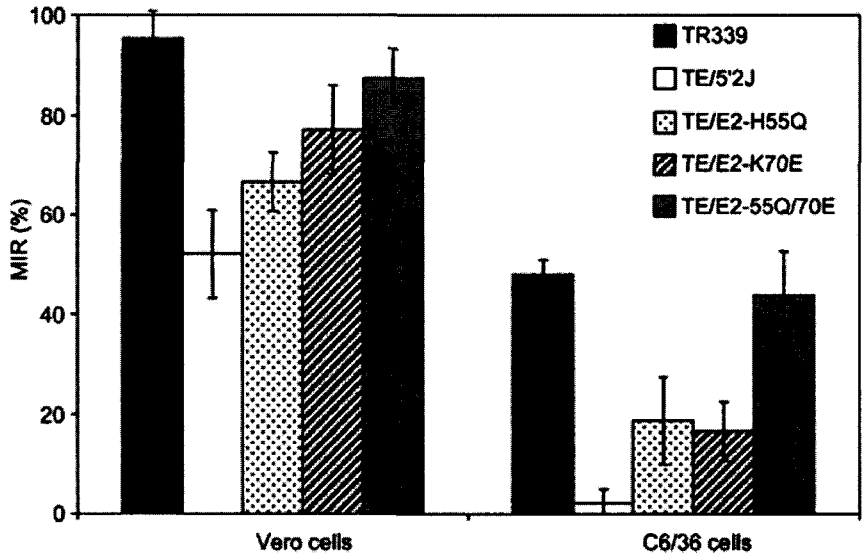


Figure 2.5. Comparison of MIR at a standardized dose. Mosquitoes were fed on an infectious blood meal with a titer of 7.5 $\log_{10}$(PFU/ml) for viruses derived in either Vero or C6/36 cells. MIR is the mean of IFA-positive midguts over n (n=21±3) from two replicates 9 days p.i. with SEM error bars.

Genetic determinants of MIR. TR339 and TE/5'2J viruses consistently had MIRs statistically different ($p<0.001$) from each other at the different bloodmeal doses (with the exception of low titered Vero cell-derived viruses; figure 2.4 on page 41). These viruses also had the highest and the lowest MIRs, respectively, of all viruses tested, at each bloodmeal dose analyzed. Changing the propagation cell type did not change the above observations.

The generation of TE/5'2J viruses containing mutations to restore the site specific E2 amino acid residues of TR339 and subsequent MIR assays revealed the genetic determinants of SINV midgut infections. Specifically, the MIR for TE/E2-55Q/70E was not statistically different from TR339 ($p>0.5$), but it was higher than the MIR of TE/5'2J ($p<0.001$) for both Vero and C6/36 cell line-derived virus at the

standardized bloodmeal dose of 7.5 $\log_{10}$ PFU/ml (figure 2.5). Using this same dose, the Vero-derived TE/E2-K70E virus had statistically higher MIRs than TE/5'2J (p=0.0105), and TE/E2-H55Q had a statistically lower MIR (p=0.0152) than the TE/E2-55Q/70E virus (figure 2.5 on page 43).

The three C6/36-derived mutant viruses all had significantly higher MIR than C6/36-derived TE/5'2J ($p<0.0142$) at a dose of 7.5 $\log_{10}$ PFU/ml (figure 2.5). Moreover the C6/36 propagated TE/E2-55Q/70E had MIRs significantly higher than C6/36-derived TE/E2-H55Q and TE/E2-K70E viruses (p=0.0082 and p=0.0039, respectively). MIRs for TE/E2-H55Q and TE/E2-K70E did not differ statistically from each other at the bloodmeal dose of 7.5 $\log_{10}$ PFU/ml for either Vero- or C6/36-derived viruses (p=0.2564 and p=0.8617, respectively; figure 2.5). The independent effect of the two TR339 residues, E2-55Q and E2-70E, on the MIR appears additive.

Extrinsic characteristics of TE/5'2J (such as competitive exclusion, viral aggregation, defective interfering particles, etc.) may negatively influence its MEC infection portential resulting in a reduced MIR. Therefore, it was hypothesized that coinfecting TE/5'2J with TE/E2-55Q/70E in a bloodmeal would lower the MIR of the latter. This was specifically analyzed by mixing 6.5 $\log_{10}$ PFU/ml TE/E2-55Q/70E-GFP (equivalent to the MID_{50} for this virus) together with either 7.5 or 6.5 $\log_{10}$ PFU/ml of TE/5'2J (all Vero cell derived), infecting mosquitoes, and calculating the MIR of the former by visualizing GFP in the midguts of *Ae. aegypti* mosquitoes 9dpi. The MIR for the negative control group (containing mosquitoes fed only TE/E2-55Q/70E-GFP) was 52.8% +/- 2.8 SEM. The mean MIRs of the two exprimental groups (containin mosquitoes fed TE/E2-55Q/70E-GFP with either 7.5 and 6.5 log10 PFU/ml of TE5'2J)

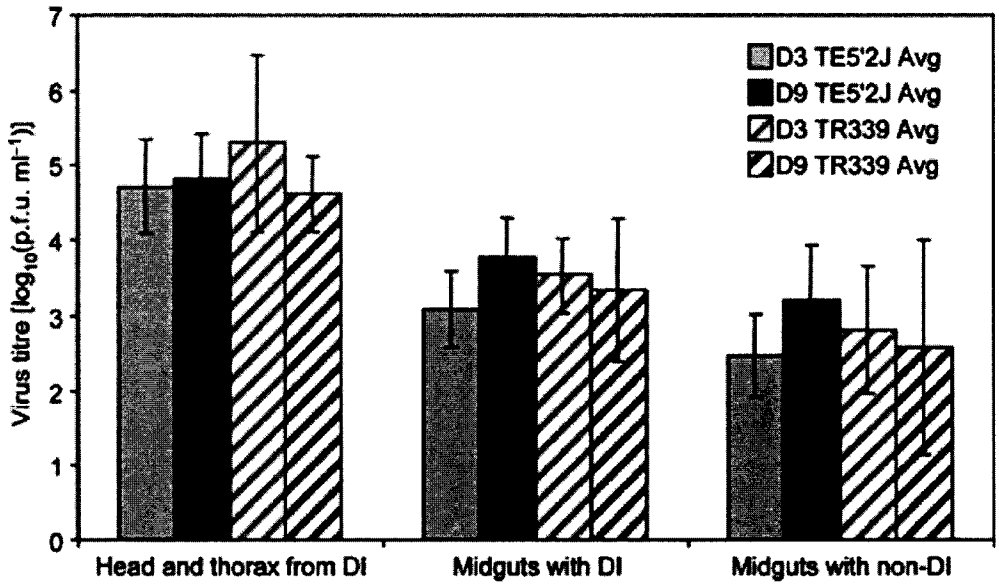


Figure 2.6. Viral titres in the mosquito 3 and 9 days p.i. Mosquitoes were orally infected with one MID_{50} dose and at either 3 or 9 dpi the body parts were isolated and virus was plaque-titrated. DI, disseminated infection. Titrated body parts are: 'Head and thorax with DI', all tissues except abdomen tissues from mosquitoes with a DI; 'Midguts with DI' and 'Midguts with non-DI', midguts from mosquitoes either having or not having a SINV titer in the 'Head and thorax with DI' group, respectively. All tissues were homogenized in 500 ml MEM, centrifuged, and supernatant was filtered (0.2 μm filter) before titraiton.

were 48.1% and 51.9%, respectively, both of which were not significantly different from the negative control. These data indicated that TE/5'2J extrinsic properties do not affect the MIR.

Midgut virus titer and dissemination rates. Viral titers in mosquito tissues were analyzed 3 and 9 dpi for mosquitoes orally infected with Vero propagated TR339 and TE/5'2J viruses (figure 2.6, above). TR339 and TE/5'2J mean titers were not statistically different from each other in the tissues of the head and thorax following a disseminated infection ($p>0.07$), in the midguts with disseminated infections ($p>0.21$) or in midguts without disseminated infection ($p>0.091$). The duration of the infection (i.e.

day 3 vs. day 9) also showed no significant difference in titer ($p>0.053$) within any dissected tissue type. In general, head and thorax tissue did have significantly higher virus titers than with the disseminated midguts ($p<0.02$) and non-disseminated midguts ($p<0.001$) at 3 and 9 dpi for all the respective viruses. Titers from midguts with a disseminated infection and non-disseminated infection were not compared because the dissected midguts from mosquitoes with a disseminated infection also had infected musculature (not seen in non-DIs; see arrows indicating infected external musculature in figure 2.3 on page 40) and tracheole cells, which would skew that data.

The virus midgut dissemination rate (MDR), characterized by the spread of virus from the midgut to other tissues, was calculated as the number of IFA positive mosquito heads over the total number of IFA positive midguts. The MDR was not sensitive to viral titer or cell propagation type (figure 2.4 on page 41). Moreover, TR339 did not have a statistically different MDR from TE/5'2J when derived in C6/36 cells ($p=0.2688$) or in Vero cells ($p=0.211$) at the 8.5 and 7.5 log10 PFU/ml bloodmeal doses, respectively.

Discussion

The mosquito midgut is a bulging tube-like structure consisting of a monolayer of epithelial cells surrounded by a basal lamina. The epithelial layer is considered a natural barrier for virus infection/transmission and the MIB is believed to be determined by the genetics of the mosquito vector. However, virus genetics also plays a significant role in determining whether a virus can successfully infect a mosquito species (Bennett,

Olson et al. 2002; Black, Bennett et al. 2002; Brault, Powers et al. 2002). The exact mechanism for alphavirus entry into susceptible cells is not yet known. In tissue cultured fibroblast cells, it is thought that the E2 glycoprotein interacts with a yet unidentified receptor(s) followed by membrane fusion via the E1 glycoprotein. In the case of alphavirus infection of mosquito midgut cells, the E2 protein sequence has been implicated in having specific genetic determinants for viral infection. In the case of SINV MRE16, a deletion of amino acid residues 200-229 resulted in significantly reduced MIR in RexD mosquitoes (Myles, Pierro et al. 2003). In VEEV, a single I→F change at position E2-207 significantly impacted midgut infection rates in *Ae. aegypti* midguts (Woodward, Miller et al. 1991). Also in VEEV, a S→N change at amino acid position 218 was shown to be a significant determinant of midgut infection rates in *Ae. taeniorhynchus* mosquitoes (Brault, Powers et al. 2004). Moreover, the amino acid residues 170 to 220 of E2 comprise a putative cell-receptor binding domain (CRBD) and studies characterizing this domain predict that the CRBD is exposed on the viral surface (Stec, Waddell et al. 1986; Davis, Pence et al. 1987; Strauss, Stec et al. 1991; Smith, Cheng et al. 1995). Anti-idiotypic antibody and virus mutant studies have also established the importance of the CRBD in cell binding (Mendoza, Stanley et al. 1988; Strauss, Stec et al. 1991; Ubol and Griffin 1991; Wang, Schmaljohn et al. 1991; Wang and Strauss 1991; Tucker, Strauss et al. 1993)

Comparison of the 423 amino acid residues of E2 from TR339 and TE/5'2J showed only 2 amino acid sites with sequence variation, E2-55 and E2-70. Neither residue relates to previously published alphavirus genetic determinants of midgut infection and both are some linear distance from those sites listed above. To further

investigate these sites, TE/5'2J viruses were generated that have the two TR339 E2 residues either individually or in combination with each other and subsequently characterized replication patterns of these viruses in cell culture and in *Ae. aegypti* mosquito assays. Growth curve analysis of all viruses had a similar pattern of growth (figure 2.2, page 39) with the exception of TE/5'2J, that did not sustain a maximum titer out to 72h in Aag2 cells (*Ae. aegypti* larval cells) as did the other viruses. This may be an indication of the TE/5'2J virus' limited ability to maintain an established infection in certain mosquito cell types.

The MIR for C6/36 cell derived TE/E2-H55Q and for TE/E2-K70E were both significantly higher than that of TE/5'2J but were significantly lower than TR339 when fed at 7.5 $\log_{10}$ PFU/ml (figure 2.5 on page 43). Vero cell derived virus followed a comparable pattern of infection. These data indicate that each of the amino acid sites has the ability to independently influence the infection of *Ae. aegypti* midguts, although these infections are less efficient than that of the TR339 virus. However, the clone TE/E2-55Q/70E generated an MIR higher than TE/5'2J, TE/E2-H55Q, TE/E2-K70E and an MIR equivalent to TR339. This indicated that the two sites together could have an additive effect in their ability to infect midguts.

In an effort to analyze viral receptor competitive exclusion to midgut infection by defective virus particles, the double subgenomic promoter element of TE viruses was used for the expression of green fluorescent protein (GFP). The TE/E2-55Q/70E-GFP virus successfully delivered GFP to approximately 50% of *Ae. aegypti* midguts when fed at the MID_{50} of the TE/E2-55Q/70E virus (data presented on page 44). Interestingly, the TE/E2-55Q/70E-GFP MID_{50} is not significantly affected by coinfection with high titered

TE/5'2J (P>0.5). This indicates that midgut infections are not affected by receptor competition restraints from other infectious, non-infectious or defective virus particles. We also observed that TR339 and TE/5'2J viruses that had been propagated in Vero cells were able to infect C6/36 cells at similar rates to each other (determined by $TCID_{50}$ assays; data not shown). The same is true for the ability of Vero cell-propagated studies viruses to subsequently infect Vero cells. In fact, no combination of tissue culture cell types used in viral propagation (i.e. mammalian or insect) had a significant effect on the subsequent $TCID_{50}$ infection rates. This indicated that niether of the parental viruses had a significant advantage over the other to infect either C6/36 (invertebrate) or Vero (vertebrate) cells.

Other publications have analyzed the SINV E2-H55Q and E2-K70E residues, although for different phenotypes. Levine and Griffin found that a Q→H change at SINV position E2-55 contributes to increased neurovirulence in 4-6 week old Balb/cJ mice and concluded that this mutation arose as a result of prolonged replication in host cells (Levine and Griffin 1993). Lee *et. al.* concluded that the E2-55 Q residue reduced virus binding to a neuroblastoma cell line but the virus replicated similarly to an E2-55 H variant virus in BHK cells (Lee, Knight et al. 2002). This group also reported that neither H nor Q at position E2-55 influenced binding of the virus to heparan sulfate (HS) and suggested that the E2-55 residue was unlikely to directly interact with the cell surface. Moreover, they repeatedly isolated neurovirulent SINV with the Q→H change at position E2-55 which suggested a strong selective advantage for neurovirulence leading to high mortality in mice. Here, TE/5'2J SINV having Q (and not H) at E2-55 significantly increased MIR in *Ae. aegypti* mosquitoes.

In this work an increased MIR was also correlated with the presence of an E at position E2-70. McKnight *et. al.* previously demonstrated that an E2-70 K residue in TR339 positively influenced BHK cell penetration rates and reduced neurovirulence in neonatal mice (McKnight, Simpson et al. 1996). Their E2-70 E→K variant was linked to higher HS binding activity; however, the predictive linear heparan sulfate interaction domain could not be found near E2-70 (Smit, Waarts et al. 2002). Moreover, Klimstra *et al.* speculated that the TR339 virus having E2-70 E utilized a weak HS-independent attachment strategy for cell entry (Klimstra, Ryman et al. 1998). The HS-binding proteins, when introduced into a model system, are quickly cleared from circulation by binding to tissue HS (Karlsson and Marklund 1988; Karlsson, Sandstrom et al. 1994). A large plaque variant of SINV with an E2-76 K→E change had a low HS affinity and slower host clearance (Byrnes and Griffin 2000). Our TE/E2-K70E virus produced the larger plaque phenotype associated with TR339 and, as stated, this site was previously characterized as having a limited HS affinity. A model for midgut infection by SINV may require dissociation from HS binding for effective receptor recognition and/or cell entry into the midgut. The E2-70 site appeared to be an important determinant that impacted viral pathogenesis in both mosquitoes and mice.

The effect of cell type used for virus propagation on MIR points to the potential influence of viral protein processing. In particular, the late stage processing of N-linked glycans in insect cells is known to diverge from that of mammalian cells by producing paucimannosidic or oligomannose structures instead of the terminal sialylated complex-type structures (Altmann, Staudacher et al. 1999; Marchal, Jarvis et al. 2001; Tomiya, Betenbaugh et al. 2003; Tomiya, Narang et al. 2004). These differences in N-

linked glycan structure are believed to affect biological activity (Prenner, Mach et al. 1992; Takahashi, Tsuda et al. 2004; Tomiya, Narang et al. 2004). Moreover, insect cell membranes are populated by sterols, as opposed to cholesterols in mammalian cells, both of which are thought to affect cell rigidity and alphavirus activity (Clayton 1964; Luukkonen, Brummer-Korvenkontio et al. 1973; Bretscher and Munro 1993; Marquardt, Phalen et al. 1993; Vashishtha, Phalen et al. 1998; Lu, Cassese et al. 1999). Functional differences have been observed previously for SINV propagated in mammalian vs. insect cell lines and, taken together, they imply that SINV maturation differs significantly between these arboviral host cell types (Lee and Brown 1994; Li, Liao et al. 1999; West, Hernandez et al. 2006).

Clearly, insect cell propagated virus propagated in an artificial bloodmeal is not a natural source or route of delivery to the mosquito midgut. This infection model using C6/36 cells for viral propagation was used to show efficiency of virus infection from midgut cell to midgut cell after the initial infection occurs. The data presented here showed that insect cell propagated alphavirus establsh an infection of midgut cells with reduced efficiency as compared to mammalian-cell propagated virus. The Δ MID_{50} data (table 2.1, page 42), which showed the genetic determinant differences in conditioning a midgut infection, together with the observation of SINV foci with limited spatial expantion (figure 2.3, page 40), indicates that viruses are not only restricted in their ability to secure a primary infection of the midgut (i.e. receptors), but that the infection progression in the midgut may be restricted by the biochemical processing of the virus in the midgut cells.

Chapter 3

Genetic determinants of Sindbis virus strain MRE16 affecting midgut infection in the mosquito, *Aedes aegypti*

Abstract

The Oriental-Australian genotype SINV strain MRE16 efficiently infects *Aedes aegypti* midgut epithelial cells (MEC) but the Paleoarctic-Ethiopian genotype SINV strain TE/5'2J poorly infects MEC. SINV determinants for MEC infection have been localized to the E2 glycoprotein. The E2 amino acid sequences of MRE16 and TE/5'2J differ at 60 residue sties. To identify the genetic determinants of midgut infection of MRE16, the TE/5'2J virus genome was altered to contain either domain gene swapping or more focused nucleotide substitutions of MRE16. The growth patterns of derived viruses in cell ulture as well as the midgut MIR in *Ae. aegypti* mosquitoes were determined. The results clearly showed that substitution of the MRE16 E2 95-96 and the E2 116-119 amino acid residues in the TE/5'2J virus increase MIR both independently and in combination with each other. In addition, a PPGDS aa motif was located between these two sites that was found to be a highly conserved sequence among alphaviruses and flavivruses, but not other arboviruses, and this motif may be involved a shared infection mechanism.

Introduction

The evolution, distribution and epidemiological characteristics of arboviruses are known to be influenced by a combination of restrictions on the mosquito, the vertebrate host, and the virus' ability to establish an infection. While the virus-human host interaction receives a significant amount of attention, the virus-mosquito interaction is also an important determinant of disease outbreaks. It is known that the majority of medically important alphaviruses and mosquito-borne flaviviruses are vectored by two

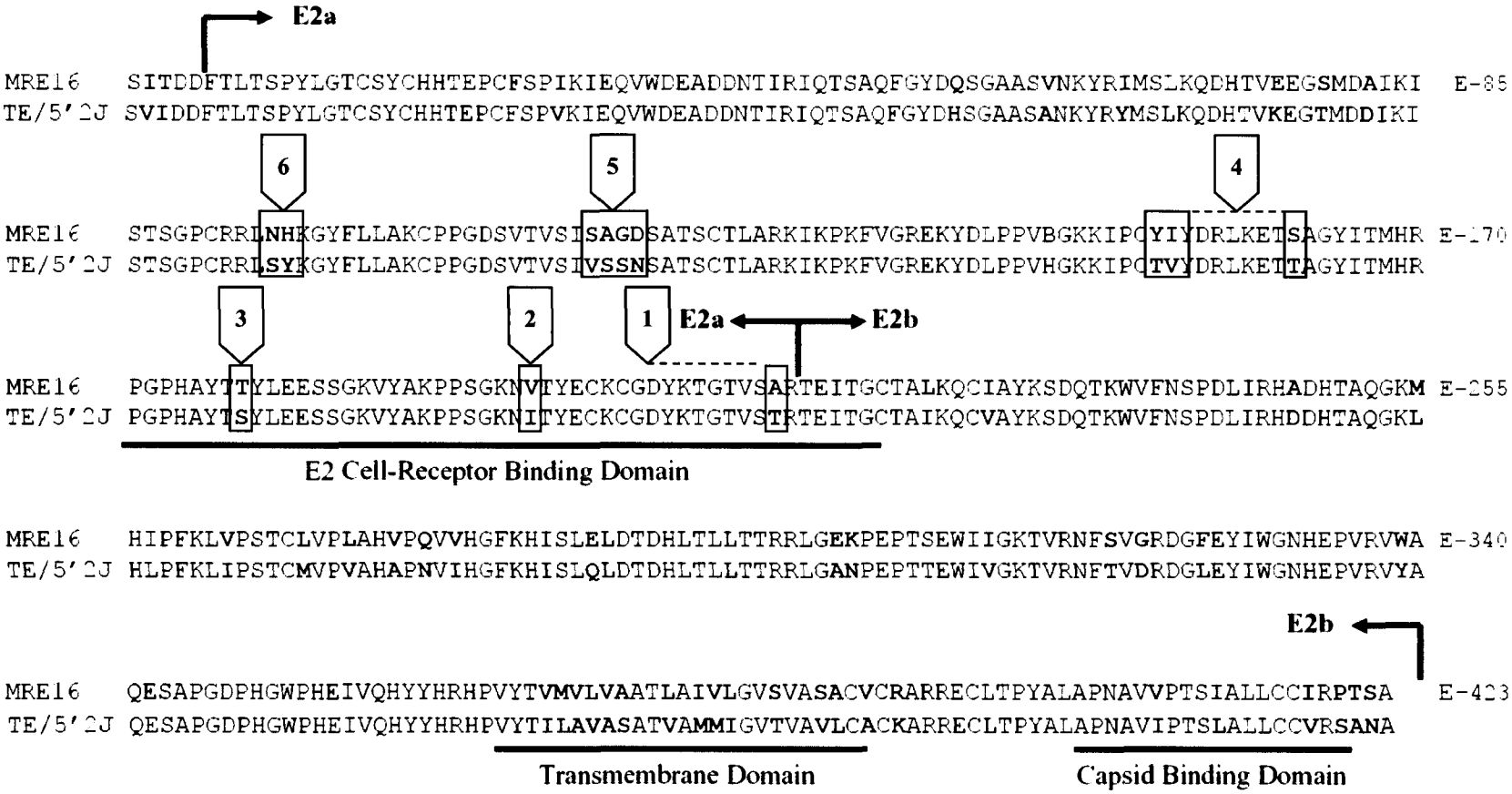
Culicinae mosquito genera: *Aedes* and *Culex* (Karabatsos 1985. Updated 2001.; Mackenzie, Lindsay et al. 1994). Interestingly, the mosquito bloodmeal feeding behavior is a key component in disease transmission. *Aedes* species feed somewhat discriminantly on mammals while C*ulex* species are more indiscriminant catholic feeders of both mammals and birds.

Ae. aegypti is a major vector in the epidemic disease cycles of dengue and yellow fever viruses as well as being a competent vector for a number of other arboviruses including SINV. As previously mentioned, there are three major genotypes of SINV; the Paleoarctic-Ethiopian (P/E) genotype, which is found in Europe and Africa, the Oriental-Australian (O/A) genotype, which is found in Asia and Australasia, and the Southwest genotype, which is found in the southwestern region of Western Australia (Rentier-Delrue and Young 1980; Olson and Trent 1985; Sammels, Lindsay et al. 1999; Saleh, Poidinger et al. 2003). The SINV strain MRE16 is an O/A genotype isolated between 1966 and 1969 from a pool of *Cx. tritaeniorhynchus* mosquitoes in Malaysia (Pudney 1979), and a full-length infectious clone (ic) of MRE16 has been generated (Myles, Pierro et al. 2003). MRE16 is currently the model O/A genotype being investigated in the laboratory in mosquito infection assays (Seabaugh, Olson et al. 1998; Olson, Myles et al. 2000; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; Foy, Myles et al. 2004; Myles, Pierro et al. 2004). MRE16 was previously characterized for its infection rate and distribution in the midgut of *Ae. aegypti* mosquitoes and found to have a 100% MIR 7 dpi (Myles, Pierro et al. 2004). In contrast, the recombinant SINV strain TE/5'2J, a double subgenomic SINV often used to deliver and express genes of interest in vector species, was constructed from a chimeric mouse neurovirulent variant

of AR339 called TE12 (Lustig, Jackson et al. 1988) and infects less than 15% of *Ae. aegypti* mosquitoes when analyzed similarly (Pierro, Myles et al. 2003).

The MRE16 E2 gene has been implicated as harboring genetic determinants of midgut infection (Pierro, Myles et al. 2003). As mentioned, several unique determinants for midgut infection have previously been identified in the E2 glycoprotein of alphaviruses. Sequence comparison between the SINV TE/5'2J and MRE16 E2 genes identified approximately 316 nt (24.9 %) differences (Seabaugh, Olson et al. 1998). Of the 423 aa residues of the MRE16 E2, 60 residues (14.2%) diverged from TE/5'2J (figure 3.1 on page 56). In contrast, SINV TE/5'2J and its parental virus strain AR339 reveals only 2 aa differences in the E2 region. *Ae. aegypti*, together with these SINVs, provide a unique model system to more specifically investigate the hypothesis that the MRE16 arboviral genetic determinants of midgut infection reside within the E2.

This chapter describes the manipulation of SINV strain TE/5'2J to generate viruses containing MRE16 sequences in the E2 gene (either as chimeras or point mutations). These viruses were characterized to determine the capacity to produce mosquito midgut infections. The results demonstrated that the TE/5'2J viruses with either the MRE16 residue motifs E2-95-6NH or E2-116-9SAGD conditioned the MIR. It was also found that both of the MRE motifs E2-95-6NH and E2-116-9SAGD, when together in TE/5'2J, had an additive effect on MIR although lower than that of the parental MRE16 virus indicating that there are additional conditioning elements to the MIR. In addition, an aa sequence upstream of the E2-116-9SAGD motif was observed that had strong conservation between alpha- and flaviviruses in their envelope genes and may be involved a shared infection mechanism among the alpha- and flaviviruses.



Figur 3.1. Amino acid alignment of the E2 proteins of MRE16 and TE/5'2J. Red letters are non conserved residues. Arrows indicate the point of delineation of the E2a and E2b in the TE/ME2a and TE/ME2b chimeras. Yellow boxes identify specific differences between TE/5'2J and MRE16 that were investigated for their effect on the MIR in *Ae. aegypti* mosquitoes.

Material and Methods

Cell culture. The African green monkey kidney Vero (ATCC CCL-81 P140-170), baby hamster kidney BHK-21 (ATCC CL-10 P60-80), rhesus monkey kidney LLC-MK2 (ATCC CCL-7), duck embryo (ATCC CCL-141), *Ae. albopictus* C6/36 (ATCC CRL-1660 P130-150) and *Ae. aegypti* Aag2 (provided by Dr. A. Raihkel, University of California at Riverside) cells were individually grown in minimal essential medium Eagle (MEM) supplemented with 8% fetal bovine serum (heat inactivated), 2mM L-glutamine, 1x non-essential amino acids for MEM, 100 U/ml penicillin, and 100 μg/ml streptomycin. Schneider's Drosophila S2 (ATCC CRL-10191) cells were grown in Schneider's Drosophila Medium supplemented with 8% fetal bovine serum (heat inactivated). Cell culture was performed at 5% atmospheric CO_2 in a 37 °C (Vero, BHK, LLC and duck embryo cells) or 28 °C (C6/36 and Aag2 cells) chamber or at room temperature with no supplied CO_2 (S2 cells).

Infectious clones. The cDNA ic pTE/5'2J was generously provided by Dr. Charles Rice (Rockefeller University, NY, NY) and its use has been previously described (Hahn, Hahn et al. 1992; Pierro, Myles et al. 2003). As mentioned, the SINV strain MRE16 was isolated between 1966 and 1969 in Malaysia from a pool of *Cx. tritaeniorhynchus* mosquitoes and was cultured in *Ae. pseudoscutellaris* (AP1) cells followed by six more passages in AP1 cells and at least three passages in C6/36 cells (Varma, Pudney et al. 1974; Pudney 1979). The cDNA pMRE16 ic, which was used as a template for sPCR, was previously developed by Dr. Kevin Myles (currently at the Virginia Polytechnical Institute and State University, Blacksburg, VA) (Myles, Pierro et al. 2003). The derived consensus sequence of MRE16 and its use has been described

previously (Seabaugh, Olson et al. 1998; Myles, Pierro et al. 2004).

Construction of TE/E2a and TE/E2b. The previously constructed plasmid pME2/5'2J (Pierro, Myles et al. 2003) used a TE/5'2J backbone that was mutated to include an Age I restriction endonuclease site (RENZ) at SINV position nt 9,902. A PCR generated MRE16 E2 gene fragment (approximately 1,229 nuecleotides) was then inserted between an Xmn I RENZ at nt position 8,673 and the 9,902 Age I RENZ to create pME2/5'2J. For this work, pME2/5'2J was mutated to include another Age I RENZ at nt 9,240 (figure 3.4 on page 66; primers in table 3.1 on page 59). For construction of TE/ME2a, primers (table 3.1 on page 59) were engineered for amplification of the 5' half of E2 (nt 8,861-9,238) of TE/5'2J, the fragment was ligated into pME2/5'2J between the Xmn I and Age I (9,240) RENZs (figure 3.4) followed by site-directed mutagenesis (sPCR) to insert E2-213A (primers "TE-M1" and "cTE-M1" in table 3.1). For construction of TE/ME2b, primers (table 3.1) were engineered for amplification of the 3' portion of E2 (nt 9,238-9,902) of TE/5'2J, and the fragment was ligated into pME2/5'2J between Age I (9,240) and Age I (9,902) RENZs (figure 3.4).

Construction of point mutations in SINV ics. SINV plasmid (p) TE/5'2J was mutated to generate the desired MRE16 aa motifs (figure 3.4 on page 66). Table 3.1 (page 59) lists the primer sequences used in sPCR with the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla,). Infectious SINV clones with multiple motifs (e.g. pTE/Mut-1-2-3-4-5-6) were generated by sequentially adding point mutations in repeated rounds of sPCR. The sPCR reaction used the following optimized conditions: 15ng DNA template, 5.0 µl 10x buffer, 1.25 µl 10mM dNTPs, 125ng base primer, 125ng complement primer, 2.0 µl Pfu turbo® DNA polymerase, and sterile, deionized

water up to 50.0 µl. The thermocycling parameters for sPCR were optimized at: one cycle 95°C for 30s, 18 cycles of 95°C for 30s, 54°C for 1min, 68 °C for 14 min. Insertion point mutation was verified by sequence analysis of the plasmid DNA as well as the derived virus RNA genome via RT/PCR.

Generation and characterization of SINV particles. The production of infectious viral particles from the cDNA of pTE/5'2J, pTE/ME2a, pTE/ME2b and the sPCR mutant clones was similar to previous descriptions (Pierro, Myles et al. 2003; Myles, Pierro et al. 2004). In summary, the production of infectious viral particles was achieved by linearizing pTE/5'2J, the derived plasmid chimeras and/or the mutant

Table 3.1. Synthetic oligonucleotides and peptides

A) Synthetic Oligonuceolotides

	ID	Sequence	Description
1	MRE-9240-Age	GCGGCGACTACAAGACCGGTACGGTGTCGGCC	sPCR to insert AgeI at 9240 in ME2/5'2J
2	cMRE-9240-Age	GGCCGACACCGTACCGGTCTTGTAGTCGCCGC	sPCR to insert AgeI at 9240 in ME2/5'2J
3	TE-8861-Xmn	CCCTACTTGGGAACATGTTCGTACTGC	PCR amplify E2a of TE
4	cTE-9238-AGE	CGAAACGGTACCGGTCTTGTAG	PCR amplify E2a of TE
5	TE-9238-Age	CTACAAGCACGGTACCGTTTCG	PCR amplify E2b of TE
6	TE-M1	CACGCTTATACAACCTACCTGGAAGAATCATCAGG	sPCR to insert E2-213/A into pTE
7	cTE-M1	CCTGATGATTCTTCCAGGTAGGTTGTATAAGCGTG	sPCR to insert E2-213/A into pTE
8	TE-M2	CCGCCATCTGGGAAGAACGTTACGTATGAGTGC	sPCR to insert E2-197/V into pTE
9	cTE-M2	GCACTCATACGTAACGTTCTTCCCAGATGGCGG	sPCR to insert E2-178/T into pTE
10	TE-M3	CCGTTTCGGCCCGCACCGAAATCACTGG	sPCR to insert E2-178/T into pTE
11	cTE-M3	CCATGTATTTCGGTGCGGGCCGAAACGG	sPCR to insert E2-178/T into pTE
12	TE-M4	CCTTGCTATATCTACGACCGTCTGAAAGAAACATCTGCAGGC	sPCR to insert E2-153-4/YI-162/S
13	cTE-M4	GCCTGCAGATGTTTCTTTCAGACGGTCGTAGATATAGCAAGG	sPCR to insert E2-153-4/YI-162/S
14	TE-M5	ACGGTTAGCATAAGTGCTGGCGACTCAGCAACGTCATGTACACTGGCC	sPCR to insert E2-116-9/SAGD into pTE
15	cTE-M5	GGCCAGTGTACATGACGTTGCTGAGTCGCCTGCACTTATGCTAACCGT	sPCR to insert E2-116-9/SAGD into pTE
16	TE-M6	CCGTGTAGAAGGCTTAACCACAAAGGATACTTTCTCCTCGCA	sPCR to insert E2-95-6/NH into pTE
17	cTE-M6	CCGTGTAGAAGGCTTAACCACAAAGGATACTTTCTCCTCGCA	sPCR to insert E2-95-6/NH into pTE

B) Synthetic Peptides

	ID	Sequence	Description
1	MRE/E2/112-125	NH-**biotin**-TVSISAGDSATSCT-COOH	AA Sequence spanning the MRE16 AVR
2	TE/E2/112-125	NH-**biotin**-TVSIVSSNSATSCT-COOH	AA Sequence spanning the TE/5'2J AVR
3	MRE-AVR	NH-VSISAGDSAT-COOH	peptide used in rabbit immunizations
4	TE-AVR	NH-VSIVSSNSAT-COOH	peptide used in rabbit immunizations

clones with *XhoI*. Full-length viral genomes were then transcribed from the plasmid encoded SP6 promotor in the presence of m^7-GpppG cap analog according to the MAXIscript® In Vitro Transcription Kit (Ambion, Inc., Austin TX) protocols. Derived RNA was electroporated (BTX ECM 630; BTX Inc., San Diego, CA) into $10^{6.4}$ Vero cells (BTX ECM630 settings: 450 kV, 1200 ohms, 150 μF) and incubated for 48 hr followed by virus expansion on C6/36 cells. First-in-cell generated virus (i.e. virus obtained by using RNA transcribed from the infections clone) was termed "P-0" and virus passaged once was called "P-1". For verification of mutation insertion, RNA from P1 virus was isolated, the E2 gene was RT/PCR amplified and fully sequenced and no adaptive mutations were identified. Plaque titrations of SINV were performed by infecting confluent monolayers of Vero cells as described previously (Miller and Mitchell 1986). Plaque sizes of P-0 were analyzed on Vero cells 5 days post infection. Growth curve analysis was performed using confluent monolayer of cells infected in triplicate with P-0 virus at a multiplicity of infection (MOI) of 0.01. For indirect immunofluoresence assay (IFA), SINV infected cultured cells were first fixed in acetone. The IFA reaction was performed using anti-SINV-E1 (30.11a) as the primary antibody (1:200) and biotinylated sheep anti-mouse antibody (1:200; Amersham Corp., Arlington Heights, IL) as the secondary antibody. The E1 protein was detected using a fluorescein isothiocyanate-conjugated streptavidin detector (1:200; Amersham). Mosquito body tissue and midguts were detected for SINV infections by IFA using the same antibodies as above and as previously described (Myles, Pierro et al. 2003).

Mosquitoes and oral infections. Maintenance of *Ae. aegypti* strain Rexville D (RexD; Rexville D, Puerto Rico), a DENV and SINV susceptable colony strain of *Ae.*

aegypti, and the infectious bloodmeal delivery mechanism were performed as previously described (Myles, Pierro et al. 2004). In summary, the propagation of virus used in bloodmeals started with confluent monolayers of either Vero or C6/36 cells that were infected with P-0 virus at a MOI of approximately 0.01 in 8% FBS/MEM and cultured in CO_2 incubators for two days at 37°C (Vero) or three days at 28 °C (C6/36). After incubation, freshly harvested P-1 cell culture supernatant (i.e. non-frozen) containing infectious virions was, when applicable, diluted in uninfected conditioned cell culture supernatant (final volume of 0.8 ml) to a predicted viral titer using the standardized growth curve data as a guide (Figure 3.5). The pre-titrated virus was then mixed with 0.8 ml of defibrinated sheep blood (Colorado Serum Co, Boulder, CO). This infectious bloodmeal mixture was warmed to 37°C and placed in a water-jacket heated (37 °C) glass membrane feeder. Mosquitoes were allowed to probe and feed through a stretched sheet of parafilm for $\leq$ 30 min. Fully engorged mosquitoes were collected and maintained in the insectary with ample food and water until assayed. The viral bloodmeal was frozen and later quantified by plaque titration. The bloodmeal titer was considered acceptable at +/-0.3 $\log_{10}$PFU/ml of the predicted viral titer. Post-bloodmeal viral titers were found to be reduced by $\leq$10% when compared with pre-bloodmeal titers (data not shown).

Statistical analysis and cluster alignment. The MIR is calculated by the number of IFA positive mosquito midguts divided by the total number of bloodfed mosquitoes, expressed as a percent, in each replicate. All mean (geometric) MIRs are reported as an average of two replicates for each virus with the (geometric) standard error of the mean (SEM) as error bars. The n value for mosquito organs in each

analyzed replicate was 21 +/-3 (total n for two replicates = 42 +/-6). Comparison of the MIR rates for statistical significance was performed by combining primary data of replicates and analyzing by Chi Square Test with an alpha cut off of 0.05 using the SAS System© statistical software (Cary, NC). The E2 amino acid cluster alignments in figure 3.8 (page 75) used the Pfam database and the color codes used for clusters are from Jalview and ClustalX (Finn, Mistry et al. 2006). The predictions for 2° structure and solvent access in figure 3.8 were performed by PredictProtein (Rost and Sander 1993; Rost and Sander 1994; Rost, Yachdav et al. 2004).

Peptide blocking assay. Synthetic peptides representing the E2 112-125 sequences of MRE16 and TE/5'2J were custom generated by a commercial source (Invitrogen, CA). Amino acid sequences are listed in table 3.1 (page 59). The peptides were tagged at the N-terminus with biotin and considered ≥70% pure. Lyophilized peptides were diluted to 5mg/ml in sterile PBS stored at –20°C until use. Peptide blocking of SINV infection of the midgut followed the standard oral bloodfeeding protocol, with the peptide added to the infectious bloodmeal to a final concentration of 250 μg/ml just prior to use.

Antibody blocking assay. Polyclonal antibodies were generated against synthetic peptides of the E2 113-122 sequence of each MRE16 and TE/5'2J by a commercial source (Bioreagents, Golden, CO). Amino acid sequences used in immunizations are listed in table 3.1 (page 59). The strategy for antibody generation was as follows: synthetically derived SINV peptides were conjugated to glutaraldehyde, which was used for enhancement of immune recognition. For each polyclonal antibody, two adult rabbits were subcutaneously injected with a high concentration of conjugated

peptide in an adjuvant cocktail. Four boosts were performed at two week intervals. Both rabbits were then sacrificed and whole body blood was collected. Total IgG antibodies were purified from the serum by precipitation. Target specific antibodies were then purified from total IgGs using a peptide affinity column by the contractor and provided as a concentrated liquid elutant. Antibody blocking of the SINV infection of the *Ae. aegypti* midgut followed the standard oral bloodfeeding protocol, with the antibody added to a final dilution of 1:4 to the diluted viral suspension that was incubated at 37C for 10 min followed by addition of the sheeps blood for mosquito feeding.

Results

Growth curve characterization of SINV. The SINV strain MRE16 is considered an O/A genotype, and the TE/5'2J and TR339 SINVs are P/E genotypes. These genotype may have a fundamental disparity in virus replication rates that influence the MIR. To investigate the potential for advantageous inherent growth characteristics in cells, an *in vitro* tissue culture growth curve assay was performed using these three viruses in three vertebrate and three invertebrate cell types (figure 3.2 on page 64).

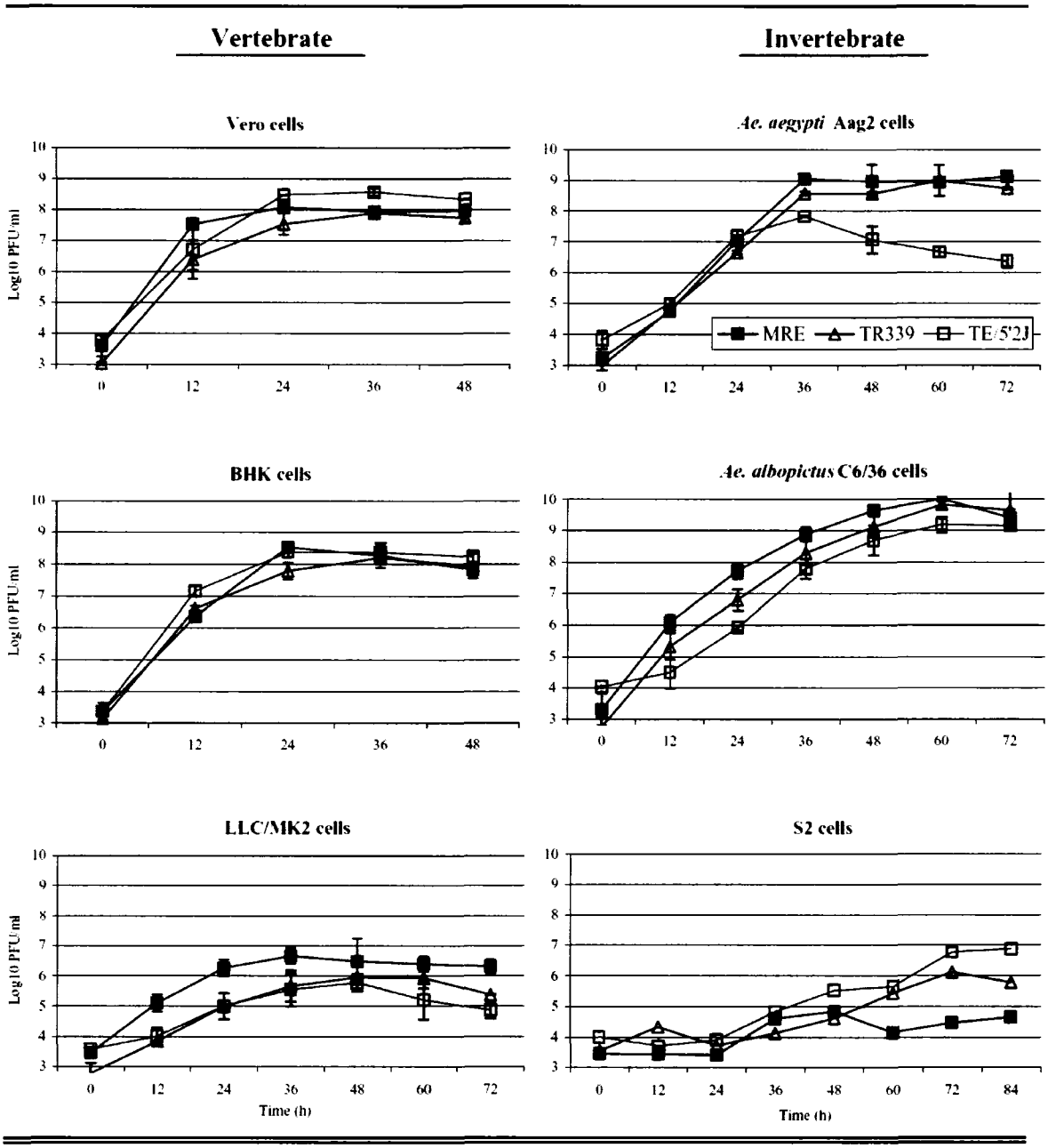


Figure 3.2. Tissue culture growth curve assays of SINVs.

These growth curve assays revealed no consistant pattern of growth advantage or disadvantage in either vertebrate or invertebrate cell types. Instead, some viruses were observed to display unique growth characteristics within a specific cell type. For example, TE/5'2J appeared to be incapable of maintaining a high viral titer in Aag2 (*Ae.*

aegypti) cell culture supernatant but this was not the case with C6/36 (*Ae. albopictus*) mosquito cells (figure 3.2, top and middle right panels). The TE5'2J virus was more restricted in the mosquito cells, but not so in drosophila S2 cells. In addition, the MRE16 virus exhibited a slightly enhanced replication efficiency over the P/E genotype viruses in LLC/MK2 (rhesus monkey kidney) cells, but not in Vero (green monkey kidney) or BHK (baby hamster kidney) cells (figure 3.2, left panels).

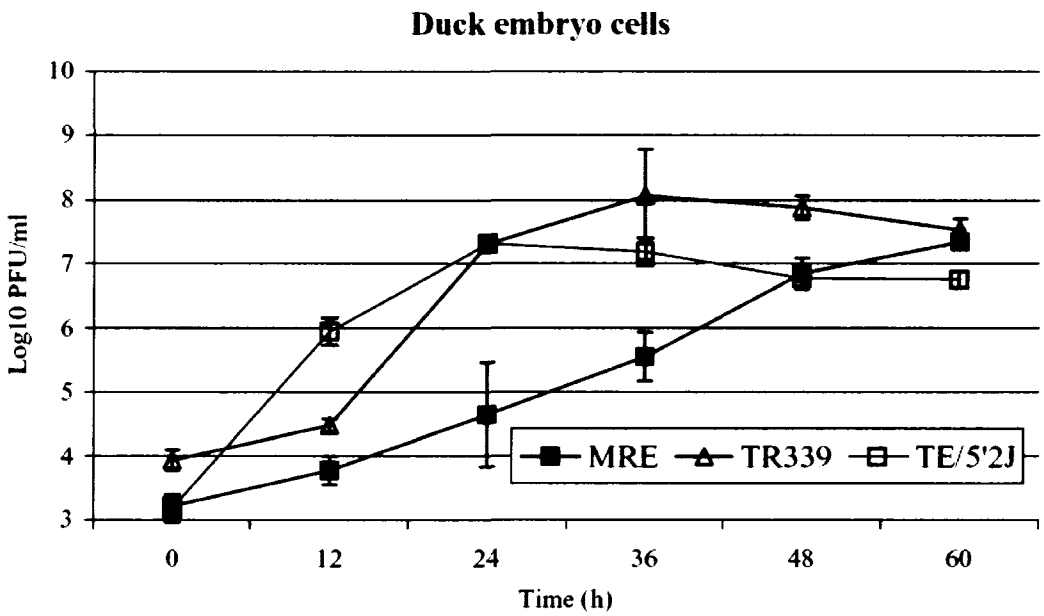


Figure 3.3. Tissue culture growth curve of SINVs in duck embryo cells

Growth of the SINVs was also evaluated in duck embryo cells and yielded significant titer differences at 24-36h ($p<0.001$) for P/E and O/A genotypes indicating replication differences (figure 3.3 above). This is interesting as the SINVs are known to use passerine birds as natural hosts in the wild. However, the observed maximum titers were statistically not different.

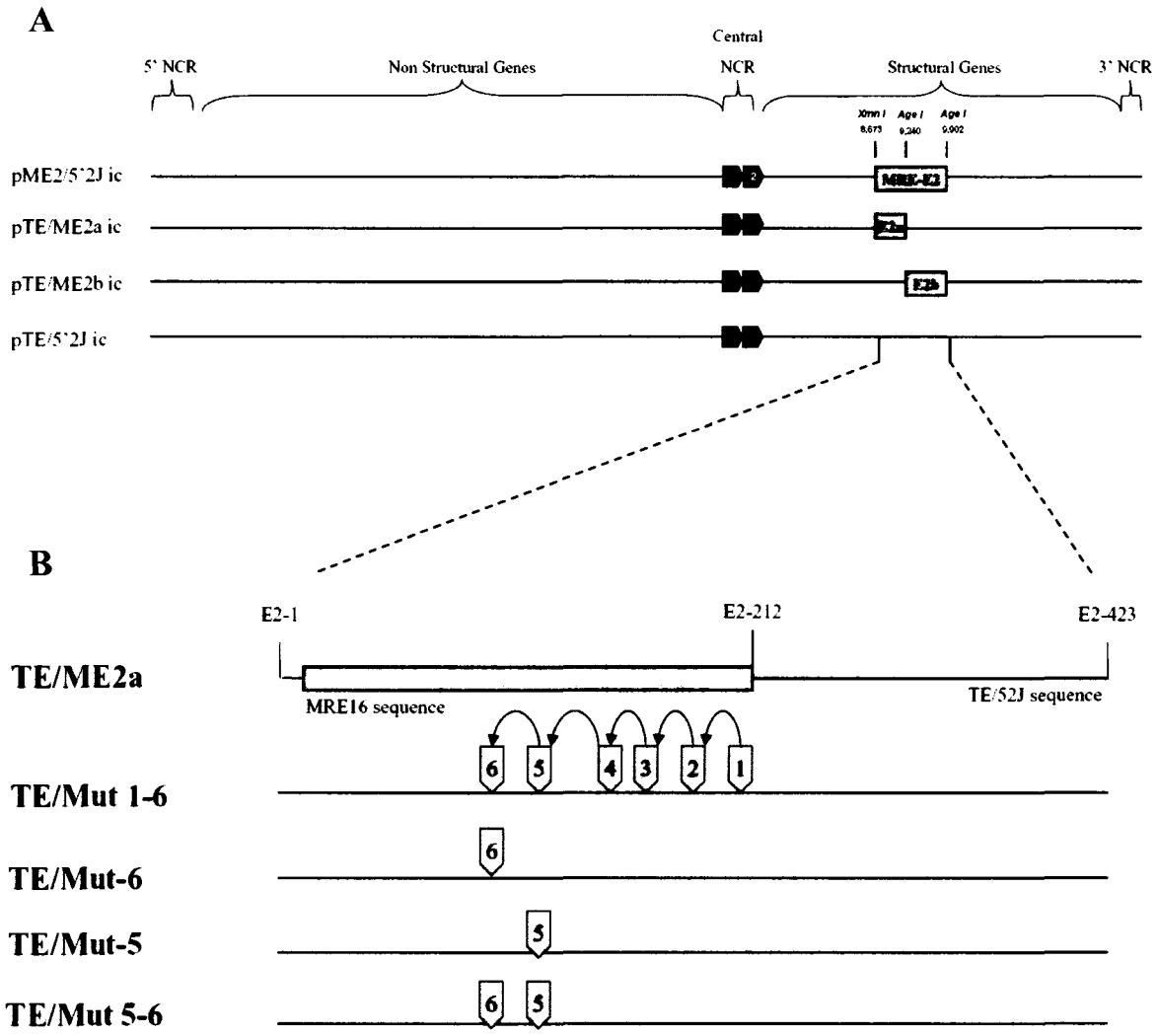


Figure 3.4. Representation of TE/5'2J clones containing MRE16 genome portions. A) Full-length TE/5'2J genome identifying chimeric MRE16 E2 regions. Solid line, TE/5'2J sequence; yellow box, MRE16 sequence; blue and maroon pentagons, 1st and 2nd subgenomic promoters, respectively. See figure 3.1 (page 56) for delineation of E2a and E2b. B) TE/5'2J E2 gene with site modifications to represent aa motifs of MRE16. Numbered yellow pentagons correlate with a MRE16 aa motif change: see table 3.1 (page59) and figure 3.1 (page 56) for specific aa residue changes. Arrows indicate the order in which each motif was added to pTE/5'2J via sPCR.

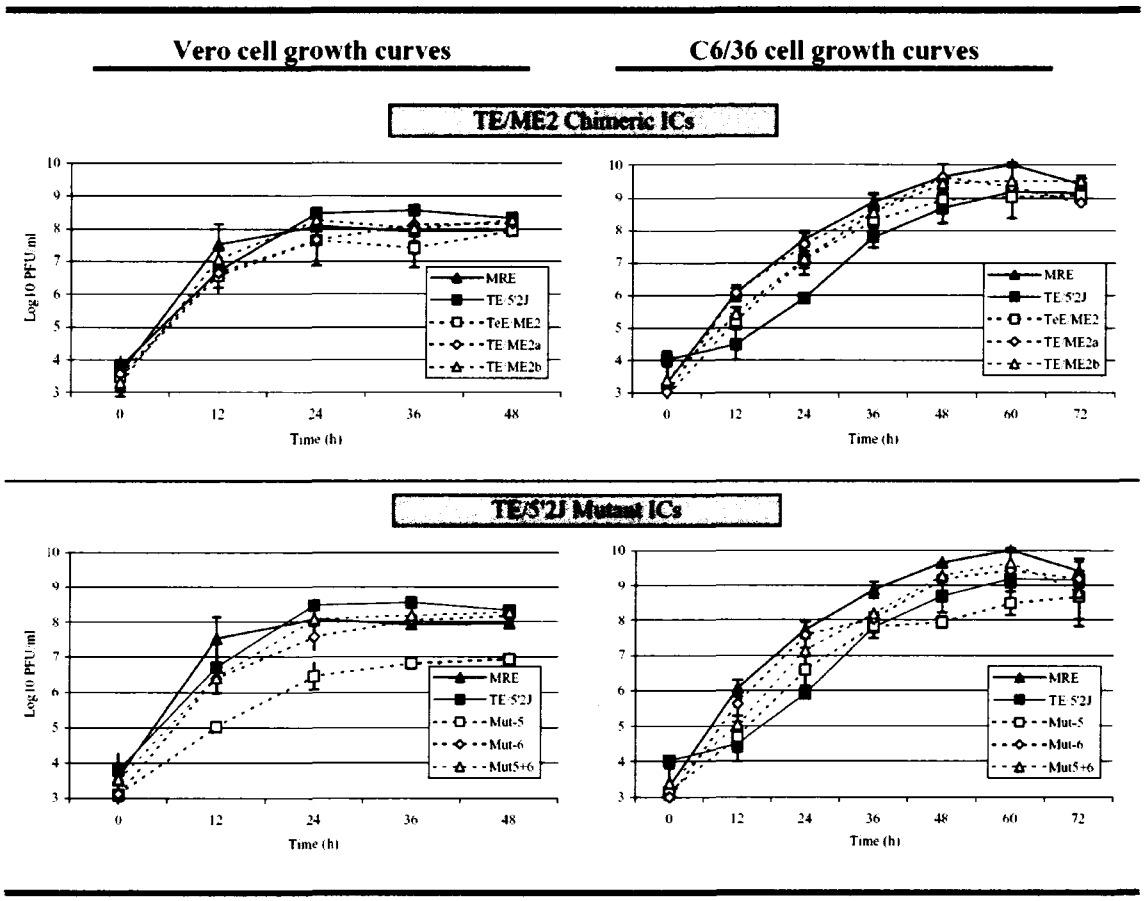


Figure 3.5. Tissue culture growth curves of SINV mutants.

Generation and characterization of SINV E2 chimeric viruses in tissue culture. Primers (table 3.1 on page 59) were designed to construct SINV chimeras containing portions of TE/5'2J and MRE16 E2 genes within the backbone genome of TE/5'2J (figure 3.4 on page 66). The chimeric plasmids pTE/ME2a and pTE/ME2b divided the E2 gene in half with each ic harboring a complete E2 chimeric gene with sequences representing either TE/5'2J or MRE16. These plasmids were used for the generation of P-0 virus from Vero cells. Virus replication rates were analyzed by growth curve analysis in both vertebrate (Vero) and invertebrate (C6/36) cell lines (figure 3.5 on page 67, top two panels).

Maximum virus titers for the SINV chimeras were achieved at approximately 24-36 hours post infection for viruses propagated in Vero cells, 48-60 hours for propagation in C6/36 cells. The maximum virus titers ranged from 7.9-8.6 and 9.1-9.7 $\log_{10}$ PFU/ml in Vero and C6/36, respectively. In addition, a large plaque phenotype was observed in MRE16, TE/ME2 and TE/ME2a viruses (table 3.3 on page 71).

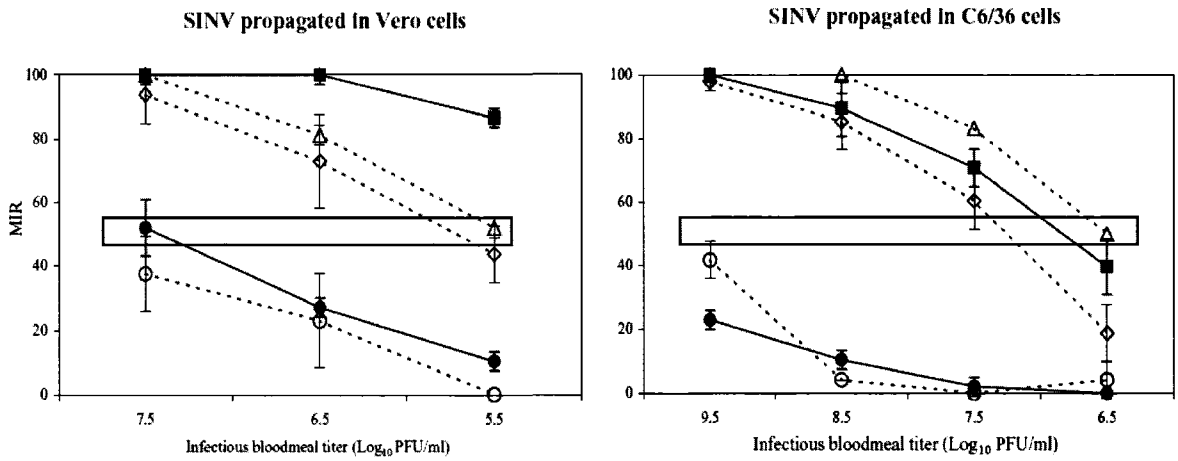


Figure 3.6. MIR of chimeric SINVs in *Ae. aegypti* mosquitoes. Viruses were propagated in either mammalian cells (left panel) or in insect cells (right panel). MIR is the mean of IFA-positive midguts over n (n=21±3) from two replicates 9 days p.i. Shaded horizontal bar is the estimated MID_{50}. Error bars, SEM. ■, MRE16; ●, TE/5'2J; Δ, TE/ME2; ◊ TE/ME2a; ○, TE/ME2b.

MRE16 E2 division harboring the genetic determinants of MIR. Parental and chimeric SINVs were analyzed in a *per os* midgut infection assay. At 9 dpi, midguts were dissected out of mosquitoes, fixed in 4% paraformaldehyde and assayed for the presence of SINV E1 antigen by IFA. The *Ae. aegypti* MIR of all the viruses were dose-dependent. In addition, MRE16 and TE/5'2J viruses consistently had MIRs statistically different ($p<0.001$) from each other at the different bloodmeal doses (figure 3.6 above). Changing the propagation cell type did not change these observations.

The TE/5'2J viruses containing N- and C-terminal halves of the MRE16 E2 gene (i.e. TE/ME2a and TE/ME2b, respectively) were assayed for oral infectivity and the subsequent MIR which revealed the region harboring the genetic determinants of SINV midgut infections (figure 3.6 on page 68). Specifically, the MIR for TE/ME2a was higher and statistically different from the MIR of TE/5'2J ($p<0.01$) for both Vero and C6/36 cell-line derived virus at all doses analyzed. In contrast, the MIR for TE/ME2b was not statistically different from TE/5'2J ($p>0.21$) with the exception of high titer ME2b (i.e. 9.5 $\log_{10}$ PFU/ml) derived from C6/36 cells ($p<0.01$). The MIRs of TE/ME2b virus were statistically lower ($p<0.5$) than those of MRE16, TE/ME2 and TE/ME2a (in figure 3.6) in both Vero and C6/36 propagated viruses (low titered C6/36 derived virus excepted).

The influence of infectious dose as well as the determinants of infection can be evaluated by comparing each of the virus' specific midgut infectious dose-50 (MID_{50}) which is the infectious virus titer at which 50% of the mosquitoes had SINV infected midguts 9 dpi. The MID_{50} can be estimated from figure 3.6 (page 68; shaded horizontal bar in MIR) and the data are presented in table 3.2 (page 70). A lower MID_{50} is an indication of an enhanced efficiency at which a virus establishes an infection in midgut cells. The MID_{50} was analyzed for viruses propagated in C6/36 cells and in Vero cells. MRE16 virus had a comparably lower MID_{50} (7.0 and <5.5 $\log_{10}$, respectively) and TE/5'2J had a higher MID_{50} (>9.5 and 7.5, respectively). SINVs with an "E2a" portion from MRE16 all had low MID_{50}s of $\leq$ 7.25 (C6/36 cell derived) and $\leq$ 5.75 (Vero cell derived) while those SINVs with an "E2a" from TE/5'2J had MID_{50}s of > 9.5 and $\geq$ 8.0 (C6/36 and Vero cell derived, respectively). The data from table 3.2 together and figure

3.6, it suggests that the determinants for enhanced midgut infection are in the E2a portion (E2 aa residues 4-213) of MRE16.

Table 3.2. Comparision of *Aedes aegypti* midgut infectious dose-50.

Virus	Primary Residues Mut-5	Mut6	*Ae. aegypti* MID_{50}† C6/36	Vero	Δ
MRE16	SAGD	NH	7	<5.5*	>1.5
TE/5'2J	VSSN	SY	>9.5*	7.5	>2
TE/ME2	SAGD	NH	6.5	5.5	1
TE/ME2a	SAGD	NH	7.25	5.75	1.5
TE/ME2b	VSSN	SY	>9.5	8	>1.5
TE/Mut-5	SAGD	SY	8.25	6.5	1.75
TE/Mut-6	VSSN	NH	9	6.75	2.25
TE/Mut-5+6	SAGD	SY	8	6.25	1.75

† from figure 3.6 & 3.7, Δ = C6/36 MID50 minus Vero MID50, * MID50 could not be accurately estimated due to virus bloodmeal titer limits (see figure 3.5).

MRE16 E2 genetic motifs that influence MIR. Of the 209 amino acid residues within the target E2a region, 19 (9%) are non-synonomous between TE/5'2J and MRE16 (see figure 3.1 on page 56). To further identify the MRE16 genetic determinants of midgut infection, these MRE16 amino acids were mutated into the TE/5'2J ic backbone, either individually or in clusters (see figure 3.4 on page 66). The goal was to spotlight those residues that positively influence the MIR in *Ae. aegypti* mosquitoes. Initially, the MRE16 amino acid codons were added sequentially to the TE/5'2J plasmid, starting at E2 position 213 (i.e. Mut-1) and moving toward the N-terminus (figure 3.4), until a virus with an enhanced MIR was identified. Specific

sPCR primers (table 3.1 on page 59) were designed to generate the desired SINV plasmids, which were subsequently used for transcription and electroporation of genome-length viral RNA, resulting in the generation of infectious P-0 virus from Vero

Table 3.3 Comparision of SINV mutant's MIR in *Aedes aegypti*.

Virus	MRE16 E2 Position	MRE16 E2 Residue	Mean Plaque Size (mm)	MIR 9dpi in *Aedes aegypti* n†	+	MIR % (SEM)
TE/5'2J	na	na	2.2	48	11	22.9 (2.9)
TE/Mut-**1**	213	T	2.3	48	12	25.0 (11.8)
TE/Mut 1-**2**	197*	V	1.9	47	9	19.1 (8.5)
TE/Mut 1-2-**3**	178*	T	2.2	48	13	27.1 (2.9)
TE/Mut 1-2-3-**4**	153-4*	YI	1.9	48	12	25.0 (5.9)
TE/Mut 1-2-3-4-**5**	116-9*	SAGD	7.9	48	15	31.3 (8.8)
TE/Mut 1-2-3-4-5-**6**	95-6*	NH	8.5	48	46	95.8 (5.9)
TE/Mut-6	95-6	NH	2.4	48	23	47.9 (14.7)
TE/Mut-5	116-9	SAGD	8.1	48	41	85.4 (2.9)
TE/Mut-5+6	95-6/116-9	NH/SAGD	8.4	48	47	97.9 (2.9)

† n = combination of 2 relicates of 24 mosquitos.
* position of **bold** mutations which were added to the previously generated mutation(s).

cells. Table 3.3 above lists the derived viruses, their specific MRE16 residues, as well the MIR of C6/36 derived P-1 virus at a bloodmeal concentration of 9.0 $\log_{10}$ PFU/ml. From table 3.3, it appears that the TE/Mut 1-2-3-4-5-6 contains a determinant of midgut infection. The Mut-6 motif was then added by itself to TE/5'2J (i.e. TE/Mut-6) and positively influenced the MIR ($p<0.03$). The subsequent addition of Mut-5 (i.e. TE/Mut 5+6) further enhanced the MIR to levels not statistically different from TE/Mut 1-6 ($p<0.03$). The Mut-5 motif was then added to TE/5'2J by itself (i.e. TE/Mut-5) and positively influenced the MIR. Interestingly, the MIR of TE/Mut-5 was not statistically different from the TE/Mut-1-2-3-4-5-6 and TE/Mut5+6. ($P<0.001$) indicating that this

mutation may be a major MRE 16 determinant in midgut infections. In summary, TE/Mut-6 alone conditions MIR as does TE/Mut-5, and together they may have an additive affect (shown statistically below). In addition, the large plaque phenotype observed in MRE16, TE/ME2, and TE/ME2a was associated with TE/5'2J clones harboring the Mut-5 MRE16 aa residues.

Characterization of Mut-5 and Mut-6 motifs. To further investigate the Mut-5 and Mut-6 residues in TE/5'2J, SINV replication rates were characterized by growth curve analysis in vertebrate (Vero) and invertebrate (C6/36) cell lines (figure 3.5 on page 67, bottom two panels). Peak virus titers for the SINV chimeras were at 24-36 hours post infection for viruses propagated in Vero cells and 48-60 hours for propagation in C6/36 cells. The maximum virus titers (log_{10} PFU/ml) for these viruses ranged from 7.9-8.6 and 8.6-10.1 in Vero and C6/36 cells, respectively, with one exception. TE/Mut-5 replicated less efficiently in Vero cells resulting in significantly lower mean titers at specific time points as well as a lower mean peak titer than the other viruses ($p<0.001$). These viruses differ only in the E2 and efficiency may be related to infection and/or E2 maturation.

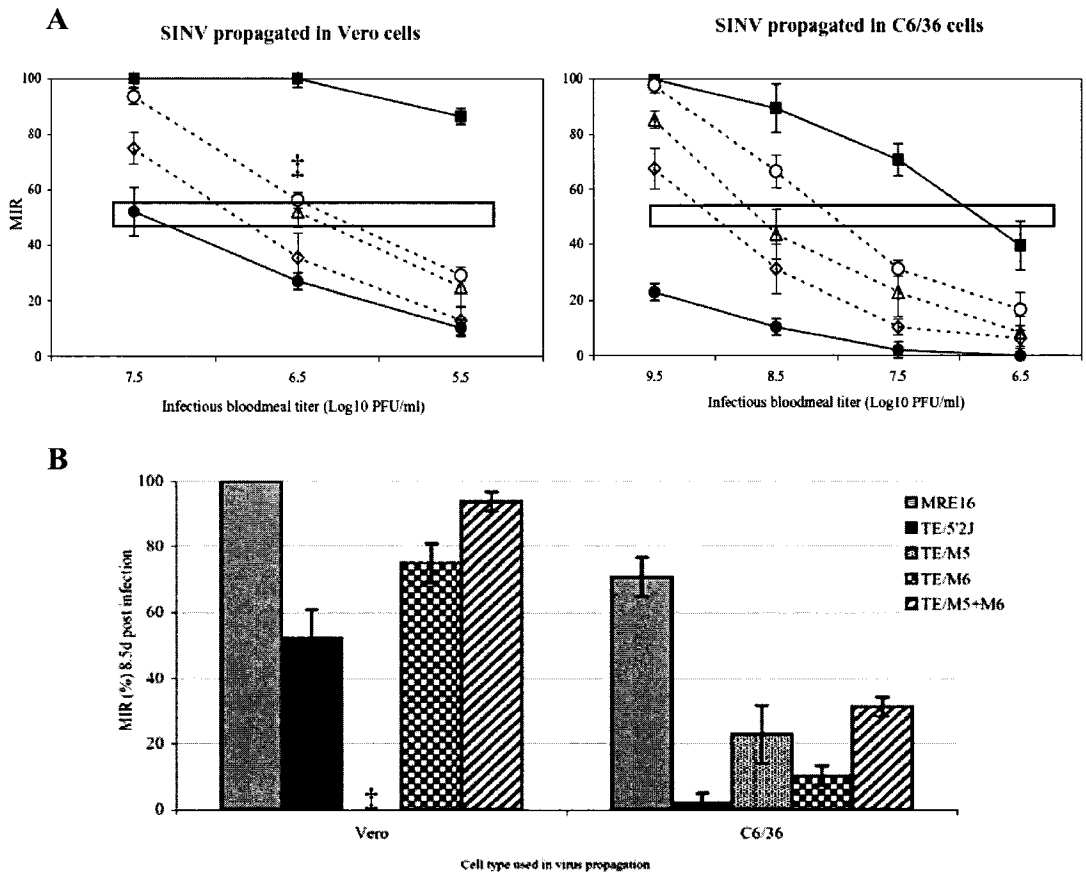


Figure 3.7. MIR of mutant SINVs in *Ae. aegypti* mosquitoes. A) Various doses of SINV that had been propagated in either mammalian cells (left panel) or in insect cells (right panel) and used to orally infect mosquito midguts. MIR is the mean of IFA-positive midguts over n (n= 21±3) from two replicates 9 days p.i. Shaded horizontal bar is the estimated MID_{50}. Error bars, SEM. ■, MRE16; •, TE/5'2J; Δ, TE/Mut-5; ◊ TE/Mut-6; ○, TE/Mut 5+6. B) MIR of SINs at a standardized titer of 7.5 Log_{10} PFU/ml. ‡, TE/Mut-5 will not generate titers above 6.5 $Logs_{10}$ PFU/ml in Vero cells (see figure 3.5).

The TE/Mut-5, TE/Mut-6 and TE/Mut 5+6 viruses were also investigated in the midgut infection assay to determine their viral infectivity (figure 3.7 on page 73). All of these viruses analyzed exhibited a dose-dependent MIR in *Ae. aegypti* mosquitoes (figure 3.7.A). MIRs for MRE16 and TE/5'2J viruses consistently differed statistically ($p<0.001$) at the various bloodmeal titers. In addition, the MIRs for TE/Mut-5, TE/Mut-6 and TE/Mut 5+6 viruses were statistically greater than that of TE/5'2J ($p<0.04$) for both Vero and C6/36 cell line-derived virus at the standardized bloodmeal dose of 7.5

log_{10} PFU/ml (Vero derived TE/Mut-5 excepted; figure 3.7.B). Using this same titer, the Vero-derived TE/Mut 5+6 viruses had statistically greater MIRs than the respective 7.5 log_{10} PFU/ml Vero-derived TE/Mut-6 SINVs ($p=0.0105$), although statistically lower than the MRE16 virus ($p<0.01$). As was the case for the chimeric E2 SINVs, the MID_{50} for the Mut-5 and Mut-6 viruses was $\geq$ 1.0 log_{10} PFU/ml greater for virus propagated in C6/36 cell as compared to Vero cells (see under Δ in Table 3.2 on page 70). In addition, both Vero and C6/36 generated TE/Mut-5, TE/Mut-6 and TE/Mut 5+6 virus's MID_{50}s were lower than for the parental TE/5'2J virus, indicating an enhanced efficiency to infect the midgut. The independent affect of the two Mut-5 and Mut-6 MRE16 E2 motifs on the MIR and in some cases the affect is statistically ($P<0.03$) additive.

Cluster alignment of SINV Mut-5+6. A span of 35 aa residues (E2 91-126) from SINV MRE16 and TE/5'2J, which included the Mut-5 and Mut-6 sites, were aligned against each other as well as a panel of alphaviruses, and the amino acids were then clustered based on residue characteristics (figure 3.8 on page 75). Neither the MRE16 Mut-5 SAGD and Mut-6 NH nor the TE/5'2J Mut-5 VSSN and Mut-6 SY residues clustered as a group with any of the other alphaviruses. Individually, two of the six (33.3%) MRE16 residues in the Mut-5 and Mut-6 motifs did cluster with other alphaviruses. Specifically, E2-116 serine (S) clustered with other hydroxyl and amine residues, and E2-119 aspartate (D) clustered with other charged residues. None of the TE/5'2J residues in the Mut-5 and Mut-6 motifs clustered with the alphavirus panel. Interestingly, the EEEV and AURAV equivalent Mut-6 SH residues (under "Mut-6" bracket in figure 3.8) incorporated one homologous residue from each of TE/5'2J and

MRE16 (i.e. S and H, respectively) possibly indicating evolutionary constraints on this region.

The predicted secondary structure of these SINVs identified only loop structures (λ) associated with both Mut-5 and Mut-6 motifs (figure 3.8 on page 75; characters flanking the SINV sequences). The residue changes in the Mut-5 motif between TE/5'2J and MRE16 had no effect on the predicted secondary structure nor the predicted solvent accessibility in this area. The aa residue changes from TE/5'2J to MRE16 in the Mut-6 motif resulted in a slight shift in the location of the loop structure and as well as the decrease in the number of loop characters (from 2 down to 1) in this motif; however, the predicted solvent accessibility was unchanged. Moreover, all 6 of the aa changes in these motifs (i.e. N-S, H-Y, S-V, A-S, G-S, D-N) could be considered as semi-conservative in nature with the most significant changes occurring in the serine residues becoming hydrophobic (i.e. to either alanine, valine or glycine) (Betts 2003). Cumulatively, this evidence leads to the hypothesis that these changes do not drastically alter the motif's physical characteristics but instead only slightly enhance or reduce each motifs' innate ability to infect the midgut.

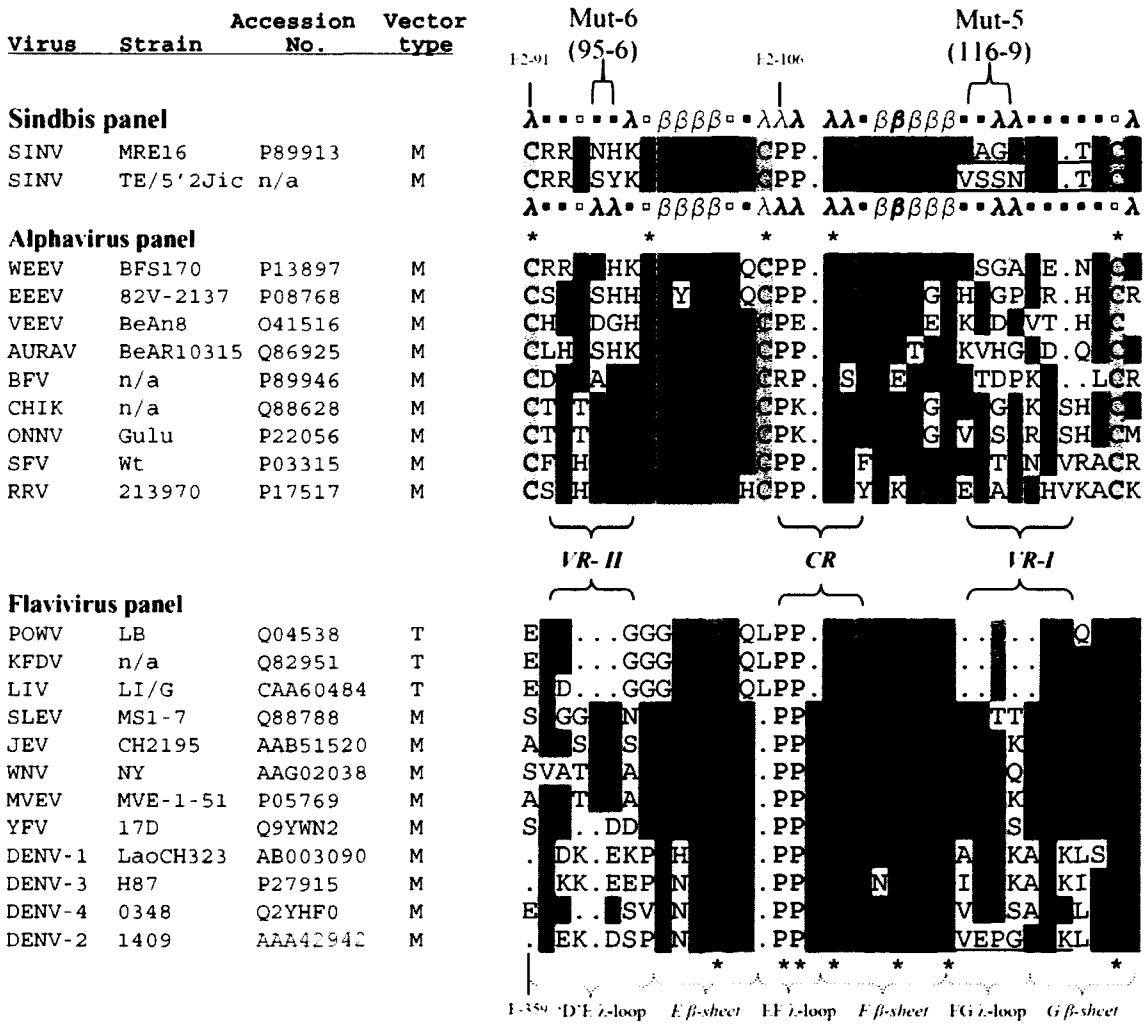


Figure 3.8. Cluster alignment of SINV Mut-5+6. The N-terminal amino acid residues were aligned and then color clustered when ≥5 residues with similar charge characteristics were present at the same location. Alignments are colored using a modified version of the ClustalX scheme in Jalview (orange: Glycine ; yellow: Proline (P); blue: small and hydrophobic amino-acids ; green: hydroxyl and amine amino-acids ; red: charged amino-acids ; cyan: Histidine and Tyrosine ; grey: Cystiene (C)). Arrows indicate TR339 residues that affect the midgut infection rate in *A. aegypti* mosquitoes. (n/a) = not available, (.) reflects no aa, (*) identifies strictly conserved aa residues (specific to each panel). The predicted secondary structure (flanking the TR339 and TE/5'2J sequences) identified loop (λ) and extended sheet (β) structures with expected average accuracy > 82% or identified no predicted structure (▫). No helixes were predicted at this threshold. **Bold** in 2° structure indicates predicted solvent accessible residues at >36%. Underline indicates synthetic peptide locations. Vector type: M, mosquito, T, tick. Varialble region, VR; Conserved region, CR.

Alphavirus and Flavivirus envelope sequence conservation associated with MIR. Figure 3.8 (page 76) shows that the aa residues in the Mut-5 and Mut-6 motifs of SINVs were not well conserved among the alphaviruses. However, a sequence stretch starting at SINV E2-98 and ending a position E2-115, which sptially separates the Mut-5 and Mut-6 motifs, appears to have clustered frequently among the alphavirus panel. The secondary structure information predicted a loop structure for the SINV, like that for both Mut-5 and Mut-6 motifs, within a subset of the intervening sequence at E2 105-109 residues (i.e. CPPGD). The function of this region is unknown, but its sequence conservation and predicted structure are noteworthy.

Database searches of SINV Mut-5 and Mut-6 sequences (plus flanking regions) did not identify other noteworthy sequences with homology. However, the intervening sequence mentioned above (i.e. CPPGD) did have a short motif that was homologous to that of the envelope protein of Powassan virus (POWV), Kyasanur Forest disease virus (KFDV), and louping illness virus (LIV), all of which are tick-borne flaviviruses. This homologous motif, having the aa sequence PPGD, was found at position 106-109 (SINV E2 positions) and overlaps the predicted loop structure sequence described above. Further alignment and clustering of this region revealed a striking homology of the SINV/alphavirus PPGDS motif (SINV E2 106-110) with that of a flavivirus motif PPF/•GDS (DENV-2 E 373-378) (figure 3.8, page 76). The motif was not conserved in other arbovirus families (e.g. Bunyaviridae, Rhabdoviridae, Reoviridae, Orthomyxoviridae and Asfarviridae), however extreme variations of the motif's residues were not compared. Thus, this motif appears to be limited to families of arboviruses with single stranded positive sense RNA genomes (i.e. Flaviviridae and Togaviridae).

For the sake of clarity, this new conserved motif (PPF/•GDS) is termed the "conserved region" (CR). The CR region is absent from the genomes of the insect-only flaviviruses (i.e. Kimiti River virus and cell fusing agent virus) and from the genome of the salmon pancreatic disease virus, which is an *Alphavirus* has no known vector.

The location of CR in flaviviruses is significant in that it is next to the RGD motif of YFV, MVEV and JEV. This RGD motif, having known affinity to cell receptors, is also spatially aligned inside of the SINV Mut-5 motif, which was shown here to be associated with SINV midgut infections. However, the specific sequences of the Mut-5 and RGD regions are not well conserved in alphaviruses and flaviviruses, respectively, and may be hypothesized to constitute an arbovirus variable region (VR) associated with mosquito infections.

The secondary structure prediction of the 35 SINV aa residues between E2 91-126 (figure 3.8 on page 76; characters flanking the SINV sequences) also had striking similarity to the known structural conformation of the flaviviruses (figure 3.8; bottom of the flavivirus panel). The CR of flaviviruses is part of the flavivirus 'EF loop' that is flanked by two beta-sheets. Interestingly, this loop structure was predicted in the SINV CR as well. In fact, the structural pattern of loop-sheet-CR_loop-sheet-loop was observed for both alphaviruses and flaviviruses in this stretch of aa. Moreover, the secondary prediction of the DENV-2 sequence containing the CR and VRs (E-359 to E-401) was nearly identical to the secondary conformation in the known DENV-2 crystal structure for this region (bottom of figure 3.8, page 76). The DENV-2 predicted secondary sequence in the region was also nearly identical to that of the SINV region of interest (E2 91-126).

Peptide and antibody antagonists of Mut-5. Based on the MIR data from the SINV ics, as well as the sequence alignment of the alphaviruses with the flaviviruses, the Mut-5 motif can be speculated to be an important region in SINV infections of the midgut. From this, I hypothesized that the Mut-5 binding affinity to the cell could be affected by competition of exogenous Mut-5 amino acids. To test this hypothesis, a homologous MRE16 synthetic VR peptide that spanned the Mut-5 motif region, plus the 4 residues both up and down stream of Mut-5, was generated for use as an antagonist in virus binding and infection assays (see underlined SINV sequence in figure 3.8 on page 76; peptide sequence listed in table 3.1 on page 59). This VR peptide, called MRE-AVR, was first assayed for binding to MRE16 virus, cell culture supernatant and sonicated cell membrane material to determine if there was unique specificity for any proteins. For dot blot assays, the MRE-AVR peptide showed efficient binding to the supernatant of TE/Mut-5 infected tissue cultures cells (table 3.4.A on page 80) as well as uninfected cell culture supernatant proteins. However, specific binding to a TE/Mut-5 E2 protein was not observed in denaturing Western blotting. In addition a significant amount of nonspecific binding was seen in the cell supernatant Western blot . Niether dot or Western blotting of the cellular membrane fraction showed binding of the MRE-AVR peptide. The equivalent peptide in TE/5' 2J, called AR339-AVR, could not be dissolved in PBS or in a $\leq$1% DEPC/PBS solution, and therefore, was unusable in cell binding assays due to its toxic effect. The MRE-AVR peptide was also tested in a PRNT-assay for infection inhibition (Table 3.4A on page 80). Cells pre-incubated with MRE-AVR did not result in a significant reduction in TE/Mut-5 infection at peptide concentrations up to 250 μg/ml.

Rabbit IgG antibodies against MRE-AVR were generated and purified by antigen affinity column. This polyclonal antibody was tested for anti-TE/Mut-5 activity. Dot blot assays showed strong binding activity and denaturing Western blot analysis showed moderate binding for E2 protein; although virus specificity was not observed possibly indicating the presence of antibodies to the homologous regions flanking the VR (i.e. the peptides used to make these antibodies were 60% homologous in thier aa sequence and thus these antiboidies maybe able to cross react; see table 3.4.B on page 80). A rabbit anti-AR339-AVR antibody was also generated and showed similar results in the dot and Western blot assays as found for the MRE-AVR antibody. IFAs on SINV infected cells, using each of these 2 new antibodies as the primary antibody, were not successful even though a control anti-SINV E2 rabbit antibody (also derived by a injection of a synthetic peptide) was capable of detecting SINV infected cells by IFA. This is probably an indication of poor affinity of the VR antibodies for viral proteins. Not surprisingly, neither the MIR assays using the MRE-AVR nor TE-AVR antibodies showed a reduction in the midgut infection rate in *Ae. aegypti* mosquitoes (Table 3.4 below).

Table 3.4. Antagonists of SINV infection.

A) Peptide antagonists of SINV infection.

Peptide	Target	Bloting Assay TE/Mut-5 Dot	Bloting Assay TE/Mut-5 Western*	PRNT TE/Mut-5 Vero	PRNT TE/Mut-5 C636	MIR assay in Ae. aegypti (% of neat MID_{50}) MRE16	TE/5'2J	TE/Mut-5	TE/Mut-6	Mut5+6
AR339-AVR	AR339 E2 112-125	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]	nd[1]
MRE-AVR	MRE16 E2 112-125	+++	++	>250μg/ml	>250μg/ml	110.7	120.0	116.0	108.3	118.5
DEN2-AVR	DENV E 380-390	nd[2]	nd[2]	>250μg/ml	>250μg/ml	96.2	100.0	104.0	94.4	107.4

B) Antibody antagonists of SINV infection.

Antibody	Type	Target	Tissue culture IFA (with different fixation) Ac	1:3 Ac	4% Para	Neat	Bloting Assay TE/Mut-5 Dot	Western*	PRNT TE/Mut-5 Vero	C636	MIR in Ae. aegypti (% of neat MID_{50}) MRE16	TE/5'2J	TE/Mut-5	TE/Mut-6	Mut5+6
α-AR339/AVR	Rabbit IgG	AR339 E2 113-120	-	-	+	-	++++	+	<1:8	<1:8	98.6	92.0	100.0	102.8	96.3
α-MRE/AVR	Rabbit IgG	MRE16 E2 113-120	-	-	+	-	++++	++	<1:8	<1:8	103.4	92.0	108.0	94.4	100.0
α-SINV E2	Rabbit IgG	SINV E2 127-143	++++	++++	++++	++++	++++	++++	<1:8	<1:8	105.8	104.0	88.0	91.7	103.7
30.11a	Mouse IgG	SINV E1	++++	++++	++++	++++	++++	-	<1:8	<1:8	nd	nd	nd	nd	nd

[1]AVR339-AVR peptide precipitated in solution
[2] DEN2-AVR peptide was not biotinylated.
* Looking for target specific signal
nd, no data, assay not performed
Ac, Acetone

Discussion

The exact mechanism of alphavirus entry into susceptible cells is not yet known. In tissue cultured fibroblast cells, it is thought that the E2 glycoprotein interacts with a yet unidentified receptor(s) followed by membrane fusion via the E1 glycoprotein. Membrane fusion is thought to be mediated through an endocytotic pH-dependent pathway, although direct cell surface penetration of membranes has been proposed. As previously reviewed in Chapter I, a receptor(s) for mediated endocytosis in vertebrate cells has not been specifically identified. However, histocompatability antigens, laminin receptors, lectin molecules, and the widely expressed glycosaminoglycan heparan sulfate (HS) have all been proposed as receptor components. This information suggests that there are multiple receptors for SINV attachment, some of which are highly conserved throughout the vertebrates (Strauss, Wang et al. 1994).

For receptor mediated endocytosis in insect cells, Ludwig et. al. have identified a 32-kDa polypeptide on mosquito cells with affinity to Venezuelan equine encephalitis virus and to laminin (Ludwig, Kondig et al. 1996). Interestingly, Ludwig et al. determined that the 32-kDa polypeptide from C6/36 cells and the 67-kDa polypeptide from BHK cells had a structural relationship and that some monoclonal antibodies could cross-react with these two polypeptides. It is this type of receptor conservation that can also help explain the broad host range of SINVs.

In the case of alphavirus infection of mosquito midgut cells, the E2 protein sequence has been implicated as having the specific genetic determinants for viral infection (Woodward, Miller et al. 1991; Myles, Pierro et al. 2003; Pierro, Myles et al. 2003; Weaver, Anishchenko et al. 2004). The work here describes the use of infectious

clones as a tool to narrow the identity of the viral E2 genetic determinants of midgut infection in *Ae. aegypti* mosquitoes of an O/A genotype of SINV, MRE16. For this, an *in vivo Ae. aegypti* mosquito midgut infection assays were used to screen for an enhanced MIR of chimeric TE/5'2J clones having substituted MRE16 residues. It was observed that the chimeric construct TE/ME2a had a MIR higher than the parental TE/5'2J. The aa sequence of the MRE16 E2a portion, which contained the putative CRBD, was 81% identical to that of TE/5'2J. Mutational exchange of the heterologous MRE16-E2a sites into the TE/5'2J ic, via sPCR, resulted in TE/5'2J viruses with specific MRE16 residues that could be analyzed in a midgut infection assay. These heterologous MRE16 sites were added sequentially to TE/5'2J until a significantly higher MIR than that of the parental TE/5'2J was observed, as was the case of the TE/Mut1-2-3-4-5-6 virus that a. To further isolate the location of the genetic determinant of infection, a TE/5'2J mutant was generated with only the Mut-6 residues that was found to have an increased MIR at all infectious doses. Subsequently, the TE/Mut-5+6 virus (having both the Mut-5 and Mut-6 motifs in a TE/5'2J backbone) exhibited an MIR equivalent to TE/Mut1-2-3-4-5-6. Interestingly, the TE/Mut-5 virus exhibited an MIR higher than the TE/5'2J and also statistically equivalent to TE/Mut-5+6 virus. However, it is likely that the Mut-5 and Mut-6 residues work cumulative to effect the virus' MIR. A cumulative effect was also seen in the TR339 genetic determinants of midgut infection (chapter 2), although at different locations.

The locations of Mut-5 (E2 116-119) and Mut-6 (E2 95-96) motifs in SINV are different from the previously published alphavirus genetic determinants of midgut infection. In chapter 2, the genetic determinants of SINV strain TR339 (P/E genotype)

were found at E2 55 and E2 70, which are some linear distance from the MRE16 (O/A genotype) sites identified to influence the MIR. MRE16 does share the E2 55-Q residue with TR339 and it can be supposed to be another genetic determinant of MRE16 MIR, although its influence was not analyzed here. In addition, amino acids in VEEV at position E2-117 and one at E2-207 each were shown to assist in the enhancement of the MIR (Woodward, Miller et al. 1991; Brault, Powers et al. 2004). Moreover, the amino acid residues 170 to 220 of E2 comprise the CRBD and studies characterizing this domain predict that the CRBD is exposed on the viral surface (Stec, Waddell et al. 1986; Davis, Pence et al. 1987; Strauss, Stec et al. 1991; Smith, Cheng et al. 1995). This information leads to the observation that multiple locations occur in the N-terminal half of E2 of alphaviruses that can influence cell binding and midgut infections.

In terms of the Mut-6 motif (E2 95-96), no published information is available on the genetic determinants specific to midgut infections in this region. However, a mutation at E2 96 of SINV, replacing Tyr with His, resulted in a neutralizing antibody escape mutant and decreased virulence in neonatal mice (Pence, Davis et al. 1990). The crystal structure of the alphavirus E2 has not been determined but recent publications have given insight into its basic conformation (Zhang, Mukhopadhyay et al. 2002; Mukhopadhyay, Zhang et al. 2006). According to those reports, the E2 glycoprotein stands perpendicular to the viral surface in 80 homotrimer units. Each E2 monomer stands like an upside-down "J" with the C-terminus (equating to the head of the "J") attached to the viral membrane and the N-terminus of E2 (equating to the bending base of the "J") protruding up from the viral membrane and bending radially

away from the homotrimer center. Using the E2 structure proposed by Mukhopadhyay et al, the SINV Mut-6 motif appears to be positioned as part of the middle portion (at the bend of the upside-down "J") of the E2 glycoprotein structure, facing away from the viral surface, with each homotrimer forming a cell surface exposed pocket of these Mut-6 motifs (Mukhopadhyay, Zhang et al. 2006).

In the case of the Mut-5 location (E2 116-119), these residues are located in an alphavirus E2 region that has been shown to influence *in vitro* cell binding and *in vivo* infections. Specifically, Brault *et al.* identified a genetic determinant in a VEEV IE subtype at position E2-117 (Glu-Lys change; SINV position 117) that assisted in enhanced MIR in *Ae. taeniorhynchus* mosquitoes (Brault, Powers et al. 2004). Interestingly, a Lys-Glu change (the inverse of above) at VEEV position 116 resulted in a virus with increased HS binding (Bernard, Klimstra et al. 2000) and draws a parrellel to the hypothesis proposed in chapter 2 of this dissertation proposing that a lower viral HS affinity is correlated to a higher MIR in mosquitoes. At a nieghboring E2 site, a residue change at SINV position E2 114 (Ser to Arg) resulted in a virus with enhanced binding to HS (Klimstra, Ryman et al. 1998). This SINV E2 114 Arg residue was shown to increase binding and penetration into BHK cells, to alter reactivity with an anti-E2 monoclonal antibody, and to attenuate SINV in mice (Davis, Fuller et al. 1986). The VEEV vaccine strain TC-83 was found to be attenuated as a result of a mutation at E2 120 (Thr to Arg; SINV position E2 121) (Kinney, Chang et al. 1993). An attenuated RRV variant was found to have five E2 mutations, one of them being at position E2 119 (SINV E2 119) (Vrati, Fernon et al. 1988). In summary, the alphavirus region encompassing all of these mutations (i.e SINV E2 114-121), which includes

SINV Mut-5, appears to affect the viral infection potentials among a variety of alphaviruses, in both mammalian and mosquito cells, and implies functional conservation in the E2 between the hosts and vector. Again, using the basic structure proposed by Mukhopadhyay *et al.* and described above, the SINV Mut-5 motif also appears to be positioned as part of the middle portion (at the bend of the upside-down "J" structure) of the E2 glycoprotein structure, facing away from the viral surface, with each homotrimer forming a cell surface exposed pocket of these Mut-5 and Mut-6 motifs (Mukhopadhyay, Zhang et al. 2006).

A significant observation concerning the location of the Mut-5 and Mut-6 aa residues along the linear E2 sequence was that they were separated by a PPGDS motif, which appears to be a highly conserved sequence in the envelope glycoprotien ectodomains of the alphavirus and flavivirus genera (PPF/.GDS in flaviviruses; figure 3.8 on page 75). The PPF/.GDS sequence, called 'CR', is located at SINV E2 position 106-110 and DENV-2 E position 371-376, respectively. The CR has not been identified in other arboviruses and this is the first known report of its existence. According to the alignment in figure 3.8, the CR is upstream of the SINV Mut-5 motif. This mut-5 motif aligns spatially with the flavivirus 'FG loop' structure, a region exposed on the distal face of domain III of the dengue virus (DENV) E glycoprotein and thought to be involved in receptor binding. The SINV Mut-5 sequence also aligns with the RGD sequence of the yellow fever virus (YFV), Japanese encephalitis virus (JEV) and Murray Valley encephalitis virus (MVEV). The RGD tripeptide is a known participant in the interaction of a number of ligands with cell receptors from the integrin superfamily (D'Souza, Ginsberg et al. 1991). Residues of the RGD motif have

previously been shown to influence YFV envelope structure as well as replication efficiency (van der Most, Corver et al. 1999). In one instance, a revertant mutation of the RGD motif was seen in the YFV-17D vaccine strain that resulted in a neuroadapted virus (Hahn, Dalrymple et al. 1987; Chambers and Nickells 2001; Nickells and Chambers 2003). Mutation of a RGD site also attenuated the MVEV and impacted cell tropism and virus entry (Lee and Lobigs 2000; Hurrelbrink and McMinn 2001). In addition, the RGD motifs from other enveloped (e.g. Epstein-Barr and blue tongue viruses) and non-enveloped viruses (e. g. foot-and–mouth disease virus [FMDV]) have been shown to participate in virus ligand binding (Fox, Parry et al. 1989; Tan, Nason et al. 2001; Tugizov, Berline et al. 2003; Storey, Theron et al. 2007). It is interesting to note that in FMDV, a number of residues that flank the RGD motif were found to be influential in virus infections (Leippert, Beck et al. 1997). Mutations that flank the flavivirus RGD region are also known to affect virus activity, including the epitopes for neutralizing antibodies to DENV, JEV and TBE (Holzmann, Heinz et al. 1990; Roehrig, Risi et al. 1994; Hiramatsu, Tadano et al. 1996; Wu, Lian et al. 1997; Serafin and Aaskov 2001). A neighboring substitution of Asp to His at residue DENV-2 E 390 was suggested to affect a functionally important structural element that could be a determinant of DENV-2 neurovirulence (Sanchez and Ruiz 1996). Moreover, a 10-mer DENV-2 synthetic peptide sequence that overlapped the loosly-termed flavivirus RGD region (equivalent to DENV-2 E position 380-389, although "RGD" is not found in DENV) inhibited binding of a DENV-2 domain III-envelope protein to mosquito but not mammalian cells, indicating that this region has more specificity for insect cells. The alphavirus Mut-5 and flavivirus RGD regions align spatially but their sequences

(plus flanking sequences) are not well conserved (figure 3.8) and even encompass a deletion region that corresponds soley to tick-borne flaviviruses (Holbrook, Shope et al. 2004). Therefore, this region (plus some of the flanking sequences) may be hypothesized to constitute an arbovirus variable region (AVR) involved in mosquito infections and is termed VR-I (see figures 3.8 on page 75 and 3.9 on page 90).

Also according to the alignment in figure 3.8, the CR is positioned proximally downstream of the SINV Mut-6 motif. This Mut-6 aligned region (i.e. SINV E2 92-97 and DENV-2 E 361-365) also had a low level of residue conservation, and the flavivirus panel indicated a deletion variability not seen in the alphavirus panel. However, as is the case for the VR-I, some evidence exists for the functionality of this region. Specifically, according to the alignment in figure 3.8 (page 75), the SINV Mut-6 motif aligns spatially with the flaviviruses '$D^{+}E$ loop' structure, a region also exposed on the face of domain III of the E glycoprotein (figure 3.9 on page 9). Moreover, as mentioned previously, a mutation at SINV E2 96 resulted in a neutralizing antibody escape mutant, and a decreased virulence in neonatal mice (Pence, Davis et al. 1990). A YFV with mutations at E 360 and 362 (DENV-2 E position 363 and 365) resulted in a virus with defective cell penetration, poor cell-to-cell spread, small plaque size and reduced growth efficiency (Vlaycheva, Nickells et al. 2005). A study by Jackson and Phillpotts suggested that a region encompassing YFV E 358-365 (DENV-2 E359-367) was involved in a conformational epitope (Jackson and Phillpotts 1997; Vlaycheva, Nickells et al. 2005). However, Roehrig *et al.* found that peptides spanning DENV-2 E 352-368 elicited only low levels of neutralizing antibody in mice (Roehrig, Risi et al. 1994). Due to the structural and functional conservation (but having a variable

sequence) of this Mut-6 region, it constitutes another VR (encompassing SINV E2 92-96 and DENV-2 E 361-365) and is termed the VR-II (figures 3.8 and 3.9).

The evidence presented here points to two functional viral glycoprotein domains, VR-I and –II, found in both the *Alphavirus* and *Flavivirus* genera, involved in mosquito and mammalian cell infections. These two regions are predicted to be loop domains and are separated by the CR, which is also a loop structure flanked by two β-sheets. The function of this newly identified CR motif is unknown. However, the sequence of CR (i.e. PPF/.GDS) is dominated by two prolines, which implies more of a passive structural role rather than an active functional one. Proline is often found at very tight turns in protein structures (i.e. where the polypeptide chain must change direction) (M.J. Betts 2003). It also functions to introduce kinks into alpha helices and often acts as a helical terminator. This is because prolines are the only amino (actually imino; NH2+ vs. NH3+ group) acid where the side chain is connected to the protein backbone twice, forming a five-membered nitrogen-containing ring. As such, proline is known to be rigid and unable to occupy the conformations easily adopted by other amino acids (Betts 2003). Prolines are also thought to position distant reactive sites for optimal accessibility within a protein (Delos, Gilbert et al. 2000). However, the proline-based conformational dependence for each of the viruses may vary slightly since the double proline sequence is not strictly conserved in alphaviruses (e.g. VEEV, BFV, CHIK; figure 3.8 on page 75).

Another component of the CR sequence is the partial use of Phenylalanine (F), which is a hydrophobic aromatic amino acid. It is persistently absent in alphaviruses as well as in tick-borne flaviviruses, but it is present in the mosquito-borne

flaviviruses and the reason for this is unclear. The F side chain is somewhat non-reactive and is rarely directly involved in protein function (Betts 2003). However, it can play a role in substrate recognition such as binding/recognition of hydrophobic ligands such as lipids (Betts 2003). The next residue in the CR sequence is a glycine (G) which is the only aa to contain a hydrogen as its side chain (rather than a carbon). This gives G more conformational flexibility and it is often found along tight turns in protein structures (Betts 2003). The G residue is strictly conserved among all of the alphaviruses and flaviviruses shown. The next CR residue in line is aspartic acid (D), which is a negatively charged, polar amino acid and quite frequently involved in protein active sites. It has a shorter side-chain than the very similar glutamate (E), a residue it exchanges with in the CR sequence. Finally is a serine (S), a slightly polar amino acid that can reside both within the interior of a protein or on the surface. Its small size helps in tight turns on the protein surface, where it is possible for the serine's side-chain hydroxyl oxygen to form a hydrogen bond with the protein backbone, effectively mimicking proline (Betts 2003). A serine-to-threonine change is not uncommon for this residue as is seen in the CR (Betts 2003). The serine-to-asparagine change in the CR alignment is noteworthy in that it is conserved in the tick-bone flaviviruses (much like the residues in the VR-I site). The above descriptions of residue characteristics for the CR appear to predict a structurally important turn. In fact, the flavivirus EF loop, containing the CR, is located at the base of the E glycoprotein's domain III (analyzed on the DENV-2 E glycoprotein (Zhang, Zhang et al. 2004)), turns the E glycoprotein strand away from the viral surface and then project the downstream FG loop outward of domain III (figure 3.9; page 92). This dramatic turn away from

the viral membrane in the flaviviruses, as well the close proximity of the of the EF loop to the viral membrane, may be an indication of a structural relationship of the EF loop with viral envelope surface. Figure 3.9 also illustrates the location of the VR-I and –II, which appear to protrude from the end of domain III with apparent easy access to cellular receptors. As mentioned above, the crystal structure of the E2 glycoprotein from an alphavirus has not been determined; however the conservation in residue characteristics between the E and E2 glycoproteins of the flaviviruses and alphaviruses in this region are an indicator that the structural format CR, VR-I and –II may be similar for that of the alphaviruses.

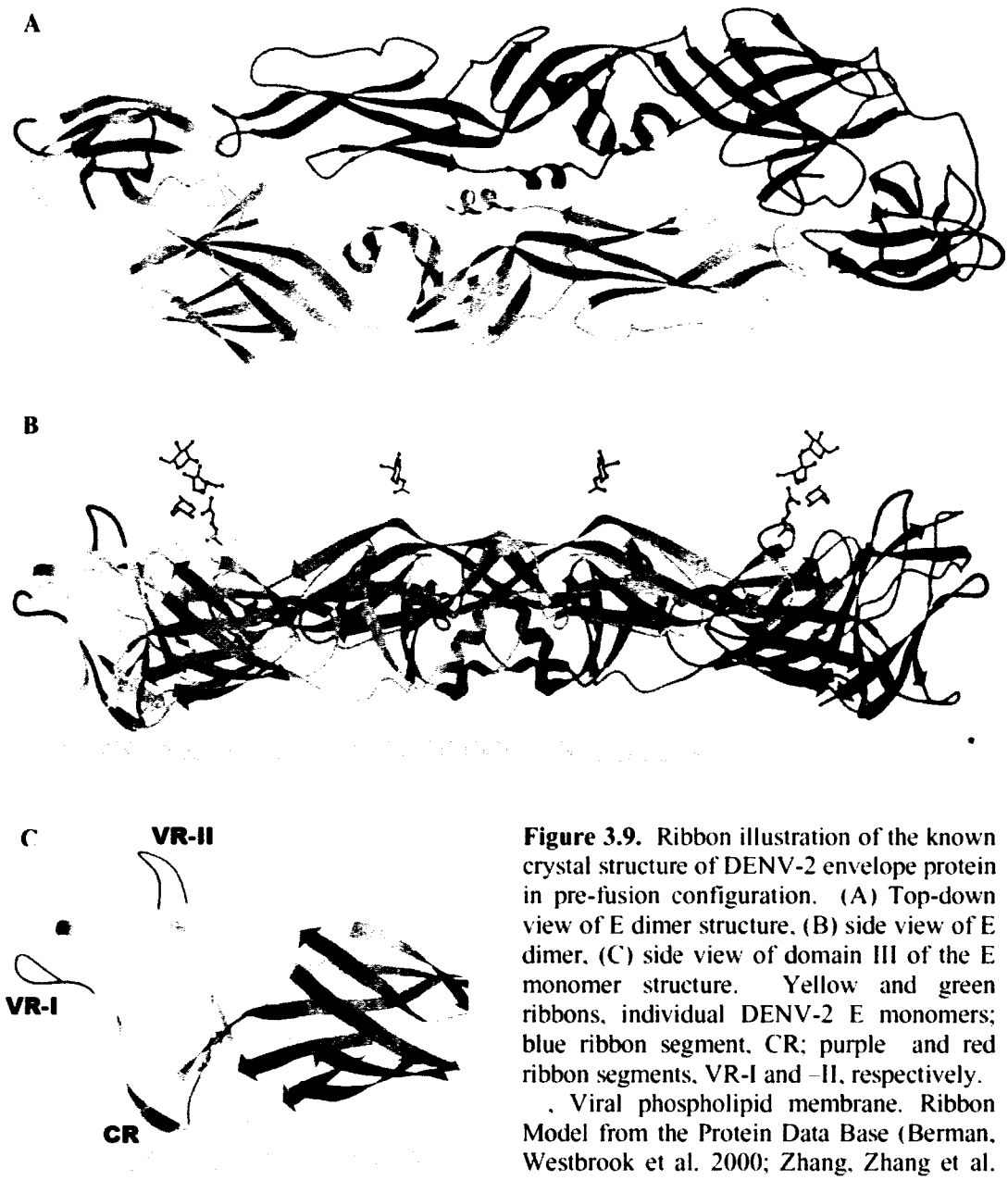


Figure 3.9. Ribbon illustration of the known crystal structure of DENV-2 envelope protein in pre-fusion configuration. (A) Top-down view of E dimer structure, (B) side view of E dimer, (C) side view of domain III of the E monomer structure. Yellow and green ribbons, individual DENV-2 E monomers; blue ribbon segment, CR; purple and red ribbon segments, VR-I and -II, respectively. , Viral phospholipid membrane. Ribbon Model from the Protein Data Base (Berman, Westbrook et al. 2000; Zhang, Zhang et al. 2004)

Unfortunately, there is very little published data on the function of CR (SINV E2 106-110, DEN2 E 371-376). Passaging of a chimeric SINV/RRV chimeric viral stock has resulted in an S-to-N change at position E2 110 which was hypothesized to be an adaptive change (Kim, Strauss et al. 2000). Navaratnarajah and Kuhn have recently

shown that insertion of a transposon linker (15-16 aa residues) into SINV at position E2 105 (just upstream of the CR) resulted in the production of infectious virus while insertions at E2 107 and 109 (in the CR) were lethal to SINV (Navaratnarajah and Kuhn 2007). Those authors also observed that various insertions of the transposon linker into the E2 107–119 region did not affect E2 expression, but did impair proper E2 transport to the plasma membrane. Published information on the flavivirus CR is limited to sequence comparisons of viral isolates. Such reports document the changes of CR residues but no functional observations have been definitively correlated (Lanciotti, Gubler et al. 1997). One noteworthy study was on DENV-3 isolates from field mosquito and human sources. That report showed a strict lack of mutational activity across the CR, VR-I and VR-II (i.e. no mutations in a 35 aa span) from DENV-3 mosquito isolates while human isolates had mutations in these regions (7 mutations in the 35 aa region). This may be an indicator of a mosquito-cell specificity for these regions (Lin, Hsieh et al. 2004).

An analysis of the data generated on genetic determinants of SINV in chapters 2 and 3 of this dissertation reveals that the amino acid sites involved in midgut infections are variable and are dispersed across the N-terminal half of E2. They are also found in loop structures interspersed by beta-sheets. Interestingly, in some group I coronaviruses (i.e human, feline, canine, etc.), the viral spike glycoprotein to binds to its recptor, Aminopeptidase N, at binding regions within a loop structures that have a highly variable amino acid sequence. Morover the binding affinity of the receptor's loop region can be affected by a single amino acid residue and has been shown to be a determinant of host range (Tussell, Schittone *et al* 2007). It could be hypothesized that

the multiple protruding loop structures on the SINV glycoprotein are working in conjunction with each other to enhance the overall affinity of the virus to cellular ligands. The data here describes a potential multi-recognition mechanism for midgut cell binding whereby the overall viral binding affinity to a cell is strengthened as each of the viral protruding loop structures (e.g VR-I and VR-II) are individually attracted to cellular ligands. Hence, the overall viral binding affinity is strengthened as a collective of protruding loop structures, much like a Velcro material analogy (i.e. the individual loops of the material have weak binding but a sheet of Velcro material binds well). This mechanism may also help explain the broad cell-host range of many arboviruses since the viral envelope proteins are belived to bind to multiple cell receptors.

Much like the previous chapter, the cell type used in virus propagation influenced the MIR. In order to achieve an equivalent MIR, mosquito cell generated virus required $\geq$1 $\log_{10}$ PFU/ml as compared to the mammalian cell derived viruses. As mentioned, these differences may be the result of the fact that insect cells are known to be populated by sterols, as opposed to cholesterols in mammalian cells, of the result of the divergent late stage processing of N-glycans in insect cells (Altmann, Staudacher et al. 1999; Marchal, Jarvis et al. 2001; Tomiya, Betenbaugh et al. 2003; Tomiya, Narang et al. 2004). The differences in N-glycan structure between mammalian cells and insect cells are believed to affect biological activity (Prenner, Mach et al. 1992; Takahashi, Tsuda et al. 2004; Tomiya, Narang et al. 2004). Such differences in biological activity have been previously observed for arboviruses propagated in mammalian vs. insect cell lines and they imply that virus maturation differs significantly between these arboviral host cell types (Lee and Brown 1994; Li, Liao et al. 1999; West, Hernandez et al.

2006). More specifically, when compared to mammalian cell propagation, the use of mosquito-derived SINV and West Nile viruses that had high-manose glycans on the envelope glycoproteins was shown to enhance viral infection of mammalian dendridic cells via interactions with the dendritic cell-specific mannose binding lectins DC-SIGN (Klimstra, Nangle et al. 2003; Davis, Nguyen et al. 2006). In addition, Shabman et al. showed that mosquito-derived Ross River virus, as opposed to mammalian derived virus, had an enhanced ability to infect murine dendritic cells as well as avoid a type I interferon response. This infection enhancement, i.e. mammalian-derived virus more efficiently infecting mosquito midgut cells (shown here) and mosquito-derived virus more efficiently infecting mammalian cells (previously published data), could constitute an optimized mechanism for efficient virus transmission between vertebrates and invertebrates. Since this effect is reciprocal between mammal and mosquito cells, it might to be an evolutionarily conserved phenotype. A model for efficient alphavirus infection of cells seems to require the use of an optimally processed virus structure (e.g. proper glycosylation processing) as well as the specific virus encoded determinants (e.g. VR-I or –II). These two features may or may not work in conjunction with each other and may even require a stepwise processing event (e.g. protease activity against a glycosylated structure to allow for the binding of VR-I and -II) for efficient cell attachment and penetration.

Chapter 4

Infectious clone construction of dengue virus type 2, strain Jamaican 1409

Abstract.

This chapter describes the construction of a full-length infectious cDNA clone (ic) from the genome of the DENV-2 Jamaica83 1409 strain, p1409ic, using a bacterial artificialt chromosome (BAC) plasmid system. Infectious virus was generated and characterized for growth in cell culture and for infection in *Ae. aegypti* mosquitoes. During construction, an isoleucine to methionine (Ile-Met) change at position 6 was found in the envelope glycoprotein sequence between low and high passage DENV-2 1409 strains. *In vitro* transcribed genomic RNA of the 1409ic with E6-Ile produced infectious virions following electroporation in mosquito cells but not mammalian cells, while 1409ic RNA with an E6-Met mutation produce virus in both cell types. Moreover, DENV-2 1409 with the E6-Ile residue produced syncytia in C6/36 cell culture, while viruses with E6-Met did not. However, *in vitro* cell culture growth curves and *in vivo* mosquito infection rates revealed that all the analyzed DENV-2 strains did not differ from each other.

Introduction

The four DENV serotypes are mosquito-borne members of the *Flavivirus* genus (family *Flaviviridae*) and are endemic in the tropical and subtropical regions of the world. Transmission to humans is by peridomestic *Aedes spp.* mosquitoes, primarily *Ae. aegypti*. Manifestations of a DENV infection range from subclinical to a self-limited fever and rash (dengue fever [DF]) to a severe and sometimes deadly illness characterized by capillary leakage, thrombocytopenia and hypovolemic shock (dengue hemorrhagic fever [DHF] and dengue shock syndrome[DSS]). These viruses are

estimated each year to cause 100 million cases of DF, 500,000 cases of DHF/DSS and 25,000 deaths, and 40% of the world population (i.e. 2.5 billion people) is at risk for infection (Monath 1994).

Epidemiological studies have identified dengue virus type 2 (DENV-2) as an important dengue virus serotype with large outbreaks occurring frequently (Diaz, Black et al. 2006). The DENV-2 genome is a single stranded positive sense RNA molecule of approximately 10,700 nt that contains a type I 5' cap structure but lacks a 3' poly-A tail. The genome is directly translated as a single polyprotein containing the three structural genes (NH_2-Capsid-prM-Envelope) fused to the seven nonstructural genes (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH) that is co- and post-translationally processed (Chambers, Hahn et al. 1990).

Molecular analysis of the envelope gene from DENV-2 field isolates originally identified five major DENV-2 genotypes: Caribbean (American), South Pacific, Middle East and Indonesia, Vietnam and Thailand (Asian), and West Africa (Rico-Hesse 1990; Lewis, Chang et al. 1993; Rico-Hesse, Harrison et al. 1997; Wang, Ni et al. 2000). Additional analysis of more DENV-2 samples has revealed a total of six DENV-2 genotypes: American, American/Asian, Asian I, Asian II, Sylvatic, and Cosmopolitan (Twiddy, Farrar et al. 2002). The new DENV-2 American/Asian genotype, which includes the DENV-2 Jamaican 1409 isolate (1409), is an Asian genotype DENV-2 introduced into the Americas, with subsequent proliferation and circulation of the virus in this region (Uzcategui, Camacho et al. 2001; Twiddy, Farrar et al. 2002; Lorono-Pino, Farfan-Ale et al. 2004). Introduction of viruses from this genotype, of which 1409 is speculated to be an early isolate, was linked to a significant increase in the

number of DHF cases across Latin America (Uzcategui, Camacho et al. 2001). DENV-2 from the Asian and American/Asian genotypes more readily infect North American *Ae. aegypti* mosquitoes than do the DENV-2 American genotypes (Armstrong and Rico-Hesse 2001). The American/Asian genotype viruses are also believed to have supplanted the American genotype in a many areas, leaving a more virulent and readily transmitted DENV-2 in its place.

The mechanisms and determinants for DENV-2 infection of the mosquito midgut are not well known. The 1409 virus has been shown to have significant differences in the MIR from geographically distinct strains of *Ae. aegypti* (Bennett, Olson et al. 2002). It is thought that bloodmeal digestion, and possibly 1409 proteolytic processing, are mediated by midgut trypsins that influence the rate of DENV-2 infection in *Ae. aegypti* mosquitoes (Molina-Cruz, Gupta et al. 2005). Bosio et al have identified two quantitative trait loci for a DENV-2 1409 midgut infection barrier that were detected on chromosomes II and III of the *Ae. aegypti* genome (Bosio, Beaty et al. 1998; Bosio, Fulton et al. 2000). Virogenesis of DEN2 1409 in the midguts of *Ae. aegypti* determined that the 1409 infection starts as an expanding focus of infection, and the infection then begins to resolve itself starting at 10 dpi (Salazar, Richardson et al. 2007). Anderson and Rico-Hesse determined that a DENV-2 from South East Asian replicated more efficiently in the midgut of *Ae. aegypti* and increased the vectorial capacity (Anderson and Rico-Hesse 2006). Currently no information is available on the specific viral genetic determinants of DENV midgut infection, however, YFV was shown to utilize elements of the nonstructural genes for midgut escape. The DENV-2 envelope amino acid position 390 has been identified as a

potential virulence determinant in mammalian cells and maybe speculated to play a role in mosquito infections as well (Sanchez and Ruiz 1996; Pryor, Carr et al. 2001).

As seen by the limited amount of information on the viral genetic determinants of DENV-2 infection of mosquitoes, more investigations are needed to identify those genetic elements that condition infection of the mosquito, particularly the midgut. Such information can be used to combat this endemic mosquito-borne disease of the tropical and subtropical regions of the world. A powerful tool for such analysis is the application of a DENV-2 full-length cDNA infectious clone (ic), which can be used to modify the viral genome at specific targeted regions. There have been a few successful constructs of DENV-2 ics, however, none of those represent an American/Asian genotype (Kapoor, Zhang et al. 1995; Kinney, Butrapet et al. 1997; Blaney, Hanson et al. 2004). Moreover, the development of full-length ics of flaviviruses has been know to be a difficult process due to an innate toxicity of the cDNA sequence of the viral genome, which is often an inhibitory step to the efficient use of flavivirus ics (Rice, Grakoui et al. 1989; Sumiyoshi, Hoke et al. 1992; Kapoor, Zhang et al. 1995; Mandl, Ecker et al. 1997; Yamshchikov, Wengler et al. 2001). The central hypothesis of this chapter was that a full-length cDNA ic of the DENV-2 American/Asian genotype, strain 1409, can be developed and manipulated *in vitro* for the production of modified, infectious 1409 virus for use in tissue culture and mosquito susceptibility infection assays.

Materials and Methods

Cells and Virus. The African green monkey kidney Vero (ATCC CCL-81

P140-170), rhesus monkey kidney LLC-MK2 (ATCC CCL-7) and *Ae. albopictus* C6/36 (ATCC CRL-1660 P130-150) were individually grown in minimal essential medium eagle (MEM) supplemented with 8% fetal bovine serum (heat inactivated), 2mM L-glutamine, 1x non-essential amino acids for MEM, 100 U/ml penicillin, and 100 ug/ml streptomycin. Cell culture was performed at 5% atmospheric CO_2 in a 37 °C (Vero and LLC-MK2) or 28 °C (C6/36) chamber. The 1409 virus was originally isolated in 1983 from a DF human patient in Jamaica. The virus was previously plaque purified on LLC-MK2 cells, stocks were prepared in C6/36 cells, and the genome sequenced (Deubel, Kinney et al. 1986; Deubel, Kinney et al. 1988). For this study, both a low passage (C6/36-passage 2) 1409 virus (1409lp), obtained from the Centers for Disease Control and Prevention (Fort Collins, CO, USA [CDC]), and high passage 1409 virus (1409hp), which has been routinely passaged (>15 passages) in C6/36 cells, were used (Bennett, Olson et al. 2002).

Genome sequencing and infectious clone (ic) construction. Five cDNA fragments (f1-f5) representing the entire 1409hp genome were generated by RT/PCR (Titan RT/PCR kit, Roche Biochemicals, Indianapolis, IN) with primers homologous to the published sequence. Each of the fragments was inserted into a modified pBR322 plasmid (Kinney, Butrapet et al. 1997), and the five plasmids (p1-p5, figure 4.1 on page 106) were transfected by electroporation into competent *Escherichia coli* XL1-Blue cells (Strategene, CA). Three individual bacterial colonies for each cloned fragment were cultured, plasmids were isolated, sequenced, and the data aligned against previously published DENV-2 1409 (1409pub) data. A significant nucleotide change in the genome between 1409hp and the 1409pub sequence was identified as a

nucleotide substitution that occurred at the same position in all three colonies. In no cases were variations found in only two of three colonies. Nucleotide substitutions that occurred in only one of three colonies are not reported here.

The strategy for ic construction was a simple 'donor-fragment ligation into a recipient-plasmid' following a specific sequential pattern, which is laid out in figure 4.1 (page 106), using standard molecular biology cloning techniques and carbenecillin for bacteria colony selection. For efficient sub-cloning and full-length clone assembly, appropriate restriction endonuclease (RENZ) sites were either engineered in the PCR primers (external of the DENV-2 1409 sequence) or native 1409 RENZ sites were used during the ic's construction. Only one intentional RENZ site was added to the 1409 ic; an intentional silent nucleotide substitution of t/6651/c in the 1409ic genome, which was generated by PCR primer engineering, to produce an N*he*I RENZ site used in full-length clone construction. Additionally, unique PCR primers attached the T7 RNA polymerase recognition sequence and the linearization S*ph*I restriction site at the 5' and 3' ends of the 1409ic, respectively, each of which are required for the A-cap analog dependent *in vitro* transcription of the clone (table 4.1 on page 104). The plasmid pBeloBac11 cloning vector was obtained from New England Biolabs, Beverly, MA. Chimeric p1409/16681 required the amplification of the f4-5 fragment from DENV-2 strain 16681 (using the same 1409 primers; figure 4.1) followed by its donation into the 1409 p1-2-3 recipient plasmid. Of note is that the p1409/16682 cDNA full-length clone is in the pBr322 backbone while the pBAC1409 and pBAC1409-I/E6/M clones are in the pBeloBac11 vector.

Construction of I/E6/M in DENV-2 ics. Site directed mutagenesis of pBAC1-

2-3 inserted a guanosine at position 954, which translates into a Met at E6, and a full-length clone was then constructed (pBAC1409-I/E6/M) as described above. The sPCR reaction used the the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla,) and the following optimized conditions: 15ng DNA template, 5.0 µl 10x buffer, 1.25 µl 10mM dNTPs, 125ng base primer, 125ng complement primer, 2.0 µl Pfu turbo® DNA polymerase, and sterile, deionized water up to 50.0 µl. The thermocycling parameters for sPCR were optimized at: one cycle 95°C for 30s, 18 cycles of 95°C for 30s, 54°C for 1min, 68 °C for 14 min. Insertion of the point mutation was verified by sequence analysis of the plasmid DNA as well as the virus derived RNA genome via RT/PCR.

Generation and characterization of DENV-2 particles. The generation of infectious viral particles from the cDNA of pBAC1409 and pBAC1409-I/E6/M clones was similar to previously described protocols (Kinney, Butrapet et al. 1997; Pierro, Myles et al. 2003; Myles, Pierro et al. 2004). Briefly, the pBAC1409ic and pBAC1409-I/E6/M were linearized with S*ph*I and full-length RNA viral genomes were transcribed from an T7 promotor in the presence of m^7-GpppA cap analog according to the T7 MAXIscript® *In Vitro* Transcription Kit (Ambion, Inc., Austin TX) protocols. Approximately 0.5 µg of RNA was electroporated into $5.0x10^6$ BHK, Vero (BTX ECM630; 450V, 1200 ohms, 150uF) or C6/36 cells (250V, 25ohms, 550uF) and 10 days later the cells were analyzed for the presence of DENV-2 E antigen by indirect fluorescent assay (IFA). For IFA, DENV-2 infected tissue culture cells were first fixed in acetone. The IFA reaction was performed using anti-DENV E (4G2) as the primary antibody (1:200) and biotinylated sheep anti-mouse antibody (1:200; Amersham Corp., Arlington Heights, IL) as the secondary antibody. The E protein was detected using a

fluorescein isothiocyanate-conjugated streptavidin detector (1:200; Amersham). Mosquito body tissue and midguts were detected for DENV-2 infections by IFA using the same antibodies above and as previously described (Myles, Pierro et al. 2003). First-in-cell generated virus (i.e. virus obtained by using RNA from the infections clone) was termed "P-0" and virus passaged once was called "P-1". For verification of the sequence of the viral genomes, RNA from P1 virus was isolated, the genomes gene fully sequenced and no adaptive mutations were identified. Plaque titrations of DENV-2 were performed by infecting confluent monolayers of LLC-MK2 cells followed by 8 incubation days as described previously (Miller and Mitchell 1986). Plaque sizes of P-0 were analyzed on Vero cells 10 days post infection. Growth curve analysis was performed using confluent monolayer of cells infected in triplicate with P-0 virus at a multiplicity of infection (MOI) of 0.01 in 10% FBS/MEM.

Mosquitoes and oral infections. *Ae. aegypti* strain Rexville D (RexD; Rexville D, Puerto Rico) use and the bloodmeal delivery mechanism was similar to as previously described (Myles, Pierro et al. 2004). In summary, the propagation of virus used in bloodmeals started with confluent monolayers of C6/36 cells that were infected with P-0 virus at a MOI of approximately 0.01 in 8% FBS/MEM and cultured in CO_2 incubators for 14 days at 28 °C (C6/36). After incubation, freshly harvested P-1 cell culture supernatant (i.e. non-frozen) containing infectious virions was mixed 1:2 with 0.8 ml of defibrinated sheep blood (Colorado Serum Co, Boulder, CO). This infectious bloodmeal mixture was warmed to 37°C and placed in a water-jacket heated (37 °C) glass membrane feeder. Mosquitoes were allowed to probe and feed through a stretched sheet of parafilm for no more than 30 min. Fully engorged mosquitoes were

collected and maintained in the insectary with ample food and water until assayed. The viral bloodmeal was frozen and later quantified by plaque titration and the titer was considered acceptable at +/-0.3 $\log_{10}$PFU/ml of the predicted viral titer.

Results

Genome sequencing. Thirteen significant nucleotide differences (either causing a coding change or in the untranslated regions) between 1409hp and 1409pub were detected (table 4.1 on page 104). Genomic 1409lp was then sequenced at these specific positions to corroborate the published sequence and seven nucleotide variations between the 1409pub sequence and the 1409lp were subsequently identified: 3934-35, 7086-87, 8241-42 and 10089 (table 4.1 on page 104). Nucleotide substitutions specific to our 1409hp laboratory strain and the 1409lp strain occurred at six positions: 954, 1101, 4107, 4596, 8562 and 10403. This resulted in only one amino acid change, located at envelope protein position 6 (E6); an Ile to Met, and one UTR change, located a 3'UTR-143 (genome position 10,403); a thymine to guanine, between 1409lp and 1409hp (table 4.1). Since goal of this chapter was to produce an ic representing the early 1409 isolate these two 1409lp sequence sites were included in our ic. Thus, the 1409ic has an Ile at E6 and a thymine at 3'UTR-143.

Table 4.1 Nucleotide and amino acid variation among DENV-2 genomes.

Genome Nucleotide Positon	Nucleotide DENV-2 1409 pub*	lp	hp	ic	Amino Acid DENV-2 1409 pub	lp	hp	ic	Amino Acid Position
954	A	A	G	A	I	I	M	I	E-6
1101	T	T	C	C	T	T	T	T	E-68
3934-35	**CT**	TC	TC	TC	**L**	S	S	S	**NS1-504**
4107	C	C	T	T	T	T	T	T	NS1-562
4596	C	C	T	T	I	I	I	I	NS3-25
6651	T	T	T	C†	S	S	S	S	NS4A-92
7086-87	**CC**	GG	GG	GG	**IH**	MD	MD	MD	**NS4B-259/260**
8241-42	**AT**	TA	TA	TA	**AS**	AT	AT	AT	**NS5-223-224**
8562	C	C	T	T	P	P	P	P	NS5-331
9792	C	C	C	T†	S	S	S	S	NS5-745
10089	**A**	G	G	G	G	G	G	G	NS5-840
10403	T	T	G	T	-	-	-	-	-

* pub, hp, lp and ic represent the DEN2 published, high passage, low passage and infectious clone residues, resp.
Bold type represents nucleotide or amino acid unique to the previously published sequence.
† Unique sequence identifier for DEN2 1409ic. ´ 3' UTR region.

IC construction. Subclones p1-2-3 (nucleotides 1-6,650) and p4-5 (6,651 to 10,724) (figure 4.1 on page 106) in the pBr322 vector were first constructed without interruption. Next f4-5 (extracted from p4-5) was to be ligated to p1-2-3 to generate p1-2-3-4-5 (i.e. p1409ic). Unfortunately, a pBR322 plasmid containing the full-length 1409 cDNA was never recovered from electroporated (BTX ECM 630; 2.5kV, 25μF and 200 ohms) XL1-Blue *E. coli.* cells. Attempts to modify growth temperature, medium type, vector construction and *E. coli* cell type all failed to yield full-length p1409ic. Transformants containing either p1-2-3 or p4-5 produced a small colony size in *E. coli* host cells on YT plates, indicating potential stress to the bacterial host. The inability of *E. coli* cells to stably accept full-length infectious clone plasmids of other

flaviviruses has been previously observed (Rice, Grakoui et al. 1989; Sumiyoshi, Hoke et al. 1992; Kapoor, Zhang et al. 1995; Mandl, Ecker et al. 1997; Yamshchikov, Wengler et al. 2001). A full-length infectious clone of the DENV-2 strain 16681 was successfully constructed previously using the pBr322 plasmid and no such lethal toxicity was observed. To determine the generalized location of the 1409 cDNA toxicity, the construction of a full-length chimeric DENV-2 1409/16681 clone was successfully generated in the pBR322 vector. This chimeric clone, p1409/16681, had a 1409 nt sequence from position 1 to 6,650 and a 16681 sequence from 6651 to the end of the genome. This indicates a cDNA associated toxicity in the 3' end of the cDNA from the 1409 genome. To minimize the toxicity associated with the 1409 cDNA, the plasmid vector was changed from pBR322 (~20 copies/cell) to the single copy/cell pBeloBac11 vector (New England Biolabs, Beverly, MA) for construction of the pBAC1-2-3 subclone [via f1-2-3's 5'-A*ge*I-site which was blunted by mung bean nuclease (figure 1B) prior to ligation to pBeloBac's S*fo*I site and via inter-ligated S*ph*I sites of f1-2-3 and vector]. The f4-5 was then successfully inserted into the pBAC1-2-3 plasmid to create the full-length pBAC1409ic (figure 4.1 on page 106).

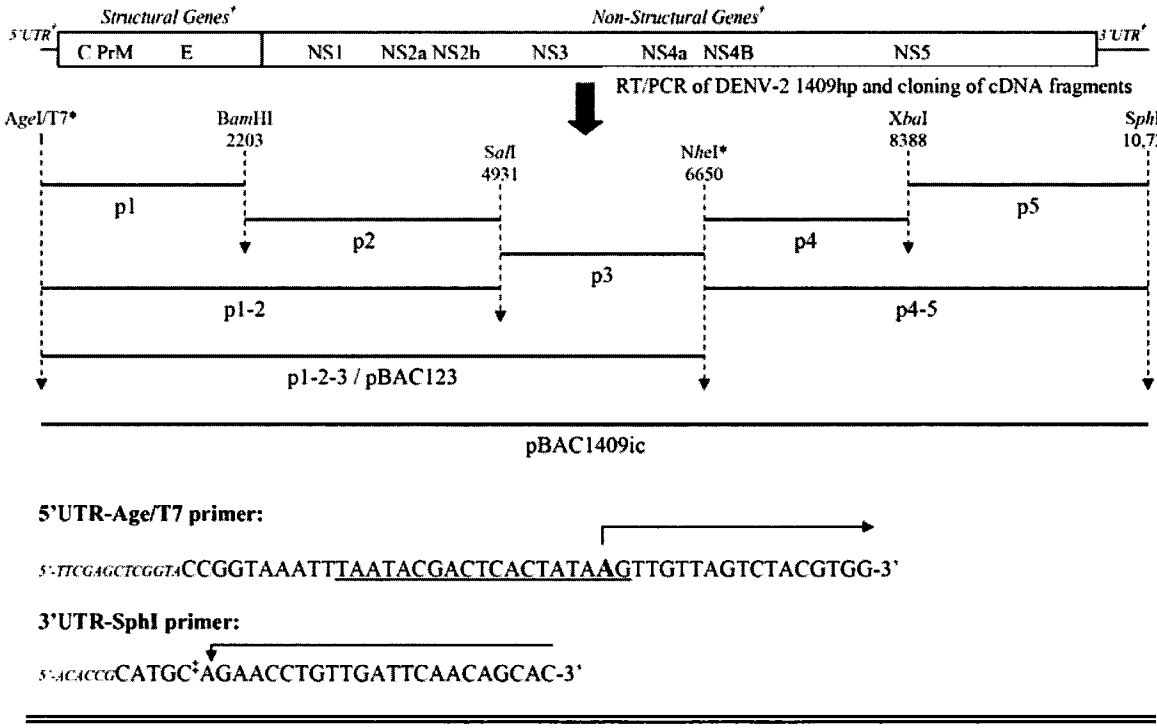


Figure 4.1. Assembly of infectious cDNA clone. Construction strategy with unique RT/PCR primers for DENV-2 1409ic. Restriction enzymes and nucleotide (nt) positions used for cloning are indicated. † Not to scale, * engineered addition, small letters in primers indicate restriction enzyme-cleaved nt lost during construction, underline T7 promoter sequence, **bold** in primer is first T7 transcript nt, → in primers represent 1409 sequence, ‡ overhanging nt resulting from *Sph*I linearization of ic.

Generation and characterization of virus. Interestingly, following electroporation of 1409ic RNA into Vero and C6/36 cells, only C6/36 cells produced DENV-2 antigen (detected by IFA), but not mammalian electroporated cells. In addition, only the C6/36 electroporated cells (but not mammalian cells) yielded 1409ic infectious virions (IVs) in cell culture supernatant (P-0). C6/36 generated P-0 1409ic virus was then passaged once (P-1) in each of three cell lines: LLC-MK2, Vero and C6/36 (MOI 0.01) resulting in DENV-2 E antigen IFA positive cells and production of IVs in all cell types. In contrast, electroporation of *in vitro* transcribed 1409-I/E6/M

RNA resulted in DENV-2 E positive cells as well as P-0 IVs from all three cell types. The influence of cell type specific electroporation efficiencies on IV production is an obvious consideration, however efficiencies are known to be higher for mammalian cells (30-40%) than C6/36 cells (2-10%) which is counter intuitive to the above DENV-2 results. Viral titers were collected over time for P-0 viruses and the growth curves patterns (data not shown) resembled that of the standardized growth curve shown in figure 4.2 as well as that of other DENV-2 viruses (Kinney et al., 1997).

Standardized growth curve analysis in LLC-MK2 and C6/36 cells (0.01 MOI, incubated in 10% FBS/MEM, ~5% CO_2 at 37°C or 28°C, respectively; titer determined by plaque titration on LLC-MK2 cells) showed similar temporal patterns of replication for all four viruses (figure 4.2A on page 108). C6/36 cells infected with 1409ic and 1409lp viruses, but not 1409hp and 1409-I/E6/M viruses, exhibited mild-to-moderate syncytia formation (figure 4.2B on page 108). DENV-2 plaques on LLC-MK2 monolayers 9 days post infection were all small sized with the average plaque size for each of the four viruses ranged from 1.07mm to 1.19mm across. Unidirectional sequencing of the P-0 1409ic or 1409-I/E6/M viral genomes revealed no unexpected changes from the parental plasmids.

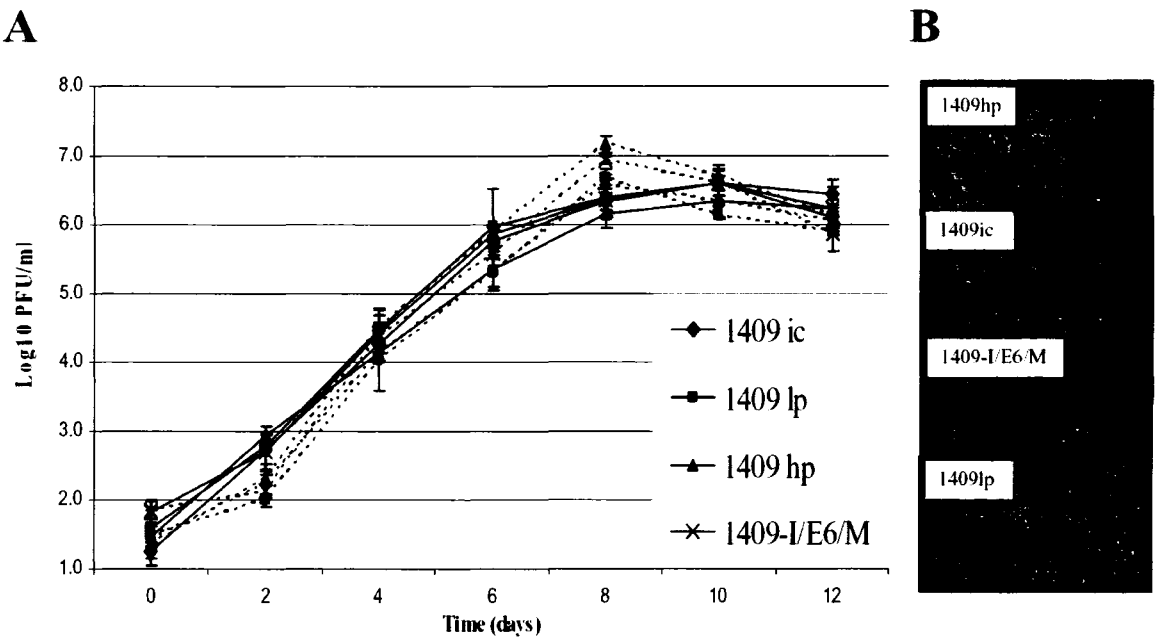


Figure 4.2. Viral phenotypes in tissue culture. A) Growth curve of DENV-2 strains in LLC-MK2 (solid lines) or C6/36 (dotted lines) cells. B) DENV-2 induced syncytia formation in C6/36 cells 8d PI.

Midgut infection assay. Mosquito midgut infection rate (MIR) and dissemination rate (MDR) of DENV-2 was determined by oral challenge of IVs in an infectious bloodmeal (6.5-6.9 PFU/ml) in *Aedes aegypti* strain Rex D mosquitoes (RexD; Rexville D, Puerto Rico, obtained from the CDC) and after an 8 day extrinsic incubation period as previously described (Bosio, Beaty et al. 1998; Myles, Pierro et al. 2004). Of the mosquitoes ingesting a bloodmeal (n = 24 mosquitoes ingested a bloodmeal, 2 replicates) containing the 1409ic virus, 66.6% had DENV-2 E antigen (Ab 4G2) in their midguts (i.e. MIR = 66.6) 8d post infection. The MIRs observed after mosquitoes ingested 1409lp, 1409hp or 1409-I/E6/M were 58.3, 72.9 and 75.0,

respectively, none of which differed significantly from each other or from 1409ic infected mosquitoes (Fischer's exact test, alpha of 0.05). The MDR, as determined by the number of anti-DENV-2 E IFA positive heads over the number of IFA positive midguts from the MIR group, for all of these viruses ranged between 78.6 and 88.5, none of which differed significantly from each other.

Discussion

Clearly the E6 residue of DENV-2 conditions viral propagation in cell culture experiments and mutational pressure may be directed at this site. Since our 1409hp stock had been repeatedly cultured in mosquito cells (>25 times), cell adapted selection may have yielded the observed mutation. Previous work has found that repeated passage of DENV-2 (strain Jamaica) in C6/36 cells resulted in an Ile to Met variation at E6 as well (Guirakhoo, Hunt et al. 1993). Those authors noted that the said DENV-2 E6-Met virus was a fusion mutant strain of DENV-2, although a direct link between a specific aa residue and the fusion phenotype was not established in that work.

Syncytium formation is the result of fusion of cellular membranes leading to the development of a polykaryocyte. DENV-2 fusion-from-without syncytium development has been demonstrated after 20 min. incubation following infection with a high MOI (Summers, Cohen et al. 1989). Additionally, low pH cell culture media have been shown to induce DENV associated syncytium progression (Randolph and Stollar 1990). These reports are consistent with our findings that a high MOI and/or a low pH medium resulted in enhanced syncytium development in C6/36 cells (data not shown).

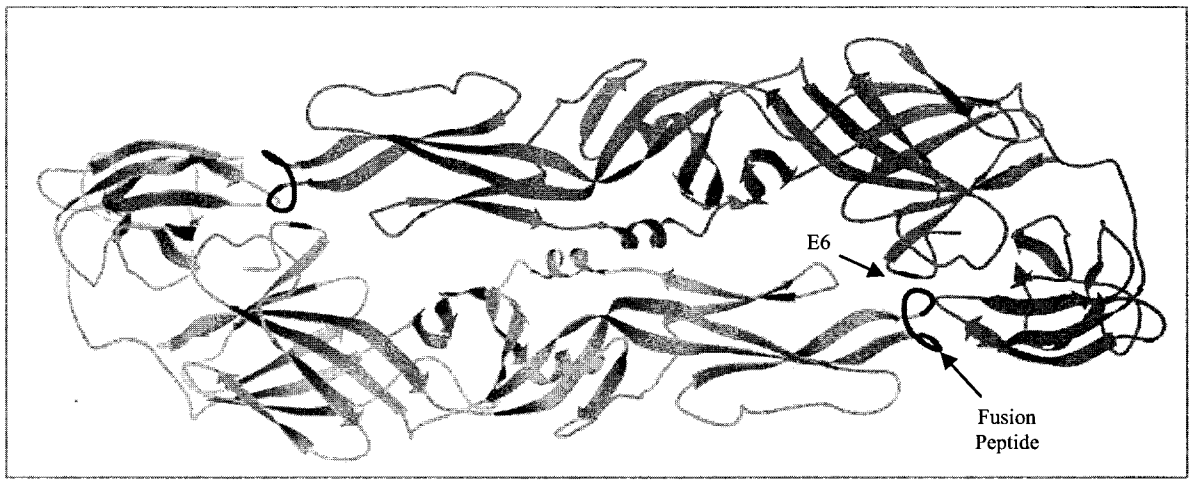


Figure 4.3. Top-down view of the flavirius envelope dimer in pre-fusion conformation. Envelope protein E6 residue (red) in in close proximity to the fusion peptide (blue) of the opposing envelope monomer (Zhang, Zhang et al. 2004). Ribbon Model from the Protein Data Base (Berman, Westbrook et al. 2000; Zhang, Zhang et al. 2004)

The flavivirus envelope glycoprotein is considered a class II type fusion protein and has a putative fusion peptide found at the tip of domain II (Rey, Heinz et al. 1995; Allison, Schalich et al. 2001). Under natural physiological states, the tip region, including the fusion peptide, is thought to be seated in a protected hydrophobic pocket between domains I and III of an opposing E monomer (figure 4.3) (Zhang, Zhang et al. 2004). As a result of exposure to low pH conditions in the endocytic vesicles, the entire E protein undergoes a conformational change resulting in the exposure of the fusion peptide to membrane surfaces (Bressanelli, Stiasny et al. 2004; Modis, Ogata et al. 2004). By analyzing the E glycoprotein structure, it was determined that the E6 site appears to be located within the domain I-III interface pocket in very close proximity to the fusion peptide (Rey, Heinz et al. 1995; Allison, Schalich et al. 2001; Modis, Ogata et al. 2003). It can be speculated that the E6 Ile residue alters the domain I-III pocket conformation, which then becomes more sensitized to pH conditions and affects the

envelope protein fusion function leading to syncytium in C6/36 cells. Potentially, this alteration could cause the premature exposure of the fusion peptide, thereby activating fusion between neighboring cell membranes. This affect may be mosquito cell type dependent as we did not observe syncytium in DENV-2 infected *Ae. aegypti* midgut cells.

The specific inhibitory effect of E6-Ile on IV production after electroporation of mammalian cells with *in vitro* transcribed mRNA points to the critical role of E6 in translation, replication, packaging, release and/or re-infection of the virus. This was an unexpected result and may be a unique aspect of DENV-2 1409 replication that warrants more investigation. This may be an effect of *in vitro* derived RNA and that this effect may not occur in nature. However, previous studies have found that low passage strains of DENV-2 show functional differences in their ability to productively infect human cells (Diamond, Edgil et al. 2000; Diamond, Roberts et al. 2000; Edgil, Diamond et al. 2003). The low passage DENV-2 strain Thai CO477 failed to produce any infectious viral particles in human foreskin fibroblast cells at an MOI below 0.1. The differences were attributed to low translational efficiency of input viral RNA. Electroporation of *in vitro* transcribed 1409ic RNA may mimic a low MOI and an inefficient translational process, which may be further comprimised through the artificial nature of the in vitro transcribed DENV-2 RNA, might prohibit virus production. It should be noted that published reports on other DENV-2 ic constructs have an Ile at E6 but were not inhibited in production of IVs in mammalian cells after electroporation (Kapoor, Zhang et al. 1995; Kinney, Butrapet et al. 1997; Blaney, Hanson et al. 2004). However, DENV-2 IV production has also been previously shown

to be influenced by the unique genetic makeup of the viral genome, specifically a nucleotide alteration in the envelope gene resulting in an aa variation at E-390 together with variations in the 3'UTR influenced DENV-2 replication in human cells (Cologna and Rico-Hesse 2003). Therefore the overall genetic makeup of the 1409 genome, and not just the E6 locus, may have an influence on translational efficiency and IV production.

The impact of quasispecies generation on IV production from transfected-RNA can also not be excluded. While the P-0 C6/36-generated 1409ic had no unexpected sequence variations, it was found that C6/36 P-3 1409ic virus had a nucleotide change in the genome that produced an Asn-to-His mutation at E-155 that resulted in the loss of a conserved glycosylation site. The loss of this glycosylation site in dengue viruses due to repeated passage in C6/36 cells has also been described by others (Guirakhoo, Hunt et al. 1993; Lee, Weir et al. 1997). Moreover, these data raise questions about the role and impact of host cell defenses to viral infection (e.g. RNAi) in mammalian vs insect cells. More analysis of the attributes of the 1409 virus in productive infection of host cells will likely provide insight into viral pathogenicity, host cell response to DENV-2 infection, and genetic determinants of efficient replication.

Chapter 5

Summary

Summary

The arboviruses, particularly the mosquito-borne viruses, are a significant public health concern in many regions of the world. Such viruses, and their mosquito vectors, do not recognize the political and geographical boundaries that define the human populations and, in fact, their dispersal is often facilitated by the transportation of goods and people across these boundaries. These viruses are also not limited to a socioeconomic status within a community, although lower standards of living do increase the likelyhood of arbovirus infection. Moreover, the evolutionary nature of RNA arboviruses facilitates a persistent and rapid mechanism for the production of new variants or arbovirus species, some of which are more virulent, more vector competent or both. The mosquito is also known to evolve in ways that affect disease transmission, such as the rise of insecticide resistance mosquitoes that can hinder vector control measures. While the control of some arbovirus diseases has been achieved within specific geographic areas for short periods of time, the sustained control of endemic arboviruses or the sterile protection from new introductions into a region is much more of an anomaly than the norm.

Research into the fundamental infection mechanisms of mosquito-borne viruses is a very important aspect to future arbovirus disease control measures. In particular, understanding the determinants and patterns of infection of a mosquito by an arbovirus will likely provide insights into the vulnerabilities of disease cycles. In fact, genetically modified mosquitoes have already been shown to take advantage of the mosquito midgut infection barrier together with the RNAi mechanism to target an incoming arbovirus and experimentally reduce vector competence in *Ae. aegypti* (Franz, Sanchez-

Vargas et al. 2006). Continued research into the dynamics of arbovirus infection of the mosquito is a necessary part of effective disease control measures for the future.

This dissertation intended to use infectious clones of SINVs to identify arbovirus genetic determinants of midgut infection in *Ae. aegypti* mosquitoes. In addition, the development of a flavivirus infectious clone with enhanced midgut infection potential was proposed for future investigations into the arbovirus determinants of infection in *Ae. aegypti*. The overall goal was to develop and use arbovirus cDNA infectious clones to evaluate the genetic determinants of midgut infections in the *Ae. aegypti* mosquito. The data presented here revealed that the genetic determinants of alphavirus infection can be found in the E2 protein at positions 55, 70, 95-96 and 116-119. Interestingly, none of the amino acid residues at these sites are well conserved among the alphaviruses implying that residue variability in the alphavirus E2 plays a role in vector competence among mosquitoes. Moreover, the SINV E2 sites 55 in conjunction with 70 and 95-96 combined with 116-119 were shown to work cooperatively to enhance the MIR implying that a simple one-viral-epitope to one-cellular-receptor mechanism may not primary mechanism for virus infection of midgut cells. It is interesting to note that the low affinity of the E2 55 and 70 sites for heparin sulfate correlated with an increase in the MIR. A disassociation for heparin sulfate is proposed as another potential determinant for enhanced midgut infection in *Ae. aegypti*.

This genetic analysis on arbovirus infection of midguts also revealed two unexpected, although very significant results. First was the observation that SINVs propagated in mammalian cells were much more efficient at infecting midguts as

compared with mosquito cell-derived viruses. This is interesting in that it is the inverse to what is reported in the literature when infecting mammalian cells with viruses propagated in these two cell types. From this, it could be hypothesized that arboviruses have evolved to enhance their infection potential when crossing species types. Protein processing, add-on structures (e.g glycosylation, cholesterols, etc), lipid raft composition or a combination of all of these cellular features may influence the determinants of this cell-propagation characteristic. The second was the identification of a highly conserved structural region (i.e. the loop-sheet-CR loop-sheet-loop secondary structure) in which a highly conserved amino acid sequence (i.e. PPF/.GDS [= CR]) was identified between the flavivirus and the alphavirus genera. Portions of this ~30 aa region are speculated to be actively involved in the infection of a cell (i.e. VR-I and -II) as well as being important in the structural support (i.e. CR) of the infectious region in the viral envelope gene. This is the first report of a highly conserved aa sequence (i.e. the CR motif) that reside within a region of known genetic determinants for cell infections (i.e. VR-I and –II) among the alphaviruses and flaviviruses. Such sequence and structural conservation between these genera helps define a potential mechanism in arbovirus infection of mosquito midguts. This mechanism emphasizes the presence of a virus envelope protein conformation that promotes the projected presentation of multiple loop regions with affinity for cellular receptors. The multiple looping regions within the viral envelope glycoprotien are proposed to work in conjunction with each other to enhance attachment to cellular ligands. This affinity determinant could impact vector competence if the binding characteristics of these variable regions are different between mosquito species.

However, much more work is needed to accurately define this mechanism.

This dissertation also reports the construction of a DENV-2 infectious clone representing the American/Asian genotype strain Jamaican 1409. This strain is thought to be an early isolate of the American/Asian genotype, and it has been observed to have a high MIR in A*e. aegypti* mosquitoes, making it an ideal candidate for this study. Unfortunately, a significant amount of technical difficulty was encountered in the development of this clone that was attributed to the toxicity of the viral cDNA sequence (a phenotype sometimes seen in other flavivirus infectious clones). In addition, genomic sequence variations between the published sequence of 1409, the in-house 1409 strain, and the sequence of a low passage 1409 virus isolate (obtained from the CDC) required the confirmation of the infectious clone's true sequence. Moreover, an E6 variation between the isolates was characterized in the clone, which influenced syncytia in mosquito cells. The secondary structure showed the E6 residue in very close proximity to the fusion peptide, and interaction between these two loci may affect the syncytium phenotype. The MIR of these viruses did not differ significantly. In summary, it was shown that the genome of an American/Asian genotype DENV-2 could be manipulated and the derived viruses could be investigated *in vitro* and in mosquito MIR assays.

This work very clearly showed the utility of cDNA infectious clone technology to highlight regions of an arbovirus envelope protein responsible for infection of midgut cells. The data generated from this technology also significantly contributed to the arbovirology body-of-knowledge by postulation of a new arbovirus midgut infection mechanism. The use of this technology will continue to be an important part

of infection determinant assays for many years to come with the expectation that the specific mechanism for infection will be accurately determined.

References

Agapov, E. V., I. A. Razumov, et al. (1994). "Localization of four antigenic sites involved in Venezuelan equine encephalomyelitis virus protection." Arch Virol **139**(1-2): 173-81.

Allison, S. L., J. Schalich, et al. (2001). "Mutational evidence for an internal fusion peptide in flavivirus envelope protein E." J Virol **75**(9): 4268-75.

Altma, R. M. (1963). "The Behavior of Murray Valley Encephalitis Virus in *Culex tritaeniorhynchus Giles* and *Culex Pipiens Quinquefasciatus Say*." Am J Trop Med Hyg **12**: 425-34.

Altmann, F., E. Staudacher, et al. (1999). "Insect cells as hosts for the expression of recombinant glycoproteins." Glycoconj J **16**(2): 109-23.

Anderson, J. R. and R. Rico-Hesse (2006). "*Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus." Am J Trop Med Hyg **75**(5): 886-92.

Anderson, R. A. and R. A. Brust (1997). "Interrupted blood feeding by *Culex* (Diptera:Culicidae) in relation to individual host tolerance to mosquito attack." J Med Entomol **34**(2): 95-101.

Anthony, R. P. and D. T. Brown (1991). "Protein-protein interactions in an alphavirus membrane." J Virol **65**(3): 1187-94.

Armstrong, P. M. and R. Rico-Hesse (2001). "Differential susceptibility of *Aedes aegypti* to infection by the American and Southeast Asian genotypes of dengue type 2 virus." Vector Borne Zoonotic Dis **1**(2): 159-68.

Barnett, H. C. (1956). "The transmission of Western equine encephalitis virus by the mosquito *Culex tarsalis Coq*." Am J Trop Med Hyg **5**(1): 86-98.

Barratt-Boyes, S. M., M. I. Zimmer, et al. (2002). "Changes in dendritic cell migration and activation during SIV infection suggest a role in initial viral spread and eventual immunosuppression." J Med Primatol **31**(4-5): 186-93.

Barrett, A. D. and S. Higgs (2007). "Yellow fever: a disease that has yet to be

conquered." Annu Rev Entomol **52**: 209-29.

Beaty, B. J. (2005). "Control of arbovirus diseases: is the vector the weak link?" Arch Virol Suppl(19): 73-88.

Beaty, B. J., B. R. Miller, et al. (1982). "Molecular basis of bunyavirus per os infection of mosquitoes: role of the middle-sized RNA segment." Proc Natl Acad Sci U S A **79**(4): 1295-7.

Bennett, K. E., B. J. Beaty, et al. (2005). "Selection of D2S3, an *Aedes aegypti* (Diptera: Culicidae) strain with high oral susceptibility to Dengue 2 virus and D2MEB, a strain with a midgut barrier to Dengue 2 escape." J Med Entomol **42**(2): 110-9.

Bennett, K. E., D. Flick, et al. (2005). "Quantitative trait loci that control dengue-2 virus dissemination in the mosquito *Aedes aegypti*." Genetics **170**(1): 185-94.

Bennett, K. E., K. E. Olson, et al. (2002). "Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States." Am J Trop Med Hyg **67**(1): 85-92.

Berman, H. M., J. Westbrook, et al. (2000). "The Protein Data Bank." Nucleic Acids Res **28**(1): 235-42.

Bernard, K. A., W. B. Klimstra, et al. (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**(1): 93-103.

Bertram, D. S. and R. G. Bird (1961). "Studies on mosquito-borne viruses in their vectors. I. The normal fine structure of the midgut epithelium of the adult female *Aedes aegypti* (L.) and the functional significance of its modification following a blood meal." Trans R Soc Trop Med Hyg **55**: 404-23.

Betts, M. J., Russell, R.B. (2003). Amino Acid properties and consequences of substitutions. Bioinformatics for Geneticists. M. R. Barnes, Grey, I.C. West Sussex, England, John Wiley and Sons Ltd. **1**.

Betts, M. J. a. R., R.B. (2003). Amino acid properties and consequences of subsitutions. Bioinformatics for Geneticists. I. C. G. M.R. Barnes, Wiley.

Billingsley, P. F. (1990). "Blood digestion in the mosquito, *Anopheles stephensi Liston* (Diptera: Culicidae): partial characterization and post-feeding activity of midgut aminopeptidases." Arch Insect Biochem Physiol **15**(3): 149-63.

Billingsley, P. F. (1990). "The midgut ultrastructure of hematophagous insects." ANNu. Rev. Ent.(35): 219-248.

Black, W. C. t., K. E. Bennett, et al. (2002). "Flavivirus susceptibility in *Aedes aegypti*." Arch Med Res **33**(4): 379-88.

Black, W. I. a. M., LE (2005). Molecular Taxonomy and Systematics of Arthropod Vectors. Biology of Disease Vectors W. Marquardt. San Diego, CA, Elsevier Academic Press: 505-528.

Blaney, J. E., Jr., C. T. Hanson, et al. (2004). "Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2." BMC Infect Dis **4**: 39.

Bosio, C. F., B. J. Beaty, et al. (1998). "Quantitative genetics of vector competence for dengue-2 virus in *Aedes aegypti*." Am J Trop Med Hyg **59**(6): 965-70.

Bosio, C. F., R. E. Fulton, et al. (2000). "Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*." Genetics **156**(2): 687-98.

Bram, R. A., J. E. George, et al. (2002). "Threat of foreign arthropod-borne pathogens to livestock in the United States." J Med Entomol **39**(3): 405-16.

Brault, A. C., A. M. Powers, et al. (2002). "Positively charged amino acid substitutions in the e2 envelope glycoprotein are associated with the emergence of venezuelan equine encephalitis virus." J Virol **76**(4): 1718-30.

Brault, A. C., A. M. Powers, et al. (2004). "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**(31): 11344-9.

Brault, A. C., A. M. Powers, et al. (2002). "Vector infection determinants of Venezuelan equine encephalitis virus reside within the E2 envelope glycoprotein." J Virol **76**(12): 6387-92.

Bressanelli, S., K. Stiasny, et al. (2004). "Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation." Embo J **23**(4): 728-38.

Bretscher, M. S. and S. Munro (1993). "Cholesterol and the Golgi apparatus." Science **261**(5126): 1280-1.

Brown, F. (1986). "The classification and nomenclatue of viruses: summary of results of meetings of the International Committee on Taxonomy of Viruses in Sendai, September 1984." Intervirology **25**: 141-143.

Brown, M. R., Lea, A.O. (1989). "Neuroendocrine and midgut endocrine systems on the adult mosquito." Adv. Dis. Vector Res.(6): 29-58.

Brown, M. R., A. S. Raikhel, et al. (1985). "Ultrastructure of midgut endocrine cells in the adult mosquito, *Aedes aegypti*." Tissue Cell **17**(5): 709-21.

Byrnes, A. P. and D. E. Griffin (1998). "Binding of Sindbis virus to cell surface heparan sulfate." J Virol **72**(9): 7349-56.

Byrnes, A. P. and D. E. Griffin (2000). "Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation." J Virol **74**(2): 644-51.

Calisher, C. H., Karabatsos, N. (1988). Arbovirus serogroups: definition and geographic distribution. The arboviruses: epidemiology and ecology. T. P. Monath. Boca Raton, FL, CRC Press. **1:** 19.

Chamberlain, R. W. and W. D. Sudia (1961). "Mechanism of transmission of viruses by mosquitoes." Annu Rev Entomol **6**: 371-90.

Chamberlain, R. W., W. D. Sudia, et al. (1959). "St. Louis encephalitis virus in mosquitoes." Am J Hyg **70**: 221-36.

Chambers, T. J., C. S. Hahn, et al. (1990). "Flavivirus genome organization, expression, and replication." Annu Rev Microbiol **44**: 649-88.

Chambers, T. J. and M. Nickells (2001). "Neuroadapted yellow fever virus 17D: genetic and biological characterization of a highly mouse-neurovirulent virus and its infectious molecular clone." J Virol **75**(22): 10912-22.

Charrel, R. N., X. de Lamballerie, et al. (2007). "Chikungunya outbreaks--the globalization of vectorborne diseases." N Engl J Med **356**(8): 769-71.

Clayton, R. B. (1964). "The Utilization of Sterols by Insects." J Lipid Res **15**: 3-19.

Cociancich, S. O., S. S. Park, et al. (1999). "Vesicular ATPase-overexpressing cells determine the distribution of malaria parasite oocysts on the midguts of mosquitoes." J Biol Chem **274**(18): 12650-5.

Cologna, R. and R. Rico-Hesse (2003). "American genotype structures decrease dengue virus output from human monocytes and dendritic cells." J Virol **77**(7): 3929-38.

D'Souza, S. E., M. H. Ginsberg, et al. (1991). "Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif." Trends Biochem Sci **16**(7): 246-50.

Davis, C. W., H. Y. Nguyen, et al. (2006). "West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection." J Virol **80**(3): 1290-301.

Davis, N. L., F. J. Fuller, et al. (1986). "A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice." Proc Natl Acad Sci U S A **83**(18): 6771-5.

Davis, N. L., D. F. Pence, et al. (1987). "Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein." Virology **161**(1): 101-8.

Day, J. F. (2005). "Host-seeking strategies of mosquito disease vectors." J Am Mosq

Control Assoc **21**(4 Suppl): 17-22.

Deen, J. L., E. Harris, et al. (2006). "The WHO dengue classification and case definitions: time for a reassessment." Lancet **368**(9530): 170-3.

Delos, S. E., J. M. Gilbert, et al. (2000). "The central proline of an internal viral fusion peptide serves two important roles." J Virol **74**(4): 1686-93.

Deubel, V., R. M. Kinney, et al. (1986). "Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype." Virology **155**(2): 365-77.

Deubel, V., R. M. Kinney, et al. (1988). "Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome." Virology **165**(1): 234-44.

Diamond, M. S., D. Edgil, et al. (2000). "Infection of human cells by dengue virus is modulated by different cell types and viral strains." J Virol **74**(17): 7814-23.

Diamond, M. S., T. G. Roberts, et al. (2000). "Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons." J Virol **74**(11): 4957-66.

Diaz, F. J., W. C. t. Black, et al. (2006). "Dengue virus circulation and evolution in Mexico: a phylogenetic perspective." Arch Med Res **37**(6): 760-73.

Dixon KL, C. J., Escribano JM, Rock DL, Vinuela E and Wilkinson PJ (1999). Family *Asfarviridae*. Virus Taxonomy. Seventh report of the International Committee on Taxonomy of Viruses. M. H. van Regenmortel, Fauquet, C.M.,Bisgop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M, et al. San Diego, Academic Press.

Doherty, R. L., J. G. Carley, et al. (1977). "Isolation of Sindbis (alphavirus) and Leanyer viruses from mosquitoes collected in the Northern Territory of Australia, 1974." Aust J Exp Biol Med Sci **55**(4): 485-9.

Doi, R. (1970). "Studies on the mode of development of Japanese encephalitis virus in some groups of mosquitoes by the fluorescent antibody technique." Jpn J Exp Med **40**(2): 101-15.

Edgil, D., M. S. Diamond, et al. (2003). "Translation efficiency determines differences in cellular infection among dengue virus type 2 strains." Virology **317**(2): 275-90.

Elizondo-Quiroga, A., A. Flores-Suarez, et al. (2006). "Host-feeding preference of *Culex quinquefasciatus* in Monterrey, northeastern Mexico." J Am Mosq Control Assoc **22**(4): 654-61.

Failloux, A. B., M. Vazeille, et al. (2002). "Geographic genetic variation in populations

of the dengue virus vector *Aedes aegypti*." J Mol Evol **55**(6): 653-63.

Favier, C., K. Chalvet-Monfray, et al. (2006). "Rift Valley fever in West Africa: the role of space in endemicity." Trop Med Int Health **11**(12): 1878-88.

Finn, R. D., J. Mistry, et al. (2006). "Pfam: clans, web tools and services." Nucleic Acids Res **34**(Database issue): D247-51.

Fonseca, D. M., N. Keyghobadi, et al. (2004). "Emerging vectors in the *Culex pipiens* complex." Science **303**(5663): 1535-8.

Fox, G., N. R. Parry, et al. (1989). "The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid)." J Gen Virol **70 (Pt 3)**: 625-37.

Foy, B. D., K. M. Myles, et al. (2004). "Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species." Insect Mol Biol **13**(1): 89-100.

Franz, A. W., I. Sanchez-Vargas, et al. (2006). "Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*." Proc Natl Acad Sci U S A **103**(11): 4198-203.

Freier, J. E. and J. C. Beier (1984). "Oral and transovarial transmission of La Crosse virus by *Aedes atropalpus*." Am J Trop Med Hyg **33**(4): 708-14.

Frolov, I., E. Frolova, et al. (1997). "Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA." J Virol **71**(4): 2819-29.

Garmashova, N., R. Gorchakov, et al. (2007). "The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff." J Virol **81**(5): 2472-84.

Gibbons, D. L., M. C. Vaney, et al. (2004). "Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus." Nature **427**(6972): 320-5.

Gould, E. A., S. Higgs, et al. (2006). "Potential arbovirus emergence and implications for the United Kingdom." Emerg Infect Dis **12**(4): 549-55.

Grakoui, A., R. Levis, et al. (1989). "A cis-acting mutation in the Sindbis virus junction region which affects subgenomic RNA synthesis." J Virol **63**(12): 5216-27.

Gratz, N. G. (2004). "Critical review of the vector status of *Aedes albopictus*." Med Vet Entomol **18**(3): 215-27.

Gresser, I., J. L. Hardy, et al. (1958). "Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus Giles*, from infected pigs and chicks to susceptible pigs and birds." Am J Trop Med Hyg

7(4): 365-73.

Griffin, D. E. (2001). Alphaviruses. Fields Virology. D. M. a. H. Knipe, P.M. Philadelphia, LIppincott, Williams & Wilkins. **2:** 917-962.

Gubler, D. J. (1998). "Dengue and dengue hemorrhagic fever." Clin Microbiol Rev **11**(3): 480-96.

Gubler, D. J. (2002). "The global emergence/resurgence of arboviral diseases as public health problems." Arch Med Res **33**(4): 330-42.

Gubler, D. J. (2004). "The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle?" Comp Immunol Microbiol Infect Dis **27**(5): 319-30.

Gubler, D. J. and G. G. Clark (1996). "Community involvement in the control of *Aedes aegypti*." Acta Trop **61**(2): 169-79.

Gubler, D. J., S. Nalim, et al. (1979). "Variation in susceptibility to oral infection with dengue viruses among geographic strains of *Aedes aegypti*." Am J Trop Med Hyg **28**(6): 1045-52.

Gubler, D. J. and L. Rosen (1976). "Variation among geographic strains of *Aedes albopictus* in susceptibility to infection with dengue viruses." Am J Trop Med Hyg **25**(2): 318-25.

Guirakhoo, F., A. R. Hunt, et al. (1993). "Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH." Virology **194**(1): 219-23.

Guzman, M. G., M. Alvarez, et al. (1999). "Fatal dengue hemorrhagic fever in Cuba, 1997." Int J Infect Dis **3**(3): 130-5.

Hahn, C. S., J. M. Dalrymple, et al. (1987). "Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it." Proc Natl Acad Sci U S A **84**(7): 2019-23.

Hahn, C. S., Y. S. Hahn, et al. (1992). "Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation." Proc Natl Acad Sci U S A **89**(7): 2679-83.

Hahn, Y. S., E. G. Strauss, et al. (1989). "Mapping of RNA- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins." J Virol **63**(7): 3142-50.

Hardy, J. (1988). Susceptibility and resistance of vector mosquitoes. The arboviruses: ecology and epidemiology. M. TP. Boca Raton, FL, CRC Press. **1:** 87.

Hayes, E. B. and D. J. Gubler (2006). "West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States." Annu Rev Med **57**: 181-94.

Hayles, L. B., J. McLintock, et al. (1972). "Laboratory studies on the transmission of western equine encephalitis virus by Saskatchewan mosquitoes. I. *Culex tarsalis*." Can J Comp Med **36**(2): 83-8.

Hecker, H. (1977). "Structure and function of midgut epithelial cells in culicidae mosquitoes (insecta, diptera)." Cell Tissue Res **184**(3): 321-41.

Hecker, H., T. A. Freyvogel, et al. (1971). "Ultrastructural differentiation of the midgut epithelium in female *Aedes aegypti (L.)* (Insecta, Diptera) imagines." Acta Trop **28**(2): 80-104.

Hecker, H., T. A. Freyvogel, et al. (1971). "The ultrastructure of midgut epithelium in *Aedes aegypti (L).* (Insecta, Diptera) males." Acta Trop **28**(3): 275-90.

Helenius, A., B. Morein, et al. (1978). "Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus." Proc Natl Acad Sci U S A **75**(8): 3846-50.

Hemingway, J., B. J. Beaty, et al. (2006). "The Innovative Vector Control Consortium: improved control of mosquito-borne diseases." Trends Parasitol **22**(7): 308-12.

Hiramatsu, K., M. Tadano, et al. (1996). "Mutational analysis of a neutralization epitope on the dengue type 2 virus (DEN2) envelope protein: monoclonal antibody resistant DEN2/DEN4 chimeras exhibit reduced mouse neurovirulence." Virology **224**(2): 437-45.

Holbrook, M. R., R. E. Shope, et al. (2004). "Use of recombinant E protein domain III-based enzyme-linked immunosorbent assays for differentiation of tick-borne encephalitis serocomplex flaviviruses from mosquito-borne flaviviruses." J Clin Microbiol **42**(9): 4101-10.

Holzmann, H., F. X. Heinz, et al. (1990). "A single amino acid substitution in envelope protein E of tick-borne encephalitis virus leads to attenuation in the mouse model." J Virol **64**(10): 5156-9.

Howard, J. J. and R. C. Wallis (1974). "Infection and transmission of eastern equine encephalomyelitis virus with colonized *Culiseta melanura (Coquillett).*" Am J Trop Med Hyg **23**(3): 522-5.

Hubalek, Z. and J. Halouzka (1999). "West Nile fever--a reemerging mosquito-borne viral disease in Europe." Emerg Infect Dis **5**(5): 643-50.

Hurlbut, H. S. (1956). "West Nile virus infection in arthropods." Am J Trop Med Hyg **5**(1): 76-85.

Hurrelbrink, R. J. and P. C. McMinn (2001). "Attenuation of Murray Valley encephalitis virus by site-directed mutagenesis of the hinge and putative receptor-binding regions of the envelope protein." J Virol **75**(16): 7692-702.

Jackson, R. E. and R. J. Phillpotts (1997). "Production of virus-specific antisera using synthetic peptides corresponding to sequences in the yellow fever E protein: brief report." Viral Immunol **10**(1): 11-4.

Johnson, B. J., J. R. Brubaker, et al. (1990). "Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein." Virology **177**(2): 676-83.

Johnson, B. W., T. V. Chambers, et al. (2002). "Growth characteristics of ChimeriVax-DEN2 vaccine virus in *Aedes aegypti* and *Aedes albopictus* mosquitoes." Am J Trop Med Hyg **67**(3): 260-5.

Johnston, L. J., G. M. Halliday, et al. (2000). "Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus." J Invest Dermatol **114**(3): 560-8.

Jupp, P. G. and B. M. McIntosh (1970). "Quantitative experiments on the vector capability of *Culex (Culex) pipiens fatigans Wiedemann* with West Nile and Sindbis viruses." J Med Entomol **7**(3): 353-6.

Jupp, P. G. and B. M. McIntosh (1970). "Quantitative experiments on the vector capability of *Culex (Culex) univittatus Theobald* with West Nile and Sindbis viruses." J Med Entomol **7**(3): 371-3.

Jupp, P. G., B. M. McIntosh, et al. (1972). "Quantitative experiments on the vector capability of *Culex (Culex) theileri Theobald* with West Nile and Sindbis viruses." J Med Entomol **9**(5): 393-5.

Kapoor, M., L. Zhang, et al. (1995). "Synthesis and characterization of an infectious dengue virus type-2 RNA genome (New Guinea C strain)." Gene **162**(2): 175-80.

Karabatsos, N. (1985. Updated 2001.). International catalogue of arthropod-borne viruses. San Antonio (TX), American Society for Tropical Medicine and Hygiene.

Karlsson, K. and S. L. Marklund (1988). "Plasma clearance of human extracellular-superoxide dismutase C in rabbits." J Clin Invest **82**(3): 762-6.

Karlsson, K., J. Sandstrom, et al. (1994). "Turnover of extracellular-superoxide dismutase in tissues." Lab Invest **70**(5): 705-10.

Kay, B. H. and C. D. Jennings (2002). "Enhancement or modulation of the vector competence of *Ochlerotatus vigilax* (Diptera: Culicidae) for Ross river virus by temperature." J Med Entomol **39**(1): 99-105.

Kim, K. H., E. G. Strauss, et al. (2000). "Adaptive mutations in Sindbis virus E2 and Ross River virus E1 that allow efficient budding of chimeric viruses." J Virol

74(6): 2663-70.

Kinney, R. M., S. Butrapet, et al. (1997). "Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53." Virology **230**(2): 300-8.

Kinney, R. M., G. J. Chang, et al. (1993). "Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein." J Virol **67**(3): 1269-77.

Klimjack, M. R., S. Jeffrey, et al. (1994). "Membrane and protein interactions of a soluble form of the Semliki Forest virus fusion protein." J Virol **68**(11): 6940-6.

Klimstra, W. B., E. M. Nangle, et al. (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**(22): 12022-32.

Klimstra, W. B., K. D. Ryman, et al. (1998). "Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor." J Virol **72**(9): 7357-66.

Kramer, L. D., J. L. Hardy, et al. (1981). "Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low viral doses." Am J Trop Med Hyg **30**(1): 190-7.

Kuno, G. and G. J. Chang (2005). "Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends." Clin Microbiol Rev **18**(4): 608-37.

LaMotte, L. C., Jr. (1960). "Japanese B encephalitis virus in the organs of infected mosquitoes." Am J Hyg **72**: 73-87.

Lanciotti, R. S., D. J. Gubler, et al. (1997). "Molecular evolution and phylogeny of dengue-4 viruses." J Gen Virol **78 (Pt 9)**: 2279-84.

Larsen, J. R. and R. F. Ashley (1971). "Demonstration of Venezuelan equine encephalomyelitis virus in tissues of *Aedes Aegypti*." Am J Trop Med Hyg **20**(5): 754-60.

Lee, E. and M. Lobigs (2000). "Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry." J Virol **74**(19): 8867-75.

Lee, E., R. C. Weir, et al. (1997). "Changes in the dengue virus major envelope protein on passaging and their localization on the three-dimensional structure of the protein." Virology **232**(2): 281-90.

Lee, H. and D. T. Brown (1994). "Mutations in an exposed domain of Sindbis virus capsid protein result in the production of noninfectious virions and

morphological variants." Virology **202**(1): 390-400.

Lee, J. H., W. A. Rowley, et al. (2000). "Vector competence of *Culex tarsalis* (Diptera: Culicidae) from Iowa for a sympatric strain of western equine encephalomyelitis (WEE-7738) virus." J Med Entomol **37**(1): 194-7.

Lee, P., R. Knight, et al. (2002). "A single mutation in the E2 glycoprotein important for neurovirulence influences binding of sindbis virus to neuroblastoma cells." J Virol **76**(12): 6302-10.

Leippert, M., E. Beck, et al. (1997). "Point mutations within the betaG-betaH loop of foot-and-mouth disease virus O1K affect virus attachment to target cells." J Virol **71**(2): 1046-51.

Lescar, J., A. Roussel, et al. (2001). "The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH." Cell **105**(1): 137-48.

Levine, B. and D. E. Griffin (1993). "Molecular analysis of neurovirulent strains of Sindbis virus that evolve during persistent infection of scid mice." J Virol **67**(11): 6872-5.

Lewis, J. A., G. J. Chang, et al. (1993). "Phylogenetic relationships of dengue-2 viruses." Virology **197**(1): 216-24.

Li, G. P. and C. M. Rice (1989). "Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication." J Virol **63**(3): 1326-37.

Li, M. L., H. J. Liao, et al. (1999). "An amino acid change in the exodomain of the E2 protein of Sindbis virus, which impairs the release of virus from chicken cells but not from mosquito cells." Virology **264**(1): 187-94.

Liang, G. D., L. Li, et al. (2000). "Isolation and complete nucleotide sequence of a Chinese Sindbis-like virus." J Gen Virol **81**(Pt 5): 1347-51.

Lin, S. R., S. C. Hsieh, et al. (2004). "Study of sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts: implications for transmission and evolution." J Virol **78**(22): 12717-21.

Lord, C. C., C. R. Rutledge, et al. (2006). "Relationships between host viremia and vector susceptibility for arboviruses." J Med Entomol **43**(3): 623-30.

Lorono-Pino, M. A., J. A. Farfan-Ale, et al. (2004). "Introduction of the American/Asian genotype of dengue 2 virus into the Yucatan State of Mexico." Am J Trop Med Hyg **71**(4): 485-92.

Lothrop, H. D. and W. K. Reisen (2001). "Landscape affects the host-seeking patterns of *Culex tarsalis* (Diptera: Culicidae) in the Coachella Valley of California." J

Med Entomol **38**(2): 325-32.

Lu, Y. E., T. Cassese, et al. (1999). "The cholesterol requirement for Sindbis virus entry and exit and characterization of a spike protein region involved in cholesterol dependence." J Virol **73**(5): 4272-8.

Ludwig, G. V., B. M. Christensen, et al. (1989). "Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus-vector infection model." Virology **171**(1): 108-13.

Ludwig, G. V., B. A. Israel, et al. (1991). "Role of La Crosse virus glycoproteins in attachment of virus to host cells." Virology **181**(2): 564-71.

Ludwig, G. V., J. P. Kondig, et al. (1996). "A putative receptor for Venezuelan equine encephalitis virus from mosquito cells." J Virol **70**(8): 5592-9.

Lustig, S., A. C. Jackson, et al. (1988). "Molecular basis of Sindbis virus neurovirulence in mice." J Virol **62**(7): 2329-36.

Luukkonen, A., M. Brummer-Korvenkontio, et al. (1973). "Lipids of cultured mosquito cells (*Aedes albopictus*). Comparison with cultured mammalian fibroblasts (BHK 21 cells)." Biochim Biophys Acta **326**(2): 256-61.

Maassen, J. A. and C. Terhorst (1981). "Identification of a cell-surface protein involved in the binding site of Sindbis virus on human lymphoblastic cell lines using a heterobifunctional cross-linker." Eur J Biochem **115**(1): 153-8.

MacDonald, G. H. and R. E. Johnston (2000). "Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis." J Virol **74**(2): 914-22.

Mackenzie, J. S., M. D. Lindsay, et al. (1994). "Arboviruses causing human disease in the Australasian zoogeographic region." Arch Virol **136**(3-4): 447-67.

Mahmood, F., R. E. Chiles, et al. (2006). "Effects of time after infection, mosquito genotype, and infectious viral dose on the dynamics of *Culex tarsalis* vector competence for western equine encephalomyelitis virus." J Am Mosq Control Assoc **22**(2): 272-81.

Mandl, C. W., M. Ecker, et al. (1997). "Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr." J Gen Virol **78 (Pt 5)**: 1049-57.

Marchal, I., D. L. Jarvis, et al. (2001). "Glycoproteins from insect cells: sialylated or not?" Biol Chem **382**(2): 151-9.

Maroli, M. and G. Majori (1991). "Permethrin-impregnated curtains against phlebotomine sandflies (Diptera: Psychodidae): laboratory and field studies." Parassitologia **33 Suppl**: 399-404.

Marquardt, M. T., T. Phalen, et al. (1993). "Cholesterol is required in the exit pathway

of Semliki Forest virus." J Cell Biol **123**(1): 57-65.

McElroy, K. L., K. A. Tsetsarkin, et al. (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**(6): 1158-64.

McElroy, K. L., K. A. Tsetsarkin, et al. (2006). "Role of the yellow fever virus structural protein genes in viral dissemination from the *Aedes aegypti* mosquito midgut." J Gen Virol **87**(Pt 10): 2993-3001.

McKnight, K. L., D. A. Simpson, et al. (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**(3): 1981-9.

McLean, D. (1955). "Multiplication of viruses in mosquitoes following feeding and injection into the body cavity." Aust J Exp Biol Med Sci **33**(1): 53-65.

Medlock, J. M., K. R. Snow, et al. (2005). "Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors." Med Vet Entomol **19**(1): 2-21.

Mendoza, Q. P., J. Stanley, et al. (1988). "Monoclonal antibodies to the E1 and E2 glycoproteins of Sindbis virus: definition of epitopes and efficiency of protection from fatal encephalitis." J Gen Virol **69 (Pt 12)**: 3015-22.

Mercado-Curiel, R. F., H. A. Esquinca-Aviles, et al. (2006). "The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and Ae. albopictus cells." BMC Microbiol **6**: 85.

Mertens, P. P., J. N. Burroughs, et al. (1996). "Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two Culicoides vector species." Virology **217**(2): 582-93.

Miller, B. R. and C. J. Mitchell (1986). "Passage of yellow fever virus: its effect on infection and transmission rates in *Aedes aegypti*." Am J Trop Med Hyg **35**(6): 1302-9.

Miller, B. R. and C. J. Mitchell (1991). "Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito *Aedes aegypti*." Am J Trop Med Hyg **45**(4): 399-407.

Modis, Y., S. Ogata, et al. (2003). "A ligand-binding pocket in the dengue virus envelope glycoprotein." Proc Natl Acad Sci U S A **100**(12): 6986-91.

Modis, Y., S. Ogata, et al. (2004). "Structure of the dengue virus envelope protein after membrane fusion." Nature **427**(6972): 313-9.

Molaei, G., T. G. Andreadis, et al. (2006). "Host feeding patterns of *Culex* mosquitoes

and West Nile virus transmission, northeastern United States." Emerg Infect Dis **12**(3): 468-74.

Molina-Cruz, A., L. Gupta, et al. (2005). "Effect of mosquito midgut trypsin activity on dengue-2 virus infection and dissemination in *Aedes aegypti*." Am J Trop Med Hyg **72**(5): 631-7.

Monath, T. P. (1994). "Dengue: the risk to developed and developing countries." Proc Natl Acad Sci U S A **91**(7): 2395-400.

Moncayo, A. C., Z. Fernandez, et al. (2004). "Dengue emergence and adaptation to peridomestic mosquitoes." Emerg Infect Dis **10**(10): 1790-6.

Mourya, D. T., S. N. Ranadive, et al. (1998). "Putative chikungunya virus-specific receptor proteins on the midgut brush border membrane of *Aedes aegypti* mosquito." Indian J Med Res **107**: 10-4.

Mudur, G. (2006). "Failure to control mosquitoes has led to two fever epidemics in India." Bmj **333**(7572): 773.

Mukhopadhyay, S., W. Zhang, et al. (2006). "Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses." Structure **14**(1): 63-73.

Munga, S., N. Minakawa, et al. (2006). "Association between land cover and habitat productivity of malaria vectors in western Kenyan highlands." Am J Trop Med Hyg **74**(1): 69-75.

Myles, K. M., D. J. Pierro, et al. (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**(16): 8872-81.

Myles, K. M., D. J. Pierro, et al. (2004). "Comparison of the transmission potential of two genetically distinct Sindbis viruses after oral infection of *Aedes aegypti* (Diptera: Culicidae)." J Med Entomol **41**(1): 95-106.

Navaratnarajah, C. K. and R. J. Kuhn (2007). "Functional characterization of the Sindbis virus E2 glycoprotein by transposon linker-insertion mutagenesis." Virology.

Nickells, M. and T. J. Chambers (2003). "Neuroadapted yellow fever virus 17D: determinants in the envelope protein govern neuroinvasiveness for SCID mice." J Virol **77**(22): 12232-42.

Ogunrinade, A. (1980). "The measurement of blood meal size in *Aedes aegypti (L.)*." Afr J Med Med Sci **9**(1-2): 69-71.

Olson, K. and D. W. Trent (1985). "Genetic and antigenic variations among geographical isolates of Sindbis virus." J Gen Virol **66 (Pt 4)**: 797-810.

Olson, K. E., K. M. Myles, et al. (2000). "Development of a Sindbis virus expression

system that efficiently expresses green fluorescent protein in midguts of *Aedes aegypti* following per os infection." Insect Mol Biol **9**(1): 57-65.

Ooi, E. E., K. T. Goh, et al. (2006). "Dengue prevention and 35 years of vector control in Singapore." Emerg Infect Dis **12**(6): 887-93.

Paredes, A. M., D. Ferreira, et al. (2004). "Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion." Virology **324**(2): 373-86.

Parola, P., X. de Lamballerie, et al. (2006). "Novel chikungunya virus variant in travelers returning from Indian Ocean islands." Emerg Infect Dis **12**(10): 1493-9.

Paulson, S. L., P. R. Grimstad, et al. (1989). "Midgut and salivary gland barriers to La Crosse virus dissemination in mosquitoes of the *Aedes triseriatus* group." Med Vet Entomol **3**(2): 113-23.

Pence, D. F., N. L. Davis, et al. (1990). "Antigenic and genetic characterization of Sindbis virus monoclonal antibody escape mutants which define a pathogenesis domain on glycoprotein E2." Virology **175**(1): 41-9.

Pereboev, A. V., I. A. Razumov, et al. (1996). "Glycoproteins E2 of the Venezuelan and eastern equine encephalomyelitis viruses contain multiple cross-reactive epitopes." Arch Virol **141**(11): 2191-205.

Pierro, D. J., K. M. Myles, et al. (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**(2): 107-16.

Pletnev, S. V., W. Zhang, et al. (2001). "Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold." Cell **105**(1): 127-36.

Polo, J. M., N. L. Davis, et al. (1988). "Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro." J Virol **62**(6): 2124-33.

Ponlawat, A. and L. C. Harrington (2005). "Blood feeding patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand." J Med Entomol **42**(5): 844-9.

Powers, A. M., A. C. Brault, et al. (2001). "Evolutionary relationships and systematics of the alphaviruses." J Virol **75**(21): 10118-31.

Prenner, C., L. Mach, et al. (1992). "The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A2. The role of alpha 1,3-fucosylation of the asparagine-bound N-acetylglucosamine." Biochem J **284 (Pt 2)**: 377-80.

Pryor, M. J., J. M. Carr, et al. (2001). "Replication of dengue virus type 2 in human monocyte-derived macrophages: comparisons of isolates and recombinant viruses with substitutions at amino acid 390 in the envelope glycoprotein." Am J Trop Med Hyg **65**(5): 427-34.

Pudney, M. L., C.J., Varma, M.G.R. (1979). Artic and Tropical Arboviruses. NYC, New York, Academic Press.

Randolph, V. B. and V. Stollar (1990). "Low pH-induced cell fusion in flavivirus-infected Aedes albopictus cell cultures." J Gen Virol **71 (Pt 8)**: 1845-50.

Reisen, W. K. and P. F. Boreham (1979). "Host selection patterns of some Pakistan mosquitoes." Am J Trop Med Hyg **28**(2): 408-21.

Reisen, W. K., Y. Fang, et al. (2006). "Effects of temperature on the transmission of west nile virus by *Culex tarsalis* (Diptera: Culicidae)." J Med Entomol **43**(2): 309-17.

Reisen, W. K., H. D. Lothrop, et al. (1997). "Time of host-seeking by *Culex tarsalis* (Diptera:Culicidae) in California." J Med Entomol **34**(4): 430-7.

Reisen, W. K., R. P. Meyer, et al. (1993). "Effect of temperature on the transmission of western equine encephalomyelitis and St. Louis encephalitis viruses by *Culex tarsalis* (Diptera: Culicidae)." J Med Entomol **30**(1): 151-60.

Rentier-Delrue, F. and N. A. Young (1980). "Genomic divergence among Sindbis virus strains." Virology **106**(1): 59-70.

Rey, F. A., F. X. Heinz, et al. (1995). "The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution." Nature **375**(6529): 291-8.

Ribeiro, J. M. (2000). "Blood-feeding in mosquitoes: probing time and salivary gland anti-haemostatic activities in representatives of three genera (*Aedes, Anopheles, Culex*)." Med Vet Entomol **14**(2): 142-8.

Rice, C. M., A. Grakoui, et al. (1989). "Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation." New Biol ogy **1**(3): 285-96.

Rice, C. M., R. Levis, et al. (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**(12): 3809-19.

Rico-Hesse, R. (1990). "Molecular evolution and distribution of dengue viruses type 1 and 2 in nature." Virology **174**(2): 479-93.

Rico-Hesse, R., L. M. Harrison, et al. (1997). "Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas." Virology **230**(2): 244-

51.

Roehrig, J. T., P. A. Risi, et al. (1994). "T-helper cell epitopes on the E-glycoprotein of dengue 2 Jamaica virus." Virology **198**(1): 31-8.

Rosen, L., L. E. Roseboom, et al. (1985). "Comparative susceptibility of mosquito species and strains to oral and parenteral infection with dengue and Japanese encephalitis viruses." Am J Trop Med Hyg **34**(3): 603-15.

Rost, B. and C. Sander (1993). "Prediction of protein secondary structure at better than 70% accuracy." J Mol Biol **232**(2): 584-99.

Rost, B. and C. Sander (1994). "Conservation and prediction of solvent accessibility in protein families." Proteins **20**(3): 216-26.

Rost, B., G. Yachdav, et al. (2004). "The PredictProtein server." Nucleic Acids Res **32**(Web Server issue): W321-6.

Ryman, K. D., C. L. Gardner, et al. (2007). "Heparan-sulfate binding can contribute to the neurovirulence of neuro-adapted and non-neuro-adapted Sindbis viruses." J Virol.

Saito, T. and M. Gale, Jr. (2007). "Principles of intracellular viral recognition." Curr Opin Immunol **19**(1): 17-23.

Salazar, M. I., J. H. Richardson, et al. (2007). "Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes." BMC Microbiol **7**: 9.

Saleh, S. M., M. Poidinger, et al. (2003). "Complete genomic sequence of the Australian south-west genotype of Sindbis virus: comparisons with other Sindbis strains and identification of a unique deletion in the 3'-untranslated region." Virus Genes **26**(3): 317-27.

Saleh, S. M., M. Poidinger, et al. (2004). "Antigenic and genetic typing of Whataroa viruses in Australia." Am J Trop Med Hyg **71**(3): 262-7.

Sammels, L. M., M. D. Lindsay, et al. (1999). "Geographic distribution and evolution of Sindbis virus in Australia." J Gen Virol **80 (Pt 3)**: 739-48.

Sanchez, I. J. and B. H. Ruiz (1996). "A single nucleotide change in the E protein gene of dengue virus 2 Mexican strain affects neurovirulence in mice." J Gen Virol **77 (Pt 10)**: 2541-5.

Schlesinger, S. (2001). "Alphavirus vectors: development and potential therapeutic applications." Expert Opin Biol Ther **1**(2): 177-91.

Schneider, B. S., L. Soong, et al. (2004). "*Aedes aegypti* salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to sindbis virus infection." Viral Immunol **17**(4): 565-73.

Scott, T. W., S. W. Hildreth, et al. (1984). "The distribution and development of eastern

equine encephalitis virus in its enzootic mosquito vector, *Culiseta melanura*." Am J Trop Med Hyg **33**(2): 300-10.

Seabaugh, R. C., K. E. Olson, et al. (1998). "Development of a chimeric Sindbis virus with enhanced per Os infection of *Aedes aegypti*." Virology **243**(1): 99-112.

Serafin, I. L. and J. G. Aaskov (2001). "Identification of epitopes on the envelope (E) protein of dengue 2 and dengue 3 viruses using monoclonal antibodies." Arch Virol **146**(12): 2469-79.

Shahabuddin, M. and P. F. Pimenta (1998). "*Plasmodium gallinaceum* preferentially invades vesicular ATPase-expressing cells in *Aedes aegypti* midgut." Proc Natl Acad Sci U S A **95**(7): 3385-9.

Shao, L., M. Devenport, et al. (2001). "The peritrophic matrix of hematophagous insects." Arch Insect Biochem Physiol **47**(2): 119-25.

Smit, J. M., B. L. Waarts, et al. (2002). "Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin." J Virol **76**(20): 10128-37.

Smith, C. E. (1956). "The history of dengue in tropical Asia and its probable relationship to the mosquito *Aedes aegypti*." J Trop Med Hyg **59**(10): 243-51.

Smith, T. J., R. H. Cheng, et al. (1995). "Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy." Proc Natl Acad Sci U S A **92**(23): 10648-52.

Stec, D. S., A. Waddell, et al. (1986). "Antibody-selected variation and reversion in Sindbis virus neutralization epitopes." J Virol **57**(3): 715-20.

Storey, P., J. Theron, et al. (2007). "A second RGD motif in the 1D capsid protein of a SAT1 type foot-and-mouth disease virus field isolate is not essential for attachment to target cells." Virus Res **124**(1-2): 184-92.

Strauss, E. G., D. S. Stec, et al. (1991). "Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants." J Virol **65**(9): 4654-64.

Strauss, J. H. and E. G. Strauss (1994). "The alphaviruses: gene expression, replication, and evolution." Microbiol Rev **58**(3): 491-562.

Strauss, J. H., K. S. Wang, et al. (1994). "Host-cell receptors for Sindbis virus." Arch Virol Suppl **9**: 473-84.

Sumiyoshi, H., C. H. Hoke, et al. (1992). "Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates." J Virol **66**(9): 5425-31.

Summers, P. L., W. H. Cohen, et al. (1989). "Flaviviruses can mediate fusion from

without in Aedes albopictus mosquito cell cultures." Virus Res **12**(4): 383-92.

Sundin, D. R., B. J. Beaty, et. al. (1987). "A G1 glycoprotein epitope of La Crosse virus: a determinant of infection in *Aedes triseriatus*." Science **235**(4788):591-3.

Tabachnick, W. J. and J. R. Powell (1979). "A world-wide survey of genetic variation in the yellow fever mosquito, *Aedes aegypti*." Genet Res **34**(3): 215-29.

Tabachnick, W. J., G. P. Wallis, et al. (1985). "Oral infection of *Aedes aegypti* with yellow fever virus: geographic variation and genetic considerations." Am J Trop Med Hyg **34**(6): 1219-24.

Takahashi, M., T. Tsuda, et al. (2004). "Role of N-glycans in growth factor signaling." Glycoconj J **20**(3): 207-12.

Tan, B. H., E. Nason, et al. (2001). "RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to Culicoides cells." J Virol **75**(8): 3937-47.

Taylor, R. M., H. S. Hurlbut, et al. (1955). "Sindbis virus: a newly recognized arthropodtransmitted virus." Am J Trop Med Hyg **4**(5): 844-62.

Tiawsirisup, S., K. B. Platt, et al. (2004). "Susceptibility of *Ochlerotatus trivittatus (Coq.)*, *Aedes albopictus (Skuse)*, and *Culex pipiens (L.)* to West Nile virus infection." Vector Borne Zoonotic Dis **4**(3): 190-7.

Titus, R. G., J. V. Bishop, et al. (2006). "The immunomodulatory factors of arthropod saliva and the potential for these factors to serve as vaccine targets to prevent pathogen transmission." Parasite Immunol **28**(4): 131-41.

Tomiya, N., M. J. Betenbaugh, et al. (2003). "Humanization of lepidopteran insect-cell-produced glycoproteins." Acc Chem Res **36**(8): 613-20.

Tomiya, N., S. Narang, et al. (2004). "Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines." Glycoconj J **21**(6): 343-60.

Tucker, P. C., E. G. Strauss, et al. (1993). "Viral determinants of age-dependent virulence of Sindbis virus for mice." J Virol **67**(8): 4605-10.

Tugizov, S. M., J. W. Berline, et al. (2003). "Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells." Nat Med **9**(3): 307-14.

Turell, M. J., T. P. Gargan, 2nd, et al. (1984). "Replication and dissemination of Rift Valley fever virus in *Culex pipiens*." Am J Trop Med Hyg **33**(1): 176-81.

Turell, M. J., G. V. Ludwig, et al. (1999). "Limited potential for mosquito transmission of genetically engineered, live-attenuated Venezuelan equine encephalitis virus vaccine candidates." Am J Trop Med Hyg **60**(6): 1041-4.

Tussel, S. M., S. A. Schittone, et al (2007). " Mutational analysis of aminopeptidase , a Receptor for several group I coronaviruses, ifentifies key determinants of viral

host range." J. Virol **81**(3): 1261-73.

Twiddy, S. S., J. J. Farrar, et al. (2002). "Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus." Virology **298**(1): 63-72.

Ubol, S. and D. E. Griffin (1991). "Identification of a putative alphavirus receptor on mouse neural cells." J Virol **65**(12): 6913-21.

Uzcategui, N. Y., D. Camacho, et al. (2001). "Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination." J Gen Virol **82**(Pt 12): 2945-53.

van der Most, R. G., J. Corver, et al. (1999). "Mutagenesis of the RGD motif in the yellow fever virus 17D envelope protein." Virology **265**(1): 83-95.

Vanlandingham, D. L., K. Tsetsarkin, et al. (2006). "Determinants of vector specificity of o'nyong nyong and chikungunya viruses in *Anopheles* and *Aedes* mosquitoes." Am J Trop Med Hyg **74**(4): 663-9.

Varma, M. G., M. Pudney, et al. (1974). "Cell lines from larvae of *Aedes* (Stegomyia) *malayensis Colless* and *Aedes* (S) *pseudoscutellaris* (Theobald) and their infection with some arboviruses." Trans R Soc Trop Med Hyg **68**(5): 374-82.

Vashishtha, M., T. Phalen, et al. (1998). "A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit." J Cell Biol **140**(1): 91-9.

Vlaycheva, L., M. Nickells, et al. (2005). "Neuroblastoma cell-adapted yellow fever virus: mutagenesis of the E protein locus involved in persistent infection and its effects on virus penetration and spread." J Gen Virol **86**(Pt 2): 413-21.

Vrati, S., C. A. Fernon, et al. (1988). "Location of a major antigenic site involved in Ross River virus neutralization." Virology **162**(2): 346-53.

Vrati, S., P. J. Kerr, et al. (1996). "Entry kinetics and mouse virulence of Ross River virus mutants altered in neutralization epitopes." J Virol **70**(3): 1745-50.

Wahlberg, J. M., R. Bron, et al. (1992). "Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein." J Virol **66**(12): 7309-18.

Wahlberg, J. M. and H. Garoff (1992). "Membrane fusion process of Semliki Forest virus. I: Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells." J Cell Biol **116**(2): 339-48.

Wang, E., H. Ni, et al. (2000). "Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses." J Virol **74**(7): 3227-34.

Wang, K. S., R. J. Kuhn, et al. (1992). "High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells." J Virol **66**(8): 4992-5001.

Wang, K. S., A. L. Schmaljohn, et al. (1991). "Antiidiotypic antibodies as probes for the Sindbis virus receptor." Virology **181**(2): 694-702.

Wang, K. S. and J. H. Strauss (1991). "Use of a lambda gt11 expression library to localize a neutralizing antibody-binding site in glycoprotein E2 of Sindbis virus." J Virol **65**(12): 7037-40.

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**(3): 624-31.

Weaver, S. C. (2006). "Evolutionary influences in arboviral disease." Curr Top Microbiol Immunol **299**: 285-314.

Weaver, S. C., M. Anishchenko, et al. (2004). "Genetic determinants of Venezuelan equine encephalitis emergence." Arch Virol Suppl(18): 43-64.

Weaver, S. C. and A. D. Barrett (2004). "Transmission cycles, host range, evolution and emergence of arboviral disease." Nat Rev Microbiol **2**(10): 789-801.

Weaver, S. C., L. H. Lorenz, et al. (1992). "Pathologic changes in the midgut of *Culex tarsalis* following infection with Western equine encephalomyelitis virus." Am J Trop Med Hyg **47**(5): 691-701.

Weaver, S. C., W. F. Scherer, et al. (1984). "Barriers to dissemination of Venezuelan encephalitis viruses in the Middle American enzootic vector mosquito, *Culex (Melanoconion) taeniopus*." Am J Trop Med Hyg **33**(5): 953-60.

Weaver, S. C. and T. W. Scott (1990). "Ultrastructural changes in the abdominal midgut of the mosquito, *Culiseta melanura*, during the gonotrophic cycle." Tissue Cell **22**(6): 895-909.

Weaver, S. C., T. W. Scott, et al. (1988). "Togavirus-associated pathologic changes in the midgut of a natural mosquito vector." J Virol **62**(6): 2083-90.

Wekesa, J. W., B. Yuval, et al. (1997). "Multiple blood feeding by *Anopheles freeborni* and *Culex tarsalis* (Diptera:Culicidae): spatial and temporal variation." J Med Entomol **34**(2): 219-25.

Wengler, G., A. Koschinski, et al. (2003). "Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents." J Gen Virol **84**(Pt 1): 173-81.

West, J., R. Hernandez, et al. (2006). "Mutations in the endodomain of Sindbis virus glycoprotein E2 define sequences critical for virus assembly." J Virol **80**(9): 4458-68.

Whitfield, S. G., F. A. Murphy, et al. (1973). "St. Louis encephalitis virus: an

ultrastructural study of infection in a mosquito vector." Virology **56**(1): 70-87.

WHO (1985). WHO Tech Rep Ser No 719. Arthropod-borne and rodent-borne viral diseases. Geneva, Switzeland, WHO.

WHO (1995). "Vector control for malaria and other mosquito-borne diseases. Report of a WHO study group." World Health Organ Tech Rep Ser **857**: 1-91.

WHO (1997). Dengue hemorrhagic fever: diagnosis, tratment , prevention and control., World Health Organization, Geneva, Switzerland.

WHO (2003). World Health Report 2003, World Health Organization, Geneva, Switzerland.**:** 154-159.

Woodring JL, H S, and Beaty BJ (1996). Natural Cycles of Vector-Borne Pathogens. The Biology of Disease Vectors. B. J. Beaty, Marquardt, W.C,. Niwot, CO, University Press of Colorado. **1:** 51-72.

Woodward, T. M., B. R. Miller, et al. (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)**: 2431-5.

Wu, S. C., W. C. Lian, et al. (1997). "Japanese encephalitis virus antigenic variants with characteristic differences in neutralization resistance and mouse virulence." Virus Res **51**(2): 173-81.

Wu, S. J., G. Grouard-Vogel, et al. (2000). "Human skin Langerhans cells are targets of dengue virus infection." Nat Med **6**(7): 816-20.

Xu, G., W. Wilson, et al. (1997). "VP7: an attachment protein of bluetongue virus for cellular receptors in *Culicoides variipennis*." J Gen Virol **78 (Pt 7)**: 1617-23.

Yamshchikov, V. F., G. Wengler, et al. (2001). "An infectious clone of the West Nile flavivirus." Virology **281**(2): 294-304.

Zeidner, N. S., S. Higgs, et al. (1999). "Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice." Parasite Immunol **21**(1): 35-44.

Zhang, W., S. Mukhopadhyay, et al. (2002). "Placement of the structural proteins in Sindbis virus." J Virol **76**(22): 11645-58.

Zhang, Y., W. Zhang, et al. (2004). "Conformational changes of the flavivirus E glycoprotein." Structure **12**(9): 1607-18 (PDB ID: 1TG8).

Zieler, H., C. F. Garon, et al. (2000). "A tubular network associated with the brush-border surface of the *Aedes aegypti* midgut: implications for pathogen transmission by mosquitoes." J Exp Biol **203**(Pt 10): 1599-611.