DISSERTATION

DETERMINANTS OF DENGUE TYPE 2 VIRUS INFECTION IN THE MOSQUITO *Aedes aegypti*

Submitted by:

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY *MA. ISABEL SALAZAR SANCHEZ* ENTITLED "DETERMINANTS OF DENGUE TYPE 2 VIRUS INFECTION IN THE MOSQUITO *Aedes aegypti*" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DETERMINANTS OF DENGUE TYPE 2 VIRUS INFECTION IN THE MOSQUITO *Aedes aegypti*

Dengue represents a public health problem in 112 countries worldwide. The control of this mosquito-borne disease has been hindered by the fact that there are neither approved vaccines nor effective drugs available. Additionally, the mosquito eradication programs have failed and the vector has reemerged in areas where it had been previously eradicated.

In this dissertation project, DENV determinants of infection in its primary vector, the mosquito *Aedes aegypti*, were investigated. Potential viral as well as vector determinants of infection were examined. The rationale for this research was that a better understanding of virus-vector interactions will enable us identification of new potential targets for virus transmission intervention.

The kinetics of infection and dissemination in the mosquito revealed the nature of midgut infection by DENV-2 and the persistence of viral RNA in midgut epithelial cells. The analysis of virus dissemination exposed the tracheal system as a potential conduit. The infection of salivary glands occurred as early as 4 days after an infectious blood meal in a dose-dependent manner. Virus exhibited striking tropisms for salivary glands and mosquito head tissues.

The correlation between DENV-2 clinical severity in humans and virogenesis in *Aedes aegypti* mosquitoes was investigated. Four viruses of the American/Asian genotype (Yucatan strains), which were isolated from cases of different clinical severity, were characterized in mosquitoes. The case severity caused by these viruses significantly

correlated with the dissemination rates to head tissues and virus titers in Chetumal (coindigenous) mosquitoes.

The phenotypic and genotypic analysis revealed surprising differences when the DENV-2 American/Asian genotypes were compared to a virus from the American genotype from the same geographic region, the Yucatan Peninsula. Dissemination rates by the DENV-2 American/Asian genotype greatly exceeded the ones caused by the member of the American genotype. Differences occurred in E protein and 3'UTR sequences. However, the 3'UTR exhibited the most significant changes, which included nucleotide substitutions in structurally crucial domains that participate in virus translation/replication.

Finally, physiological and anatomical conditions could also be determinants of DENV-2 infection and dissemination in the mosquito vector. These include the pH in the mosquito midgut and the ingestion of cell-associated DENV-2.

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can fly"

Ivan Petrovich Pavlov

"El lenguaje de los hechos es más elocuente que el lenguaje

de las palabras"

Ivan Petrovich Pavlov

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Chapter I

Literature Review

1.1. Dengue- The disease

Dengue infection can cause illnesses ranging from inapparent or mild disease to a severe and occasionally fatal disease (111). The two main clinical manifestations of dengue are: classic dengue fever (DF) and dengue hemorrhagic fever (DHF). The latter can progress to dengue shock syndrome (DSS). DHF and DSS are severe forms of the disease that can be life-threatening.

1.1.1 History and global importance

The first known cases of DF reportedly occurred in 1779, in Batavia (Jakarta), Indonesia and Cairo, Egypt. Another important epidemic of DF took place in Philadelphia in 1780, when Benjamin Rush described the disease, referring to it as "break-bone fever" (252). However, records of an infectious disease similar to dengue appear in Chinese documents dated from the Chin Dynasty (265 to 420 A.D.), which were compiled during the Tang (610 A.D.) and the Northern Sung Dynasties (992 A.D.). The condition was described as water poison connected with flying insects. Outbreaks of illness in the French West Indies in 1635 and in Panama in 1699 may have been dengue as well (104). It was during an epidemic in Cuba in 1928, when the term "dengue" became well-known.

DHF cases first arose in Manila, Philippines, between 1953 and 1954. Within 20 years DHF spread throughout Southeast Asia and became a leading cause of admission to the hospitals and death among children in that area (104). Currently, the dengue pandemic affects more than 100 countries, including nations in Africa, the Americas, the Indian subcontinent, Southeast Asia, the Eastern Mediterranean, and the Western Pacific (61). The World Health Organization (WHO) estimates 50 million cases of dengue infection worldwide, about 500,000 hospital admissions, and 22,000 dengue-related deaths occur every year. Globally, more than 2.5 billion people are at risk of this disease. Although epidemic DF and DHF are major public health problems worldwide,

its emergence has been most dramatic in Latin America. A striking increase in DHF cases has happened in this region since 1989 (104, 105). The medical importance of dengue may increase since neither reliable vaccines nor effective antiviral drugs are available to prevent or treat the disease. In addition mosquito eradication programs have failed, and the vector has resurged in most tropical and subtropical areas.

1.1.2. Characteristics and risk factors for the disease

Albert Sabin successfully isolated dengue virus in 1944 (253, 254). Dengue virus includes four genetically related, but antigenically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Although these viruses infect almost solely humans, sylvatic cycles occur among nonhuman primates in Southeast Asia and Western Africa (303). In endemic areas of dengue, two or more serotypes may circulate simultaneously or successively, increasing the risk of DHF and DSS cases.

DF is a mild form of the disease. It starts with abrupt onset of high fever that lasts for 2-7 days (often biphasic), chills, headache with frontal and retro-orbital pain and rash. Clinical symptoms include severe myalgia and arthralgia, therefore the term, "break-bone" fever. In DHF bleeding occurs in the skin (petechiae) and occasionally in the gums and nose. Increased vascular permeability can result in leakage of plasma into extravascular spaces, leading to DSS. Plasma leakage, hemorrhages and complement activation are hallmarks of DHF and DSS (104, 105, 115, 250). The WHO has classified clinical DHF/DSS. Disease can be mild (grades I and II) or severe (grades III and IV). Grade III and IV may be associated with DSS (Table 1.1) (WHO, 1975).

Grade I	Fever, constitutional symptoms, positive tourniquet test
Grade II	Grade I + spontaneous bleeding (skin, gums, GI tract)
Grade III*	Grade II + circulatory failure and agitation, clammy skin, hypotension
Grade IV*	Grade III + profound shock (unrecordable blood pressure)

Table 1.1. WHO classification of DHF. Grades I to III also include hepatomegaly, low platelet counts and high hematocrit levels. *Classification associated with DSS.

The incubation period for dengue in humans ranges from 3 to 6 days, but occasionally reaches 15 days. Historically, DHF in Southeast Asia has been predominantly a disease of children (below 15 years old). Capillary fragility varies by age, providing partial explanation for the high number of DHF and DSS cases in children (Figure 1.1) (82, 155). However, in the Americas DHF commonly occurs in older patients. During the 1980's and the 1990's, an increase in the number of DHF cases in adults occurred in countries such as Malaysia and the Philippines (111). Infection with dengue virus results in lifelong homotypic immunity, but only temporary immunity to other serotypes (243).

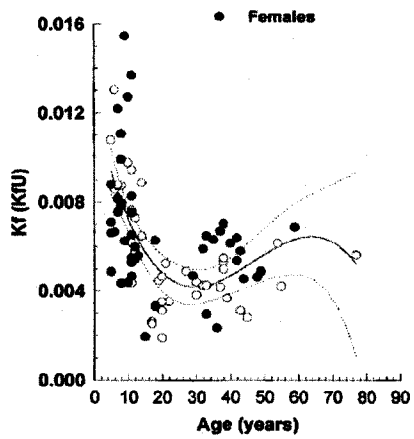


Figure 1.1. Age-related changes in microvascular permeability. The filtration capacity (Kf) is an index of microvascular hydraulic permeability. Solid circles represent female subjects and empty circles male subjects. The solid line represents a third-order polynomial, best fit for these data From: Gamble *et.al.*, 2000 (82).

A preexisting immunity in the host to any of the four dengue serotypes is a risk factor to develop DHF and DSS in secondary infections with a different serotype. Antibody dependent enhancement (ADE) may explain this feature (78, 114, 115, 117). However, primary DENV infections can also result in severe disease (315). Therefore the exact pathogenesis of DHF remains a controversy.

DHF in infants occurs between 6 and 10 months of age. The titers of homologous maternal neutralizing antibodies (at birth) and at the onset age negatively correlate with

disease severity (Figure 1.2). Maternal dengue antibodies may play a dual role by first reducing and later increasing the risk for DHF and DSS in patients (116, 152). Mortality associated with severe dengue is consistently greater in infants than in children (143).

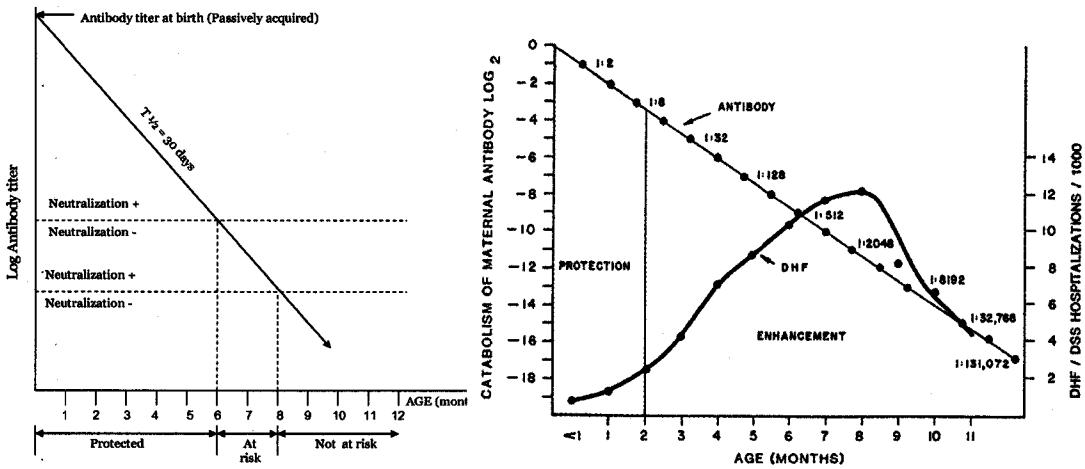


Figure 1.2. Correlation of titers of homologous maternal neutralizing antibodies (at birth) and the infant age at onset. A) Schematic demonstration of the temporary dependency of disease outcome and maternal antibodies degradation (152). B) Schematic representation of the dual role of maternal antidengue antibodies on disease severity in infants (116, 308).

The cytokine profile in infants differs between primary and secondary infections. In addition, secondary dengue infections are more often associated with development of DHF in children ($P < 0.0001$) than in adults (315). Overproduction of IFN-γ and TNF-α, (proinflammatory cytokines), and IL-10/IL-6 (anti-inflammary cytokines) in severe disease suggests a role for these cytokines in DHF and DSS pathogenesis (132). Genetics plays a role in susceptibility to severe forms of dengue in humans as well (164, 181, 182, 224, 276, 328). Humans of Caucasian and Asian genetic makeup are more susceptible than those of African ancestry.

HLA typing has revealed that alleles such as HLA-A1 and HLA-Cw1 are significantly more common in patients that develop DHF than in controls with DF (192, 276). A study of Thai patients infected with any DENV serotype revealed HLA class I associations with disease severity in secondary infections. HLA-A*0203, HLA-B*51, and HLA-DRB1*04 were associated with relative resistance to DHF, regardless of the

secondary infecting virus serotype (164, 276). In contrast, HLA-A*0207 and HLA-B*52 were related to susceptibility to DHF in secondary infections caused by DENV-1 and DENV-2. HLA-B44, B62, B76 and B77 were associated with protection against developing clinical disease in secondary infections (276).

Because of the significance of flaviviruses as human pathogens, understanding better their genetic nature, pathogenesis, and transmission mechanisms is critical to find effective ways to control them. Individual, epidemiological and viral factors participate in dengue transmission and disease outcome (Figure 1.3).

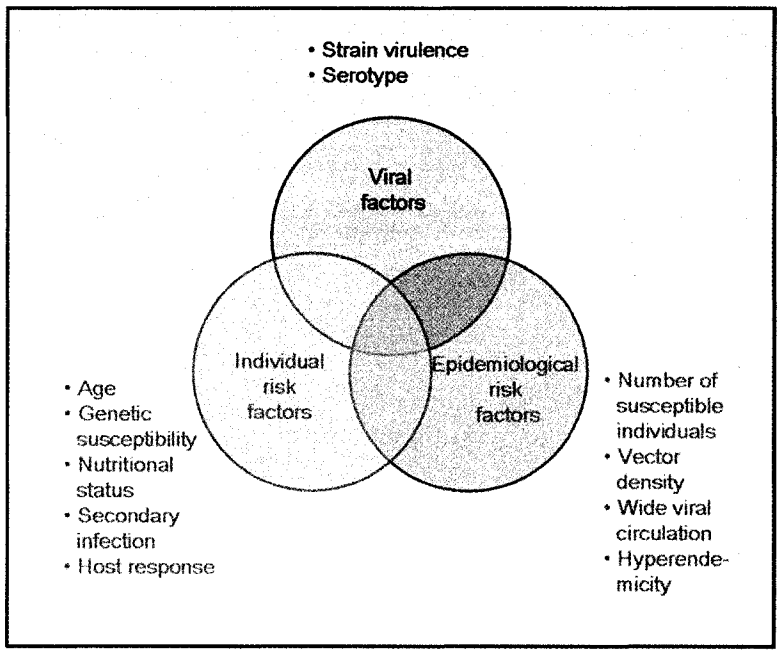


Figure 1.3. Factors that influence dengue disease outcome (Adapted from: Guzmán and Kourí, 2002).

1.1.3. Control of dengue

Dengue and yellow fever virtually vanished in the Americas from 1946 to the late 1970s as a result of successful *Aedes aegypti* eradication programs conducted by the Pan American Health Organization (PAHO). These programs focused on mosquito larval control using source reduction and insecticides spraying, primarily dichlorodiphenyl-trichloroethane (DDT). The program was abandoned in the early 1970s, since the

problem was no longer perceived as a threat to public health. As consequence, *Aedes aegypti* re-infested all of the countries in which it had been eradicated and dengue reappeared in Central and South America (103).

There are no vaccines or effective therapies available against DENV. A viable way to decrease incidence is to control the mosquito vector, *Aedes aegypti*. Source reduction is by far the most important measure, which focuses on decreasing or removing breeding sites or stopping mosquitoes from laying eggs. Larviciding using temefos is the principal control measure, but resistance is becoming prevalent in many areas.

Since the mosquito is anthropophilic and endophagic, indoor residual spraying is used against *Aedes aegypti* too. Space sprays for the control of adult mosquito populations are widely used. In the Southeast Asia and Western Pacific regions, adulticides are especially used to control vectors during outbreaks of mosquito-borne arboviral diseases such as dengue and Japanese encephalitis.

1.2. Dengue virus- The pathogen

Dengue is the most important mosquito-borne viral disease affecting humans. DENV is a member of the family *Flaviviridae*, which consists of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. The genus *Flavivirus* includes important human pathogens such as DENV, Yellow fever virus (YF), West Nile virus (WNV), and Japanese encephalitis virus (JEV). DENV as other arboviruses must infect and successfully replicate in two phylogenetically distant organisms, vertebrates and arthropods, to sustain their transmission cycle.

1.2.1. Flaviviruses

The *Flavivirus* genus includes about 73 viruses, forty of which have been associated with human diseases. Of these, 34 are mosquito-borne, 17 tick-borne, and 22 are zoonotic agents with no known vector (176) (Table 1.2). Significant and important

differences exist between the genomes of mosquito and the tick-borne flaviviruses (Figure 1.4) (177).

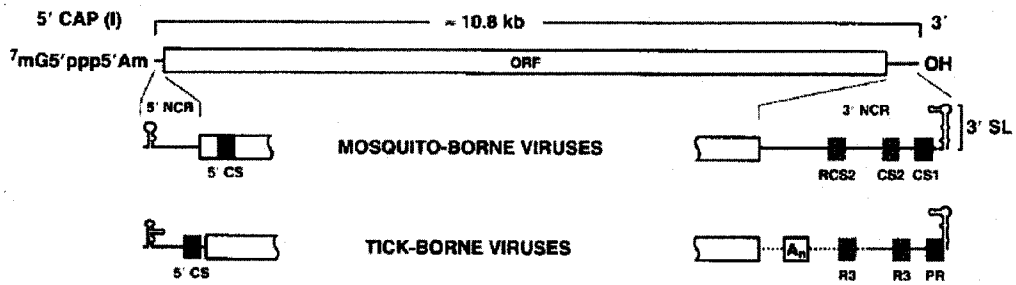


Figure 1.4. Flavivirus genome organization. Comparable genomic motifs for mosquito- and tick-borne flaviviruses are shown. These include the 5' NCR, 5' CS (conserved sequence) and the 3' SL (stem loop). Mosquito-borne viruses contain CS1 and one or two CS2 motifs (CS2 and RCS2) in the 3' NCR. The PR sequence in tick-borne viruses may interact with the 5' CS overlapping the 3' SL. From: Lidenbach and Rice, 2003 (177).

Members of the genus *Flavivirus* transmitted by mosquitoes share common antigens that permit grouping them in serocomplexes. These flaviviruses are classified in three major groups: the dengue, the Japanese encephalitis, and the Yellow fever serocomplexes. The dengue serocomplex includes all four serotypes of DENV. The Japanese encephalitis serocomplex contains viruses such as West Nile, Kunjin, Murray Valley encephalitis, St. Louis encephalitis and Japanese encephalitis. The tick-borne flaviviruses consists of Powassan, Louping ill and Tick-borne encephalitis viruses among others (176).

	Flaviviruses		
Vector	**Total number**	**Human pathogens**	**Diseases**
Mosquito-Borne	**34**	**22**	**Dengue, Yellow fever, West Nile, Japanese encephalitis, St. Louis encephalitis, Murray Valley encephalitis, Ilheus.**
Tick-Borne	**17**	**13**	**Russian spring-summer fever encephalitis, Omsk hemorrhagic fever, Powassan.**
No known Vector	**22**	**5**	**Rio bravo encephalitis, Rocio encephalitis.**

Table 1.2. Flaviviruses: number, vectors and diseases passed on humans. (From Fields, Virology) (176).

The mosquito-borne group includes different kinds of flaviviruses: those associated with encephalitis and those associated with fevers or hemorraghic fevers. The

first group includes St. Louis encephalitis, Japanese encephalitis, West Nile, Murray Valley, and Ilheus viruses. The second group includes Omsk hemorrhagic fever, Kyasanur Forest disease, DENV, and Yellow fever virus (176).

1.2.2. Virus Genome and Replication

The DENV genome is a single-stranded positive-sense RNA of about 11 Kb in length. It contains a single open reading frame (ORF) and a 5'-type I cap, but lacks a 3'-poly (A) tail. In the cytoplasm, the host cell translational machinery translates the viral RNA into a polyprotein. Host and viral proteases break down the polyprotein posttranslationally resulting in a 10 mature viral proteins: C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Appendix 7.1) (Figure 1.5).

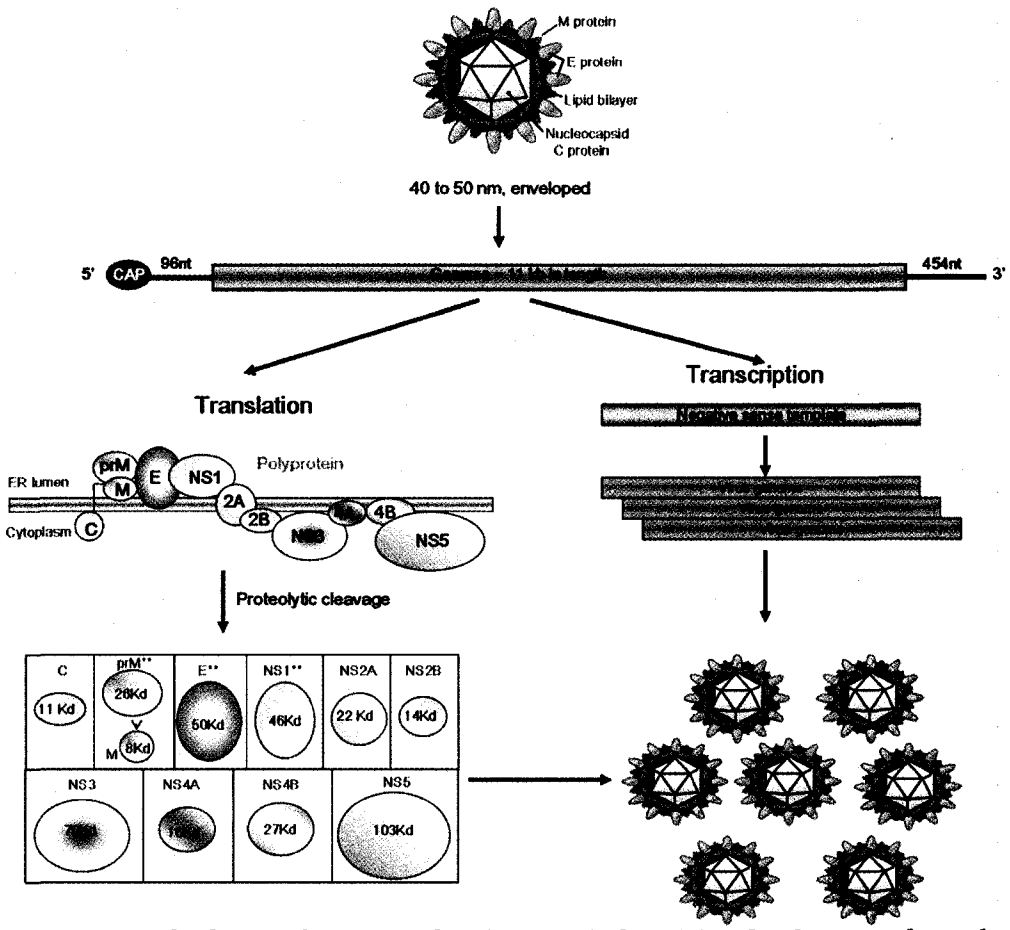


Figure 1.5. General scheme of DENV replication. ** Viral proteins that become glycosylated in the host cell endoplasmic reticulum.

A host signal peptidase is responsible for the cleavage between C-prM, prM-E, and E-NS1. The virus-encoded NS2B-NS3 serine protease is responsible for the cleavage among NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B, NS4B/NS5, and also an internal cleavage in C protein. The enzyme responsible for NS1-NS2A cleavage remains unknown (177).

Formation of intracellular prM:E heterodimers occurs rapidly after translation and is important for the assembly and secretion of virus particles (227). Virus particles assemble by budding into the RER (62, 192, 216). The assembled virus particles pass through the host secretory pathway where maturation takes place (38, 120). Vesicles may fuse to the plasma membrane and release virions from the host cell.

Several of the nonstructural (NS) proteins associate to form the viral replicase complex. The positive-strand viral genome binds to the viral replicase complex by the 3' NCR and produces a minus-strand intermediate. This intermediate serves as the template for the synthesis of positive-strand genomes. Specific RNA motifs in the 3' terminus of the positive-strand genome are not in the 3'-terminus of the complementary minus strand. These motifs may regulate transcription or translation initiation (225). As for Hepatitis C virus (HCV), EF-1α (translation elongation factor- 1α), La autoantigen, and PTB (polypyrimidine tract-binding protein) host proteins probably take part in DENV replication (56, 83). Early in the viral infection negative and positive-strand RNA synthesis occurs at similar rates, but the ratio eventually becomes asymmetric, favoring positive-strand synthesis as the infection progresses. Extensive expansion of membranous organelles (vesicle packets) is characteristic of flavivirus-infected cells. The vesicle packets (VP) harbor and protect the virus replicative forms (RF) (295).

Flavivirus genomes carry conserved and essential motifs important for virus replication (48, 225, 288). Cis-acting elements in the 5' and 3' untranslated regions anneal, causing RNA cyclization and allowing the genome to replicate (112, 148, 179,

326). The short and long stem-loops at the 3' NCR (3'-SL) are critical for infectivity in DENV (325). These same motifs in West Nile virus (WNV) have a role in RNA synthesis but not in translation (289). Leitmeyer *et al.*, important differences in the 3' NCR of American and Southeast Asian genotypes of DENV-2 (171). However, these results are controversial since further studies showed that the predicted structures conflict with the essential cyclization domains, thus they were not likely to be formed (288).

1.2.3. Virus Particle Structure

Dengue virions are spherical, between 40-50 nm in diameter, and have a lipid bilayer envelope. Multiple studies have provided a detailed description of the viral particle structure (158, 198, 326) (Figure 1.6). Significant differences exist between the immature and mature DENV particles. The immature particle displays abundant spikes, which consist of three prM:E protein heterodimers. The prM protein in each spike protects the fusion peptide of the E protein; this results in a larger diameter (about 15 %) of the immature particle (326).

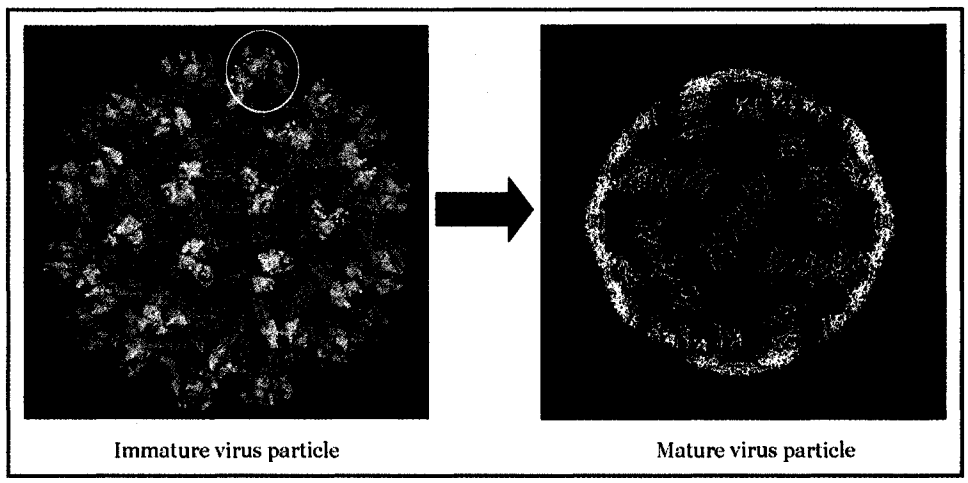
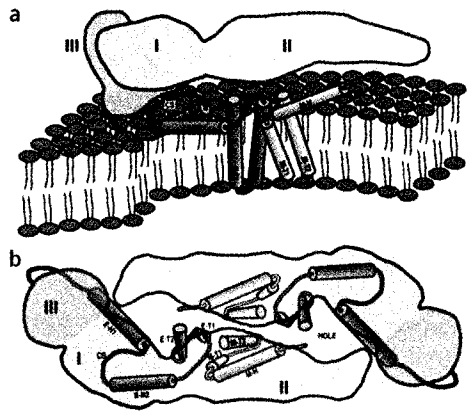


Figure 1.6. Diagram of DENV immature and mature particles (From: Kuhn *et al.*, 2002; Zhang *et al.*, 2003) (158, 326). Circle shows spikes, which are composed by three prM:E heterodimers.

In the mature particle, there are no spikes and E and M proteins are buried in the virus membrane (202). DENV particles purified from Vero cell culture contain a mixture of prM and M proteins, probably caused by an inefficient cleaved of prM during the late

stages of maturation. Whether differences exist between the viral particles produced in mosquito and mammalian cells is not clear. The prM protein in the viral particles is cleaved by the host convertase furin during export through the secretory pathway. The cleaved causes rearrangement of the E glycoprotein and probably plays a role in virus infectivity. In Vero cells, virions containing prM are infective but their export is affected (146, 304).

1)



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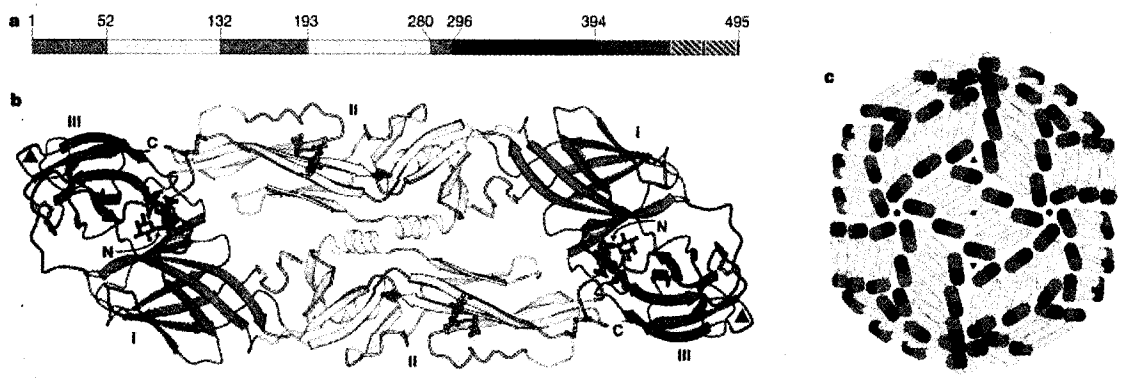


Figure 1.7. DENV E protein conformation on the surface of a mature virus particle. 1) Diagram of the ectodomain and transmembrane domain proteins (From: Zhang *et al.*, 2003) (326). 2) Domain locations in the E viral glycoprotein. Domain I contains the 8-stranded central β-barrel. Domain II contains the fusion peptide as its tip and contains 12 β-strands and two α-helices. Domain III has an Ig-like motif with 10 β-strands (From: Modis *et al.*, 2004) (196).

The E glycoprotein of DENV mediates binding and fusion during host cell infection and is the major surface protein in the viral particle. It lies in a flat orientation

on the envelope surface of the virus. In the endosomal compartment, after entry, the E protein reclusters irreversibly from its homodimeric native state into a homotrimeric fusogenic complex. This change in conformation occurs at a pH below 6.3, exposing the fusion peptide to the cell membrane (2, 120, 269, 302). In its native state the fusion peptide remains buried in the E glycoprotein dimer (195, 196, 232, 278, 279). The protein consists of 495 amino acids (1485 nt), which are functionally grouped into three domains (Figure 1.7).

All the domains enclose numerous beta sheet folds (130). Domain I contains the molecular hinge region involved in the conformational changes occurring at low pH. Domain II is involved in dimerization and contains the fusion peptide. Finally, domain III may take part in virus attachment to its receptor and contains the carboxy-terminal Ig-like section. There are two glycosylated asparagines (Asn) on each DENV E subunit: Asn-153 on domain I and Asn-67 on domain II (195). Domain III undergoes the most significant displacement in the dimer-to-trimer transition. This transition is irreversible, since trimers are structurally more stable than dimers (196).

1.2.4. Virus evolution

The ability of DENV of RNA viruses to adapt results from the remarkable evolutionary potential of their RNA genomes. Although genetic diversity is common in arboviruses, several of them clearly evolve more slowly than other RNA and DNA viruses (Figure 1.8) (127). In general, virus genome evolution measured by nonsynonymous changes occurs at a smaller rate for vector transmitted (arboviruses) than for nonvector transmitted viruses. Thus, the alternation between hosts may constrain arbovirus evolution (311).

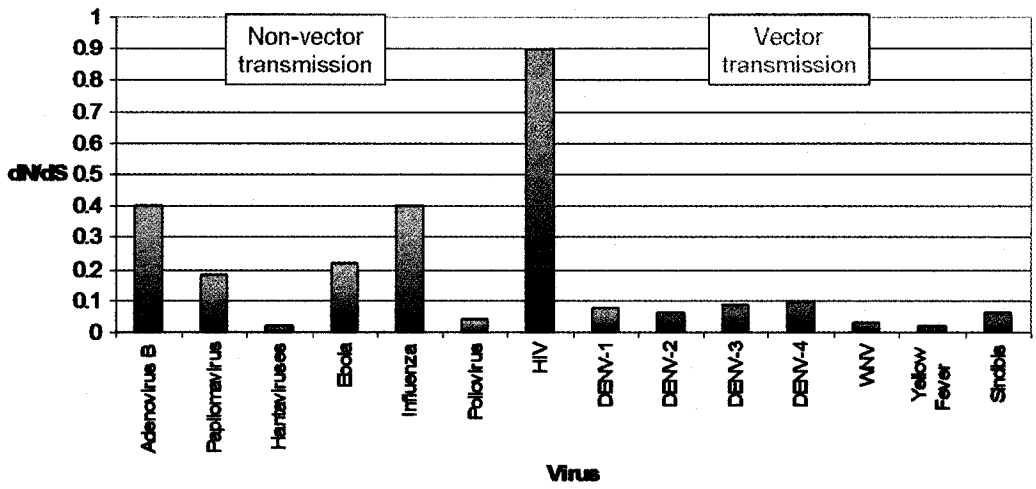


Figure 1.8. Evolutionary potential measured by d_N/d_S ratio. Clearly some nucleotide substitutions may result in amino acid replacements caused by transitions (T↔C, A↔G) or transversions (T↔A, T↔G, C↔A, C↔G). Nucleotide substitutions resulting in amino acid changes are nonsynonymous (d_N), while substitutions that do not cause an amino acid replacement are synonymous (d_S). The d_S substitutions are usually neutral and appear at a much faster rate than d_N substitutions because they do not affect the protein role. Nonvector and vector transmitted viruses obviously differ in restraints for amino acid changes in the virus sequence. DENV evolutionary potential may be low because of these constraints (127).

Flavivirus polymerases, like all RNA-dependent RNA polymerases (RdRps), are error prone and lack proofreading capacity. Consequently, there is continuous generation of mutant genomes within each replication cycle. Mutation rates can also depend on intracellular environmental changes such as relative concentrations of nucleotide substrates, intracellular ionic composition, and presence of mutagens. Since tolerance to different mutations varies, mutation rates are not necessarily an accurate predictor of mutant frequencies (67). Substitution rates for dengue range from 4.55×10^{-4} (DENV 1) to 9.01×10^{-4} (DENV 3) substitutions per site a year (293). In contrast the average substitution rate for other RNA viruses is approximately 10^{-3} substitutions per site a year.

The most frequent amino acid changes created by mutation in the DENV genome occur in the E glycoprotein, suggesting that these regions are constantly under selection. The E protein of DENV is the major determinant of antigenicity, tropism, binding, entry, and fusion. Other regions under positive selective pressure include amino acids in NS2B

and NS5 proteins of DENV-2. The capsid, prM and nonstructural genes (NS1, NS2A, NS3 and NS4) seem to be constrained by their role in infectivity (291, 294).

Additional distinctive features of RNA virus replication include high yields of viral particles, short replication times and occasional recombination events. Reports on dengue recombination are based only on phylogenetic analysis; no direct laboratory evidence has been obtained (53, 128, 290, 292, 296, 319). Therefore, rather than being homogeneous, RNA virus populations consist of a complex distribution of closely related, but not identical, mutant and sometimes recombinant viral genomes known as *quasispecies* (69).

The quasispecies model best explains the diversity and dynamics of RNA viral populations. Quasispecies enable viral populations to persist in their hosts and allow them at the same time to exploit new hosts or conditions (68). In a quasispecies pool, the RNA viral genome that is most fit in a respective environment becomes the predominant sequence (master sequence) among progeny by selection. If the environment remains constant, this master sequence increases in numbers representing a large peak in the adaptive landscape. Hence, if a viral population is in a constant environment the master sequence is isolated repeatedly, despite the high mutation rates in that population. Clearly, viral populations harboring many mutants with large differences in fitness would allow rapid adaptation to new environmental conditions. The "Red Queen" theory of evolution explains some of the characteristics in the dynamics of viral quasispecies (273). The Red Queen, Carrol's character in "Through the looking glass", states *"in this place it takes all the running you can do, to keep in the same place"*. Indeed, the virus keeps interacting with the host through constant change, "co-evolving".

Two other characteristics associated with the quasispecies model are error-catastrophe and quasispecies memory. A prediction for the quasispecies is the co-existence of both: 1) an organized mutant variety and 2) random mutant sequences

lacking information (69). However, high rates of mutation conduct the population of quasispecies to approach error-catastrophe. Once there even a slight increase in mutation frequency should cause a dramatic fall in virus infectivity (70, 98), by forcing some viral quasispecies into extinction. The quasispecies distribution is also limited by purifying selection. Therefore, mechanisms working to limit harmful mutations, such as the so-called "mutational robustness" or recombinational events are valuable to preserve the viral fitness. The selective advantage of a robust system is to decrease susceptibility to deleterious mutations (300).

"Memory" in viral quasispecies explains the composition and behavior of a viral population in response to a selective constraint. The past evolutionary history has influence in this response in a way not predictable by examining the consensus nucleotide sequences in that population. Nature and intensity of the virus response to a selective constraint also influence the molecular memory of viral quasispecies (251).

1.2.5. Virus determinants of virulence

The broad range of clinical outcomes in dengue relate not only to the interaction with the host immune response but also to the genetic make up of the virus. Remarkable differences in virulence of flaviviruses result from minor changes in viral proteins (34, 71, 167, 180, 188, 193, 271, 320). Genetic determinants associated with virulence in dengue are frequently amino acid changes in viral E, prM and NS3 proteins (71, 171). Also certain nucleotide changes in 3' nontranslated region (NTR) associate with virulence (171, 213, 214, 226). Thus, virulence relates importantly to viral sequences affecting both tropisms and replication efficiency (Appendix 7.2). The viral E protein participates in binding, entry and fusion to host cells. Mutations in domain I and II of the E protein that alter the fusion pH threshold can affect virulence as well (195).

Among all dengue serotypes, DENV-2 is the most virulent and widely distributed serotype. Phylogenetic analysis reveals five major genotypes for DENV-2: Asian 1, Asian

2, American, American/Asian and Cosmopolitan genotypes (64, 127, 184, 291). The Asian 1 genotype contains many of the most virulent DENV-2 strains. Replacement of the old American DENV-2 genotype by new Southeast Asian ones and introduction of a DENV-3 serotype virus (Sri Lanka genotype) contributed to the emergence of DHF and DSS in the Americas (6, 7, 50, 184, 238, 296).

In Mexico DHF did not appear until 1995. This event was associated with the introduction of a new DENV-2 genotype, introduction of the DENV-3 serotype, and the co-circulation of DENV-1 and DENV-4 (Figure 1.9).

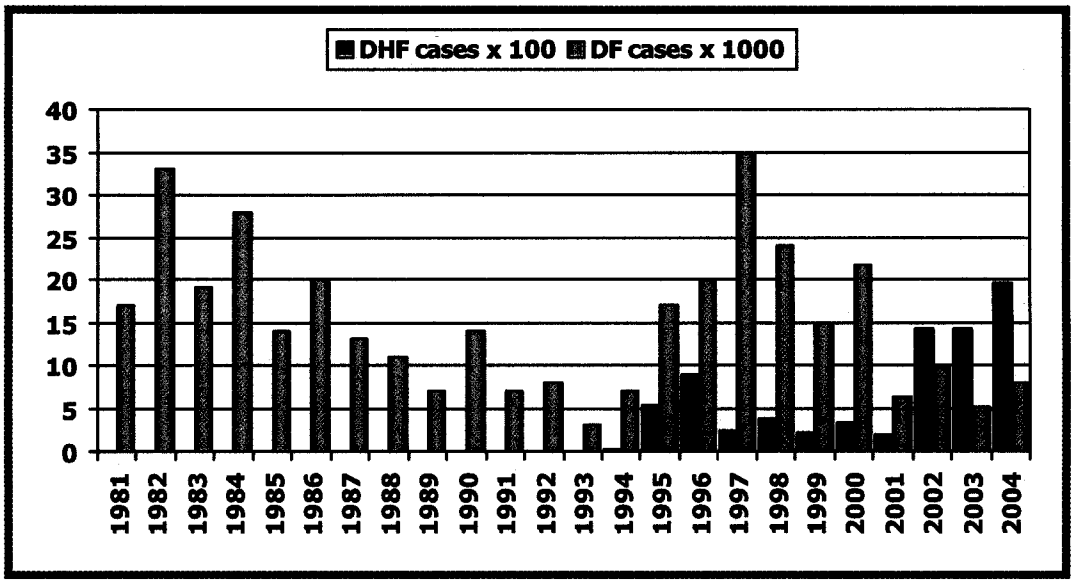


Figure 1.9. DF and DHF cases in Mexico in the past 23 years. A significant increase in the number of DHF has occurred since 2001 (PAHO).

The increased number of DHF cases in the Yucatan Peninsula (South East of Mexico) during 2001-2002 coincided with the more frequent isolation of DENV-2 viruses that clustered in the American/Asian genotype (184). **In Chapter III**, the correlation between clinical severity in humans and virogenesis in mosquitoes was analyzed for different DENV-2 strains from the Yucatan Peninsula in Mexico. These viruses were compared to a member of the American genotype (QR0094) in their growth characteristics in cell culture and dissemination in mosquitoes. At the end of this chapter potential genotypic changes underlaying the phenotypic differences were investigated.

1.2.6. Pathogenesis

DENV infection in humans starts with the bite of an infected mosquito. Viral particles are deposited with saliva in the incision site during feeding (Figure 1.10).

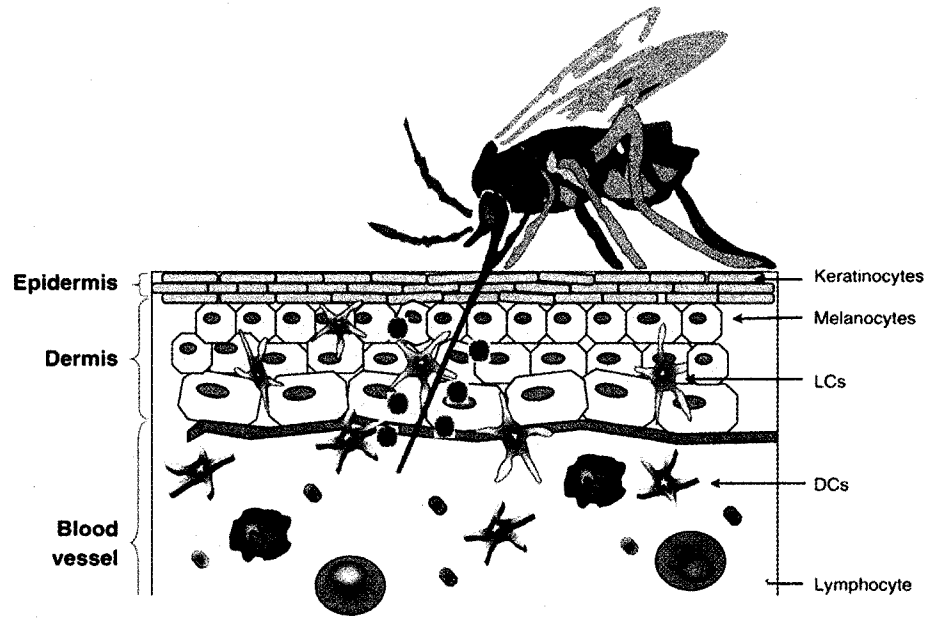


Figure 1.10. DENV infection of humans. DENV is passed onto humans while an infected mosquito salivates to obtain blood from an individual. From: Navarro-Sanchez *et al.*, 2005 (205).

Saliva from hematophagous arthropods contains potent proteins that modulate the host's hemostatic, inflammatory and immune responses that promote the blood flow and allow the feeding. These immunomodulatory molecules induce or increase pathogen transmission (89, 113, 162, 174, 258). The role of arthropod saliva in pathogen infection is of such importance that anti-arthropod saliva vaccines represent a feasible way to prevent transmission of some human pathogens like *Leishmania* (42, 94). Mosquito saliva alone can skew the host immune response to a Th1 type (324). This may have important implications in viral infection.

In mammalian cells DENV is likely to bind host cell receptors via domain III of the E protein, leading to endocytic uptake. In cell culture, both glycoproteins and glycosaminoglycans participate in binding of DENV to the cells (88, 175, 203, 255).

However, the most receptors in the different cell-types infected by DENV remain unknown (124, 133). Exceptions to the endocytic mechanism of entry could occur in some insect cells. Some reports suggest that DENV-2 gets into the cytoplasm after direct fusion of the virion and cellular plasma membranes when infecting at acidic pH (280). The acidification inside the endosome induces rearrangement of E protein exposing its internal fusion peptide and triggering membrane fusion (Figure 1.11). This enables the release of virus nucleocapsid into the cytoplasm (195, 196, 232).

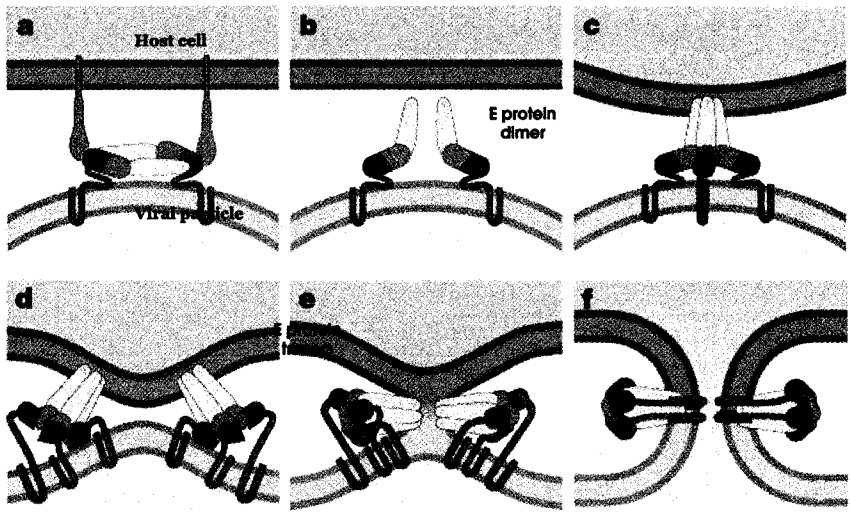


Figure 1.11. Proposed mechanism for fusion mediated by class II viral fusion proteins. **A)** E glycoprotein binds to a receptor on the cell surface and the virion is internalized to an endosome. **B)** reduced pH in the endosome causes domain II (yellow) to hinge outward from the virion surface, exposing the fusion loop, and allowing E monomers to rearrange laterally in virion. **C)** the fusion loop inserts into the host-cell membrane, promoting trimer formation. **D)** formation of trimer contacts spreads from the fusion loop, then domain III (blue) shifts and rotates to create trimer contacts. **E)** creation of extra trimer contacts. **F)** formation of a lipidic fusion pore. From: Modis, *et al.*, 2004 (196).

Langerhans-type dendritic cells (DCs) are the first cells infected by DENV in humans; these cells are central in eliciting the host antiviral immune response and in transporting the virus to the lymphoid tissues. The ICAM3 nonintegrin (DC-SIGN) molecule seem to be the receptor that mediates DENV binding to these cells (204). DC-SIGN is an endocytic receptor that participates in internalizing several pathogens via glycoprotein interactions (185). Viral infection converts DCs from immature sentinels

that accumulate foreign and self-antigens in tissues into mature cells that migrate and present viral antigens to *naïve* T lymphocytes. The *naïve* T lymphocytes are stimulated by Toll-like receptors (TLRs), which provide the critical signals responsible for linking the innate and adaptive immune responses against the pathogen (142, 150, 212, 321).

Once in the lymph node, an unidentified cell type becomes infected. Candidate cell types include macrophages, B cells, and follicular DCs. A new round of replication occurs in these lymphoid cells, inducing the entry of viral particles to the circulation through the efferent lymphatic and thoracic duct then resulting in the primary viremia (63). Virus replicates efficiently in B cells, which naturally circulate between lymph nodes and circulation (175), and are likely to traffic the virus between these two systems.

In the blood, DENV infects monocytic cells (PBMC) including: B and CD20 cells (133) as well as CD14+ (281). DENV triggers TNF-α secretion in macrophages and IFN-γ, IL-1 and IL-8 in monocytes (40, 76, 125, 160, 306). DENV particles may be recovered from the blood of infected individuals from 2 days before the onset of fever to 5-6 days after (111). Reports on viremia titers of DENV in blood vary depending on the method used for the quantification. Reports indicate viremia ranges from 10^3 to 10^8 $TCID_{50}$/ml (305), or 10^2 - 10^7 PFU/ml (266), or 10 - 10^9 MID_{50}/ml (297). Virus particles circulating in the bloodstream reach and infect other cell types in secondary organs. These cell types include: hepatocytes (175, 189), bone marrow cells (156), and endothelial cells (4, 36, 306). These infected organs release viral particles that can cause a secondary viremia.

The host innate nonspecific immune response to DENV infection includes activation of macrophages, IFN, TNFs, natural killer (NK) cells and T cell mediated immunity (312). The cytokines produced by macrophages have long-range changes on host defense including body temperature elevation. Fever is caused by endogenous pyrogens such as TNF-α, IL-1 and IL-6, and aids to control the infection (136). Two other cellular factors, MIP-1α and MIP-1β, likely have a role in immunopathology and may

contribute to the fever production and the bone marrow suppression noted in patients (274). The adaptive immune response includes production of antibodies and an antigen specific T-cell response (312). Antibodies produced by the adaptive immune system against DENV augment the NK-mediated lysis. DENV-specific $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes are present in the blood of donors previously infected by DENV. Nevertheless, DENV acute infections cause depression of *in vitro* T cells (191).

DENV infection also mediates an aberrant activation ratio of $CD4^+/CD8^+$ T cells in some patients by inducing cytokine overproduction. A shift from a Th1-type response in mild illness to a Th2-type response might result in more severe disease. The relative levels of IFN-γ, IL-10, IL-12 and TGF-β regulate this shift (201). Also, disease severity correlates with viral load circulating in the blood during the viremic stage: a mean titer of $10^{7.6}$ for those with DF versus $10^{8.5}$ MID50/mL for patients with DHF (P= 0.01) (297). High plasma viral loads detected by quantitative RT-PCR during defervescence also correlate with DHF (53).

Increased vascular permeability during DHF and DSS results in sudden and massive plasma leakage into extravascular sites including the pleural and abdominal cavities. Complement activation, chemokine induction, and apoptotic cell death in presence of anti-dengue antibodies may play a role in the vascular leakage characteristic of DHF and DSS (9). Induction of apoptosis by DENV may be also important in the pathogenesis of hepatic failure associated with DHF and DSS (189).

DSS is characterized by a rising of hemocrit, hypoproteinemia, serum effusion, hypotension, narrow pulse pressure and hypovolemic shock. DHF and DSS outcomes could result from a direct change of endothelial tissue physiology. Human endothelial cells infected with DENV induce transcriptional up-regulation and secretion of RANTES and IL-8. DENV infected human umbilical vein endothelial cells (HUVECs) produce large amounts of IL-6 and IL-8 but not IL-1β (131). DENV infection of HUVECs induces

different genes including inhibitor of apoptosis-1, the 2'-5' oligoadenylate synthetase (OAS), galectin-9, myxovirus protein A (MxA), regulator of G-protein signaling, endothelial and smooth muscle cell-derived neuropilin-like protein, and phospholipid scramblase-1 (306).

The pathogenesis of DHF and DSS has not been clarified. However, it appears to be multifactorial (312). Several hypotheses have been proposed to explain it, although none of them adequately explains clinical DHF and DSS. Likely, a complex combination of events determines the severe outcomes. The most important of these hypotheses include: 1) Antibody-dependent enhancement (ADE) (discussed in section 1.1.2), 2) Virus virulence, 3) Molecular mimicry and 4) Original antigenic sin.

Virus determinants of virulence. Phylogenetic and epidemiological analysis of DENV isolates from diverse epidemics have associated specific viral genotypes with severity (235). Some changes in E protein amino acids and the 5'- and 3'-terminal nucleotide regions have a significant effect on virus entry, replication, and probably on virulence (50).

Molecular mimicry with DENV. Mimicry occurs when a protein fragment of an infectious agent closely resembles part of a self-protein. In DENV, a 20 amino acid sequence in the E protein shares similarity with a sequence found in the human clotting factor plasminogen. This sequence is largely conserved within flaviviruses (47, 190). Antibodies against viral NS1 protein may be involved in the cross-reactivity with endothelial cells, which are observed in a significantly higher level in serum specimens from patients with DHF than with DF (173, 267). Some authors hypothesize that platelets are destroyed during DENV infection by cross-reactive antiplatelet autoantibodies. This event increases chances of hemorrhagic outcomes because of the imbalance between coagulation and fibrinolysis activation (170).

The original antigenic sin theory. This theory proposes that a large fraction of T-cells are activated during a heterologous secondary infection and antibodies of poor affinity to antigenic peptides of the second virus are produced. However, these antibodies are highly reactive to the previous infecting virus. This "inappropriate" response may contribute to the immunopathology of the disease while hindering clearance of the second infecting virus (198).

1.3. *Aedes aegypti* - The vector

The *Aedes aegypti* mosquito is the primary vector for DENV worldwide, although *Aedes albopictus* is also a competent vector for dengue and other arboviruses (100). *Aedes aegypti* is an early morning or late afternoon feeder, but also bites at night under artificial illumination. *Aedes (Stegomyia) aegypti* is a member of the subfamily Culicinae and is perhaps the most domesticated of all mosquito species. It breeds in artificial containers and females feed almost exclusively on humans. They live near or in human habitation and are anthropophilic and endophagic. In Asia and Africa, sylvatic cycles of dengue involving nonhuman primates and mosquitoes in the *Aedes* subgenera *Diceromyia, Stegomyia* and *Finlaya* occur in the forest (104, 303). However the urban cycle of DENV, in which humans are the amplifying hosts, is by far the most important.

1.3.1. Host seeking and blood feeding

The means by which female mosquitoes locate suitable vertebrate hosts is still poorly understood. The best explanation is that the human body's warmth, moisture, and scent attract mosquitoes. The CO_2 produced by hosts and other substances, such as lactic acid, may be the major attractants (20, 57, 99, 119). *Aedes aegypti* host-seeking behavior is inhibited after a large bloodmeal (full- engorgement) and resumes only after oviposition has taken place (154, 194). A female mosquito is able to ingest two to four times her body weight in blood. The size of the bloodmeal depends on several conditions including: temperature, age, mating status, previous feeding history, source of the blood,

and stage of the gonadotrophic cycle (209). In *Ae. aegypti*, nutrients for oogenesis and energy requirements are mostly obtained from blood (49).

In nature, *Ae. aegypti* female mosquitoes feed almost exclusively and rather frequently on humans (118). Multiple feedings until complete a large bloodmeal during a single gonotrophic cycle is characteristic of *Ae. aegypti*. This behavior varies according to the geographical location and changes under different climate conditions (262). Mosquitoes seeking multiple meals make them an efficient vehicle to spread pathogens from host to host (8). On average, an individual mosquito takes 0.63 - 0.76 human bloodmeals a day (261). Female mosquitoes that fed on human blood alone have greater rates of net replacement and intrinsic growth than conspecific mosquitoes feeding on human blood plus sucrose (264). Blood ingestion and continuity of feeding are governed by abdominal stretch receptors, and added stimuli come from the blood itself. A critical stimulus is the break-down products of blood-proteins produced by early trypsin (10, 209, 210).

Ae. aegypti mosquitoes become infected by feeding on a dengue viremic individual. The mosquito midgut is the first organ to be in contact with the ingested blood and the virus (49, 318). The mosquito midgut plays a crucial role in the blood digestion and nutrients uptake. Once the virus infects the mosquito, an extrinsic incubation period (10-14 days) is required before DENV is passed on to a new host (104). Environmental conditions including temperature, humidity and viral dose affect the extrinsic incubation period (24). Infected female mosquitoes may also transmit the virus to the next mosquito generation by vertical transmission, albeit at a low rate (37, 140, 244).

1.3.2. Bloodmeal digestion

With a few hours following bloodmeal ingestion, a type 1 peritrophic matrix (PM1) is formed. The PM-1 is made of proteins, glycoproteins, and chitin microfibrils in

a proteoglycan matrix (169). Proteoglycans are glycosylated proteins covalently linked to sulfated glycosaminoglycans, (i.e., chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate). The PM1 effectively divides the midgut lumen into an endo- and ecto-peritrophic space, with an intra-PM-1 compartment. Digestive enzymes released from the midgut epithelium traverse the PM-1 to reach the bloodmeal. The digestion products cross in the opposite direction where they are absorbed by midgut epithelial cells.

Thus compartmentalization by the PM-1 modulates blood digestion in the mosquito midgut (169, 299). In *Ae. aegypti*, PM1 is first detected within 5 h and it fully matures within 5 to 15 h (60). The thickness of PM1 typically ranges from 1 to 20 μm. The PM1-secreting cells are epithelial cells found in the posterior midgut region. Bloodmeal ingestion causes marked morphological changes on these cells (Figure 1.12).

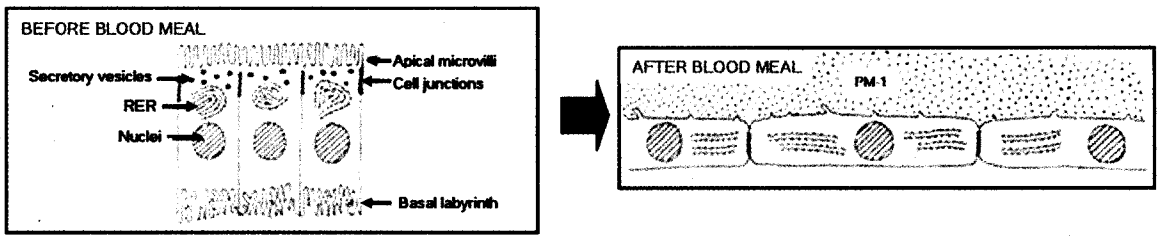


Figure 1.12. Ultrastructural changes in PM-1-secreting cells take place after a bloodmeal. The columnar cells become stretched out and flattened; on the luminal side, microvilli decrease in size and number to allow the epithelium to distend. On the basal side, unwinding of the extensive labyrinth network allows expansion of the surface (Denvenport and Jacobs-Lorena, 2005) (60).

After a bloodmeal, a large amount of heme results from red cell digestion in the midgut of mosquitoes (217). Heme contains iron, which in its pure form is toxic for most organisms. However, hematophagous insects seem unaffected by the high iron loads released during bloodmeal digestion. The resistance to iron toxicity in these insects comes from the presence of iron-binding proteins, such as ferritin (87). The PM-1 sheath not only compartmentalizes the bloodmeal digestion, but it also binds toxic heme, preventing its contact with midgut epithelia (217).

Bloodmeal digestion involves proteolytic enzymes secreted in the mosquito midgut. Early trypsin appears within 2 hours, and disappears 8 hours after the bloodmeal. Transcription of the early trypsin gene first occurs under the control of the juvenile hormone after adult emergence. The transcript remains untranslated and translation only happens after a blood or protein meal ingestion (208, 209). Early trypsin mRNA levels decrease rapidly during the first 24 h after the feeding. However, the transcripts increase again at the end of the blood digestion (~60 h), as the mosquito prepares for a second bloodmeal. This enzyme acts not only in the first phase of proteolytic processing of a bloodmeal, but is also essential to induce other enzymes. Late trypsin, which is the major endoprotease involved in the blood digestion, is activated due to the signal cascade started by early trypsin (10, 209, 210). Late trypsin is detected in the midgut 12 hours after the bloodmeal. Trypsin-modulating oostatic factor (TMOF) is a hormone secreted from the ovary that circulates in the hemolymph 18 h after the bloodmeal. TMOF binds to a gut receptor and stops trypsin biosynthesis in the mosquito gut by exerting a translational control on the mRNA (26).

Some other proteolytic enzymes are important in the blood digestion, these include: chymotrypsins and amino- and carboxy-peptidases (10, 209, 210). Amino acids resulting from blood digestion are transferred by transmembrane transporters situated in the apical and basal membrane of the epithelial midgut cells. Dietary lipids are transported into the hemolymph as part of a lipoprotein complex called lipophorin. In mosquitoes triacylglycerol is the primary lipid transported by lipophorins. To transport glucose, insects create a concentration gradient between the midgut lumen and the hemolymph that ensures its passive transport (209).

Female mosquitoes obtain the nutrients for yolk egg protein production from bloodmeals. A bloodmeal induces vitellogenesis and secretion of yolk protein precursors (YPPs) in the fat body. The oocyte grows rapidly, accumulating the yolk needed for the

embryo development. *Ae. aegypti* is usually an obligatory anautogenous mosquito, and thus cannot undergo oogenesis without one (or multiple) bloodmeal. Thus, the reproductive biology of mosquitoes augment their potential to transmit pathogens (8). Most of the yolk nutrients are not produced by the oocyte or nurse cells, but are taken up from the hemolymph by receptor mediated-endocytosis (240).

The initial intake of a bloodmeal adds two to four times the female mosquito body weight (about 1.3 mg), limiting dramatically its mobility and compromising its ability to fly. A significant amount of the bloodmeal is water. Hence, an effective way for a mosquito to deal with this difficulty is to remove promptly water through diuresis. The diuresis occurs at the distal part of the malpighian tubules and starts even before the mosquito has finished engorging the bloodmeal. Diuresis removes excess Na^+, Cl^-, water and weight. Powerful epithelial transport mechanisms are driven by V-type H^+–ATPase and diuretic hormones that enhance diuresis in the mosquito (21).

Bloodmeal uptake has profound physiological effects and imposes a significant impact on midgut gene expression patterns (Figure 1.13). Gene expression patterns during the life of the adult female mosquito are intimately related to bloodmeal digestion and oogenesis. In *Ae. aegypti*, distinctive sets of genes are expressed before and after each blood feeding. This gene expression is under strict control of a hormonal cascade started and finished by the bloodmeal digestion. After a bloodmeal, expression of genes related to protein digestion, amino acid degradation, PM-synthesis, fatty acid metabolism and some stress-response-related genes increase significantly. Interestingly, aquoporin, which is a water transporter shows a huge decrease in its transcription (257). In *Ae. aegypti* aquoporin has been found in the tracheoles of the malpighian tubules (72). Secretion in Malpighian tubules is induced by diuretic hormones released in hematophagous insects as a response to the abdomen distension during feeding (229).

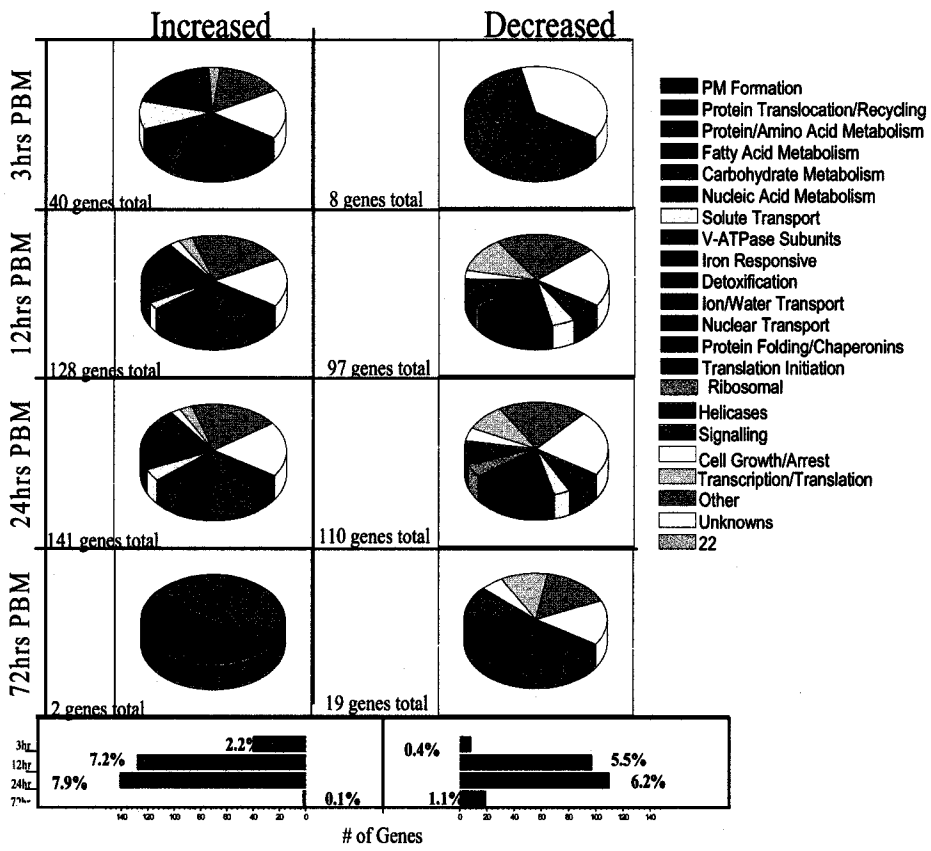


Figure 1.13. Microarray analysis of genes in midgut after a bloodmeal. *Ae. aegypti* female mosquitoes were used in this study From: Sanders *et al.*, 2003 (257).

The malpighian tubules of *Rhodnius prolixus* can increase within minutes their secretion rates by over 1000 times (186), which precipitously decline several hours after the feeding (229). The physiological significance of these changes is not completely understood.

1.3.3. Vector infection by arboviruses

The dengue viremia level in patients that effectively initiates infection in mosquitoes is unknown. Nonetheless, it may be several orders of magnitude lower than the viral titers routinely used to infect mosquitoes in artificial bloodmeals. Natural infections with La Crosse virus (LACV) demonstrate that viremias $\geq 6.3 \times 10^4$ MID_{50}/ml result in ≥50% of *Aedes triseriatus* mosquitoes becoming infected and able to transmit the virus. In contrast, if membrane feeding is used, 2-3 Logs higher viral titers are

required to infect the same percentage of mosquitoes (218). However, due in part to the lack of an animal model for dengue, the minimal necessary viremia to infect mosquitoes has not been established. Dissemination rates for different virus doses in artificial bloodmeals and a detailed study of DENV-2 virogenesis in field-relevant mosquitoes were conducted in this dissertation project (presented in **Chapter II**).

Successful transmission of pathogens to vertebrate hosts by mosquitoes is the result of a complex series of anatomical and molecular interactions in the vector. Participating events include genetics and innate immunity, as well as viral dose and environmental conditions, all of which determine vector competence. To maintain their life cycles, viruses must effectively overcome the natural anatomic barriers and innate immune defense mechanisms encountered in the vector (23).

Epithelial cells in the midgut are infected by the virus. The mosquito midgut comprises a single layer of columnar epithelial cells with microvilli facing the luminal side; regenerative cells are also present (Figure 1.14). The midgut has a porous basal lamina (about 10 nm pore size) surrounding the hemocoel side and continuous junctions laterally spread between the cells (129, 230). Contractile muscle fibers run longitudinally and latitudinally forming a grid-like structure that surrounds the entire organ (215). Respiratory tracheoles permeate all tissues in the mosquito (Appendix 7.3) (242).

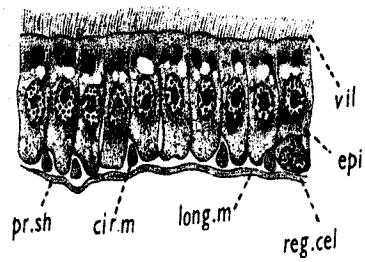


Figure 1.14. Anatomy of the mosquito midgut epithelium. vil=microvilli, epi=columnar epithelial cells, reg.cel=regenerative cells, long.m=longitudinal muscles, cir.m=circular muscles, pr.sh=peritoneal sheath (138).

The exact mechanism used by arboviruses to enter midgut epithelial cells is unknown, but it may involve direct fusion of virus to the cells or attachment to specific

receptors (265). The infection of the midgut in field-relevant mosquito populations by DENV has been shown to be dose dependent from 4 to 8 Log 10 $TCID_{50}$/ml (17).

DENV as other arboviruses can persistently infect insect and human cell lines and its vectors (74, 159, 161, 206). Microenvironment and physiological conditions, such as pH and proteolytic activity in the vector midgut, may affect virus entry of arboviruses. Some potential physiological determinants of vector infection by DENV-2 were examined in **Chapter IV**.

Once the viruses have replicated and disseminated out of the midgut, they infect different organs and tissues. Depending on the infecting virus, these may include the salivary glands, Malpighian tubules, muscle, neural tissues, reproductive organs, pericardium, and fat body (12, 19, 65, 263, 275). Virus escaping from the midgut might directly infect the salivary glands, or secondary amplification may be necessary when only small amounts of virus escape the midgut (231). However, detailed knowledge about virus-vector interactions is limited. DENV as other arboviruses must eventually make its way to the vector salivary glands, from where it may be transmitted to secondary hosts during further feedings.

A female mosquito has two salivary glands; each one consists of two lateral and one medial cylindrical lobes (137). Each lobe contains primarily a single layer of cuboidal epithelial cells surrounded by a basal lamina, and a chitinous central duct that converges with the salivary gland duct connected to the hypopharynx (231). Infection patterns of salivary glands vary for different viruses (108, 283). Events related to temporal and spatial virus replication in salivary glands require more detailed analysis. Studies in **Chapter II and III** had a particular emphasis and examined temporal and anatomical distribution of DENV-2 in mosquito salivary glands.

1.3.4. Genetic mapping in mosquito

With only a single exception, all mosquitoes species contain a genome of 2n= 6 chromosomes (23). *Ae. aegypti* was the first mosquito species for which a genetic linkage map was composed (52). This map was expanded using restriction fragment length polymorphism (RFLP) cDNA-based markers (35). A detailed intensive linkage map of *Ae. aegypti* was also constructed by using single-strand conformation polymorphism (SSCP) analysis (80).

The absence of well resolved polytene chromosomes in Culicine mosquito hampered the physical mapping of their genomes. The first physical map for *Ae. aegypti* was developed using fluorescent *in situ* hybridization (FISH) (35). Thus linkage and physical maps could be integrated (Figure 1.15). The centromere region of each of the chromosomes contains heterochromatin (tightly condensed DNA) with few active genes. The distal chromosome arms contain euchromatin, which is readily decondensed DNA and encloses highly active genes (25).

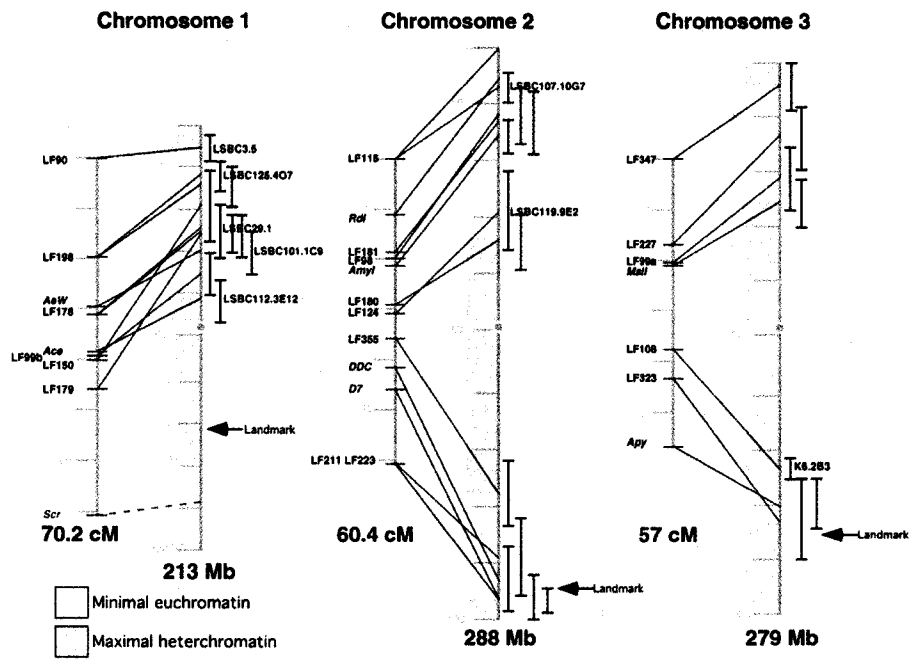


Figure 1.15. Graphic representation integrating the genetic linkage maps and the physical map of *Ae. aegypti* (Brown *et al.*, 2001) (257).

The genome of *Ae. aegypti* is both large and complex. The haploid genome size was estimated to be between 0.81-1.32 Gb (or pg). The genome contains between 13-20% repetitive sequences, between 20-24% moderately repetitive sequences, and 60-64% unique DNA sequences (78, 307). Comparative linkage analysis revealed significant areas of synteny between *Drosophila melanogaster* and *Ae. aegypti* (80).

In May 2005, the *Ae. aegypti* genome project carried out jointly at "The Institute for Genomic Research (TIGR)" and "The Broad Institute" was completed and the data were deposited in Genbank.

1.3.4. Vector susceptibility to dengue virus

The presence of receptors on the cell surface controls cell susceptibility to virus infection. However, the cell permissiveness for virus replication also depends on some other specific intracellular conditions. Therefore, cells must be both susceptible and permissive to allow successful virus replication and productive infection. Host cell-type susceptibility and permissiveness to a virus governs tropisms in an organism (79).

Several *Ae. aegypti* subspecies and strains from different geographic areas have been shown to vary in their vector competence to dengue infection (17, 106, 286). Vector competence is the intrinsic permissiveness of a vector for infection, replication, and transmission of a virus. The vector competence for arboviruses is associated with anatomic barriers that impede productive vector infection. These include a midgut infection barrier (MIB), a midgut escape barrier (MEB), and a salivary gland barrier. In vectors with a MIB, virus cannot infect or replicate in the mosquito midgut cells. Vectors with a MEB may allow significant virus replication in the midgut, but virus is unable to disseminate. Barriers to infection can vary widely among *Ae. aegypti* mosquito populations, thus vector competence for DENV may be variable as well (18, 28). Mosquito natural barriers to virus infection influence perhaps DENV infection patterns in the vector.

Quantitative trait loci (QTL) that condition DENV infection in mosquitoes have been identified. Genes such as *carboxypeptidase, early trypsin, apolipophorin* 2, and *late trypsin* are genetically linked with MIB and MEB for DENV in *Ae. aegypti* mosquitoes (Figure 1.16) (16, 28, 95). In spite of this, association mapping showed no consistency between segregating sites in early trypsin and susceptibility to DENV-2 (97).

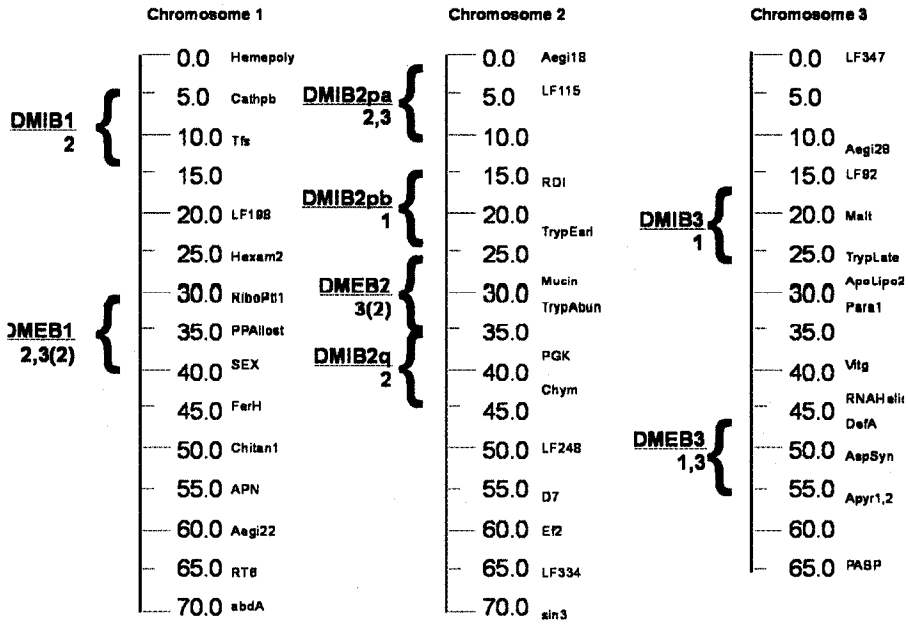


Figure 1.16. QTLs associated with DENV-2 MEB or MIB in *Ae. aegypti* genome (Courtesy of Dr. William Black).

Importantly, RNAi-mediated innate immune response as well as apoptosis may restrain and define virus infection patterns in the mosquito vector (147, 256). The innate immune response is the first line of defense against pathogens in both insects and mammals. In insects, multiple self-defense mechanisms contribute to innate immune response against pathogens. These mechanisms include: bacterial killing by either antimicrobial peptides or reactive oxygen forms, engulfment of microorganisms by hemocytes, and entrapment of pathogens by melanization (285). Thus, more studies are necessary to understand the mechanisms that determine DENV dissemination in mosquitoes.

1.4. Dissertation Project

Dengue is a significant cause of morbidity and mortality in humans worldwide (187). Main clinical manifestations of dengue in humans are DF and DHF, the latter causing fatalities due to DSS. Since pathogenesis of the disease is complex, prevention of the disease greatly involves control of the mosquito vector. However efforts to control the vector have often failed. A better understanding of virus-vector interactions could provide new potential targets for virus transmission intervention.

The first endeavor of this research was to establish the landmarks of DENV-2 replication in *Ae. aegypti*. The kinetics and tropisms of DENV-2 infection in different organs and tissues in the mosquito were examined, the results are provided in Chapter II. In Chapter III, the correlation between clinical severity of dengue and virogenesis in the mosquito was investigated. Potential genetic determinants in the E protein and 3'UTR, which could condition the observed differences, were identified. Finally, the effects of midgut physiology in the infection of the vector were explored in Chapter IV. The role of pH in the mosquito midgut and the presence of DENV-2 in cells in the bloodmeal were evaluated. These investigations revealed some determinants of DENV-2 infection of the mosquito vector and raised important questions that must be addressed in future research projects.

Chapter II

Virogenesis of DENV-2 Jam 1409 in orally infected *Aedes aegypti* mosquitoes.

2.1. Introduction

There is extensive experimental evidence of intraspecific variability in vector competence to arboviruses in different mosquito populations or strains. Variations in vector virus susceptibility have been shown for multiple vector-virus associations. These include: YFV in *Aedes aegypti* (1, 282, 301), La Crosse virus (LACV) in *Aedes triseriatus* mosquitoes (101, 219, 220), and Dengue virus in *Ae. aegypti* (18, 101, 106, 286, 298).

Analysis of laboratory-adapted mosquitoes revealed significant genetic divergence from their field equivalents (123, 144). Events such as founder effects, inbreeding and genetic drift importantly impact mosquito genetics. As a consequence, the laboratory-reared mosquito populations could genetically differ from their field counterparts, especially those with a long colonization history.

How genetic changes occurring during colonization affect mosquito susceptibility to arboviruses remains unclear. Colonization in *Aedes albopictus* mosquitoes results in significant but nondirectional changes in the allele frequencies at most of the examined loci. Loss of low frequency alleles occurs, while the overall genetic variation within and among populations does not change substantially (144). Laboratory colonization of *Aedes triseriatus* has diverse and unpredictable effects on both infection and transmission of LACV (101). The infection and transmission rates of Rift Valley Fever virus (RVFV) change significantly during colonization of *Culex pipiens* mosquitoes with no effects on viral titers in individual mosquitoes (85). *Ae. aegypti* infection rates with YFV vary significantly between generations, correlating with changes at some specific loci (183). There are no reports of *Ae. aegypti* colonization effects on DENV susceptibility.

Different laboratory techniques have been valuable in investigating virogenesis in mosquitoes. Indirect immunofluorescence has made it easy to examine viral antigen

distribution in mosquito midguts at various times after *per os* infection with Japanese encephalitis virus (JEV), LACV and DENV (11, 12, 65, 66). Electron microscopy has been used to characterize St. Louis encephalitis (SLE) virus infection in *Culex pipiens* (314), Eastern equine encephalitis virus (EEEV) in *Aedes triseriatus* (313), and VEEV in *Culex* mosquitoes (310). Salivary gland infection by Chikungunya virus (CHIKV) virus was examined in *Ae. aegypti* also using electron microscopy (137). Mosquito infection by Japanese B encephalitis virus (JEV), Bunyamwera virus (BUNV), and Rift Valley fever virus (RVFV) has been analyzed by plaque titrations (77, 165, 221).

Detailed information on tropisms in the vector is available for DENV-3 in intrathoracically injected mosquitoes (178). Intrathoracic injections used to examine viral tropisms do not represent the rates and times of the natural route of infection. Virus taken in a bloodmeal encounters and must overcome midgut infection and escape barriers (MIB, MEB) (29), which vary among mosquito populations (17). Viruses are able to overcome these barriers and efficiently replicate in both vertebrate and invertebrate hosts, probably through selection of fit viral quasispecies. Selection of quasispecies could allow virus to replicate in both hosts and permeate anatomic barriers. LACV selection of quasispecies takes place at midgut level in *Aedes triseriatus* (27). Infection of organs such as midguts is dose dependent (18), thus, the number of viral particles that reach the hemolymph may vary depending on the virus and mosquito strain.

In this chapter, the aim was to establish the progression, tropisms and levels of DENV infection in a recent colonized field strain (Chetumal) mosquitoes after oral challenge. Determination of virogenesis of DENV-2 in field-relevant mosquitoes would provide a better understanding of transmission potential, entomological associations, and events underlying dengue epidemiology. Questions addressed in this chapter included: What are the dynamics of DENV-2 replication in orally infected mosquitoes? What are the viral tropisms to different tissues and organs? What are the outstanding

differences in kinetics of virogenesis? What is the effect of a second bloodmeal on virus infection? Tropisms and virogenesis of DENV-2 Jam1409 were examined in two susceptible mosquito populations: one recently colonized (Chetumal) (17, 234) and a laboratory adapted (RexD) from larvae originally collected from Rexville (Bayamon), Puerto Rico (107). Recently colonized or field collected mosquitoes better reflect the actual infection rates and epidemic potential for dengue.

2.2. Materials and Methods

2.2.1. Study design

The general approach for these studies is presented in Figure 2.2.1. Briefly, virus suspensions obtained from infected cell cultures at 12 dpi were mixed 1:1 with washed sheep red blood cells and provided to mosquitoes using a membrane blood feeder (12, 286). Mosquitoes were either collected or processed at different time points according to the objective of each study. Virus infection evaluation included qualitative and quantitative analysis. Qualitative analysis was performed on mosquito tissues assayed by IFA, while plaque assay, end point titration, quantitative RT-PCR, and microscopic analysis, were used to quantify virus infection levels.

2.2.2 Mosquito rearing

Aedes aegypti Chetumal (18, 234) mosquitoes (F3 to F6) were originally collected from Chetumal city, Yucatan Peninsula, México. The Rex D and D2S3 (15) mosquitoes were used as reference strains for control purposes in the study. Mosquito egg liners were hatched by immersion in water containing 0.01% brain-heart infusion (BHI) medium. Newly emerged larvae were separated in plastic pans the day after hatching and 90 to 100 individuals placed in one liter of water with 0.5 gram of catfish food.

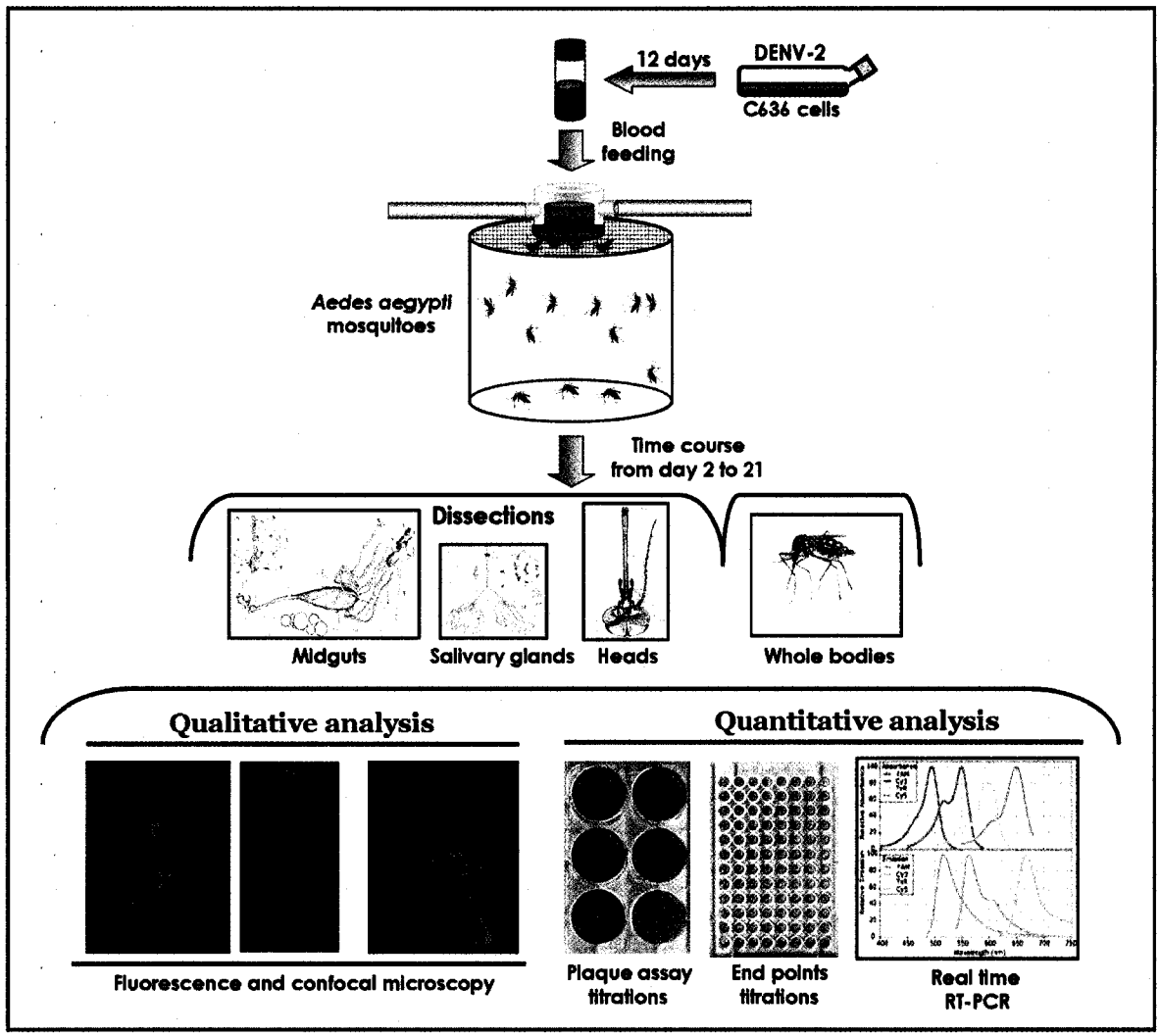


Figure 2.2.1. General protocol to evaluate infection in mosquitoes. *Aedes aegypti* mosquitoes orally challenged with 1.7 x10^7 ± 0.7 PFU/ml of high passage DENV-2 Jam 1409.

Female pupae were then sorted by size, transferred to cardboard cartons and allowed to emerge in containers. After 5 to 7 days of emergence, mosquitoes were allowed to feed on artificial bloodmeals. Mosquitoes were reared and maintained at 28°C, 70-80% relative humidity, with a 12-12 hour light-dark photoperiod.

2.2.3. Virus propagation

The high passage DENV-2 Jamaica 1409 strain was used for these studies. The virus stock was maintained at -80°C. C6/36 mosquito cells were grown to confluency in 25 cm^2 flasks at 28°C using Leibowitz L-15 medium with 10% fetal bovine serum, 2 mM

L-glutamine (Gibco), 1X nonessential amino acids solution (Cellgro), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cell culture was infected at a multiplicity of infection (MOI) of 0.001. The virus inoculum was suspended in 1 ml of L-15- 2%FBS medium and incubated on the C6/36 cell monolayers. The incubation took place at room temperature for 1-hour with constant rocking. Subsequently, 4 ml fresh medium were added, and the cell cultures were incubated for 7 days at 28°C. At day 7, the medium was replaced with fresh medium. At 12 days post-infection virus was harvested to prepare an artificial bloodmeal, variation of the original protocol which allowed infection until 14 dpi (18). The infected cells were scraped, the suspensions transferred to a 15 ml tube, and mixed with blood. After this time, the cell monolayers were damaged and viral titers obtained by plaque assays had decreased.

2.2.4. Oral infection of mosquitoes with DENV-2 Jam 1409

Artificial feedings of mosquitoes followed the protocol originally reported by Beaty and Thompson (12), with some variations. Briefly, 100-150 *Ae. aegypti* female mosquitoes between 4 and 7 days post-emergence were deprived of sugar for 14 hours and of water 8 hours prior to the blood feeding. The infected cell monolayer was scraped from the flask surface and viral suspension was collected in a 15 ml tube. Infectious bloodmeals contained a mixture (1:1) of DENV-2 Jam1409 suspension and washed sheep erythrocytes. The bloodmeal was added to a glass feeder covered with a hog gut membrane and kept at 37°C by circulating warm water though an external jacket (81).

Mosquitoes were allowed to feed no longer than about 45 minutes to prevent a decrease in viral titers in the bloodmeal. Aliquots of bloodmeals were collected before and after the feeding for titration by plaque assay. Bloodmeals contained viral titers of 1.0-2.4 x10^7 PFU/ml. Engorged mosquitoes were sorted, placed in cartons, and kept in an insectary environment with a consistent supply of sugar and water.

2.2.5. Mosquito dissections

Thirty mosquitoes were dissected to analyze midguts, salivary glands, thoraxes, heads, and abdomens at each time point. Midguts were dissected in PBS to prevent tissue desiccation, fixed in 4% paraformaldehyde, and kept at 4°C until staining. Salivary glands were also dissected in PBS, fixed in 4% paraformaldehyde and 1% glutaraldehyde or acetone, and kept at 4°C until staining. Abdomens, thoraxes and heads were squashed on glass slides pretreated with Vectabond (Vector) and fixed for 15 minutes in cold acetone for subsequent immunostaining and analysis.

2.2.6. Indirect immunofluorescence assays

Tissues fixed to slides were assayed by indirect immunofluorescence to detect DENV-2 antigen. The serotype-specific monoclonal DENV-2 antibody 3H5-1 (ATCC number HB-46) from mouse ascites fluid was used for this test. The primary antibody diluted 1:200 in PBS was incubated for 1 hour on the tissue, followed by two washes of 5 minutes with PBS. Then a 1:200 dilution of goat-anti-mouse avidin-conjugated secondary antibody (Amersham Biosciences) was applied to slides. After an incubation of 40 minutes, two more washes in PBS were followed by final 20 minutes incubation with 1:200 of fluorescein-streptavidin (Amersham Biosciences) and 0.01% Evans blue in PBS. Two additional washes of 5 minutes each with PBS and one more with distilled water followed. DABCO oil was added to the tissues, and coverslips were then placed onto them. All the incubations took place at 37°C. An Olympus BH2-RFCA fluorescence microscope was used to examine slides.

2.2.7. Plaque Assay Titrations

Mosquitoes were collected from three independent experiments at 4, 7, 10 and 14 days post-blood feeding and preserved at -70°C. Levels of virus infection were quantified by plaque assays following the technique originally reported by CDC in its *Manual on dengue diagnostic laboratory procedures for the Americas* (1981). Briefly, LLC-MK2

cells were grown on 6-well plates in MEM with Earl's salts. The medium also contained 10% fetal bovine serum (Gemini), 2 mM L-glutamine (Gibco), and 1X nonessential amino acids solution (Cellgro), 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were maintained in a CO_2 incubator at 37°C until they reached confluency. Mosquitoes were individually triturated in 1 ml of MEM containing 2% FBS using a pestle and mechanical grinder and centrifuged at 15,000 rpm for 30s using a bench microfuge (ISCBioexpress).

The supernatant was filtered through a 0.22 µm syringe filter and collected in a sterile 1.5 ml microtube. Then, 150 µl from sequential 10-fold dilutions were applied onto the LLC-MK2 cell monolayers respectively. The 6-well plates were incubated for 1 h at 37 in a CO_2 incubator with constant rocking. The first overlay was added at 42°C and contained 1% agar, 1X Earl's balanced salts solution, 4% YE-LAH solution, 2% FBS, 0.22% sodium bicarbonate, 50 µg/ml of gentamycin and 2 µg/ml of fungizone (Appendix 7.4). Following the first overlay, plates were inverted and incubated for 7 days at 37°C in a CO_2 incubator. Then, a second overlay containing 0.125% neutral red to aid in virus plaque visualization was added. After an extra 48 hours of incubation at 37°C in a CO_2, the number of plaques was quantified and scored.

2.2.8. End Point Titrations

End point titers were determined using the microtiter plate assay described by Schoepp and Beaty (259). Each sample was assayed in quadruplicate. Briefly, 10-fold serial dilutions of sample in 100 µl of L-15 medium containing 4% FBS were added to wells in a 96-well plate. Then, 100 µl of a C6/36 cell suspension in L-15 (4%FBS) containing $1x10^6$cells/ml was added. The plates were incubated for 7 days at 28°C in a closed plastic container. After incubation, the supernatant in the 96-well plates was removed and inactivated. Subsequently the cells attached to the plate were fixed for 15 minutes at room temperature by adding 150 µl/well of a 3:1 mixture of cold acetone:PBS.

Fixed cell monolayers were assayed by indirect IFA using the 3H5 monoclonal antibody and a fluorescein conjugate. Wells were examined for virus using an epifluorescence microscope. Endpoints were calculated by the Karber method (145), and expressed as log10 $TCID_{50}$/ml.

2.2.9. DENV- 2 quantitative real time RT-PCR

DENV-2 in mosquitoes was also quantified by RT-PCR (q-RT-PCR) based on the method described by Richardson *et al.* (197). RNA for PCR assays was extracted using QIAamp® Viral RNA Kit from Qiagen (Section 3.2.5). The RT-PCR mixture contained 1X DyNAzyme buffer (Finnzymes, Espoo, Finland), 0.2mM dNTP mix, 0.5X SYBRGreenI, 2.5mM MgCl2, 10μM forward and reverse primers, 0.4U DyNAzyme II Recombinant DNA Polymerase (Finnzymes), and 2μL cDNA in a final volume of 20μL. The SYBR Green I 10,000X stock was diluted in DMSO to a working solution of 100X, which was stored at -20°C in 15 μL aliquots to minimize exposure to light and freeze-thaw cycles. Plasmid standards and cDNA samples were vortexed before setup.

Reactions took place in microseal 96 Microplates covered with optically clear caps (MJ Research) spun at 3000 rpm for 5 minutes to remove bubbles that interfere with fluorescence readings. Opticon 2 thermal cycling settings were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 sec, 64°C for 20 sec, 72°C for 30 sec, and 84°C for 1 sec for fluorescence measurement. After a final extension at 72°C for 10 minutes, a melting curve was ascertained using the program: 70°C to 95°C, 0.2°C/read, 1 sec hold to confirm product specificity. The assay measured (+) and (-) strand DENV-2 RNA and standard curves were generated on each plate by analyzing $2x10^2$ to $2x10^8$ copies/reaction of DENV-2 from plasmid standards. Primers targeted a 177 bp region of the DENV-2 NS5 gene (Forward 5' ACAAGTCGAACAACCTGGTCCAT 3' and Reverse 5' GCCGCACCATTGGTCTTCTC 3'). All samples and standards were assayed in triplicate.

2.2.10. Immunostaining assay of Mosquito Midguts

Infected or control mosquito midguts were dissected and placed in a microtube containing paraformaldehyde (4%, 1X PBS) for at least 2 hours. The paraformaldehyde solution was removed and the midguts were rinsed once in PBS. The midguts were then incubated for 1 hour at room temperature with constant rocking in a PBT-0.2 solution containing: 0.2% Triton X-100, 1% BSA in 1X PBS-Ashburners (1X Ashburners-PBS solution contains 13 mM NaCl, 0.7 mM Na_2HPO_4, and 1 mM NaH_2PO_4 at pH=7.2). The subsequent incubations and rinses used a solution containing 0.1% Triton X-100 detergent. For fluorescence assay the primary antibody used was either the polyclonal human anti-dengue specific antibody (Biodesign International) or the serotype-specific mouse 3H5 monoclonal antibody. Midguts were incubated in a 1:250 dilution of primary antibody for 60 minutes and rinsed twice in a PBT-0.1 solution with constant rocking for 30 minutes at room temperature.

Midguts were then incubated with 1:400 dilution of secondary goat antimouse antibody conjugated to Alexa-488 fluorochrome (Molecular Probes). Midguts were washed extensively twice with PBT-0.1 with constant rocking at room temperature for 2 hours, then incubated with a 1:40 dilution of Phalloidin-Alexa 546 (Molecular Probes Inc). After rinsing with PBS for 20 minutes, midguts were incubated with a 1:500 dilution of TOPRO-3 (Molecular Probes Inc) in PBS for 5 minutes. Midguts were placed onto slides with vectashield (Vector) to reduce photobleaching, and coverslips were added, and sealed to the slide with nail polish. Midguts were examined using a fluorescent microscope (Olympus BH2, with 10X, 20X and 40X objectives) or a confocal microscope (FVX-IHRT Fluoview Confocal, LSM, Olympus).

2.3. Results

2.3.1. Susceptibility to DENV-2 infection in studied mosquito populations.

Viral infection rates in the *Aedes aegypti* Chetumal (field collected), RexD (long-term laboratory adapted) (107) and D2S3 (15) were compared. Bloodmeals used to challenge orally mosquitoes contained 1.7± 1.0 x10^7 PFU/ml of DENV-2 Jam1409. Values of midgut infection and dissemination rates represent the average of multiple independent experiments. The strains were susceptible to DENV-2 Jam 1409 infection (Table 2.3.1). Dissemination rates in Chetumal, field-relevant mosquito population were higher (75% ± 10%) than the laboratory-adapted RexD strain (60% ± 10%). The highest midgut infection and dissemination rates were exhibited by the DENV-2 highly susceptible D2D3 mosquito reference strain (87.5% ± 7.5%).

	Midgut infection rates*	**Dissemination rates****
Chetumal (Field-relevant population)	87.5% ± 7.5%	75% ± 10%
RexD (Laboratory adapted)	70% ± 10%	60% ± 10%
D2S3 (Reference strain)	90%± 10%	80%± 10%

Table 2.3.1. Parameters of overall DENV-2 susceptibility displayed by the three *Aedes aegypti* mosquito populations. * Midgut infection rates= Number of mosquitoes with virus antigen in the midgut/number of engorged mosquitoes examined. ** Dissemination rate= the number of mosquitoes with detectable virus antigen in head squash tissue/ number of engorged mosquitoes examined. Approximately 20-30 midguts and 40-50 heads from 3 to 5 experiments were analyzed to obtain the overall rates.

Detection of virus antigen in the midgut revealed that mosquitoes did not have an MIB, since an active infection occurred. Viral antigen in the head squashed tissue showed dissemination from midgut, so there was no MEB in the examined mosquitoes.

2.3.2. Temporal DENV-2 Jam1409 tropism in midguts.

Viral infection progression in midguts was examined at different time points by IFA and contrasted between Chetumal and RexD mosquitoes (Figure 2.3.1 and 2.3.2). A few individual midgut epithelial cells infected with DENV-2 were distinguishable in

dissected midguts as early as 2 days after an oral challenge. At day 3, infection foci in Chetumal mosquitoes involved several cells, but in Rex D mosquitoes only individual cells showed infection. In RexD the infection started more often in the posterior portion of the midgut, but in Chetumal mosquitoes infection foci were more randomly distributed.

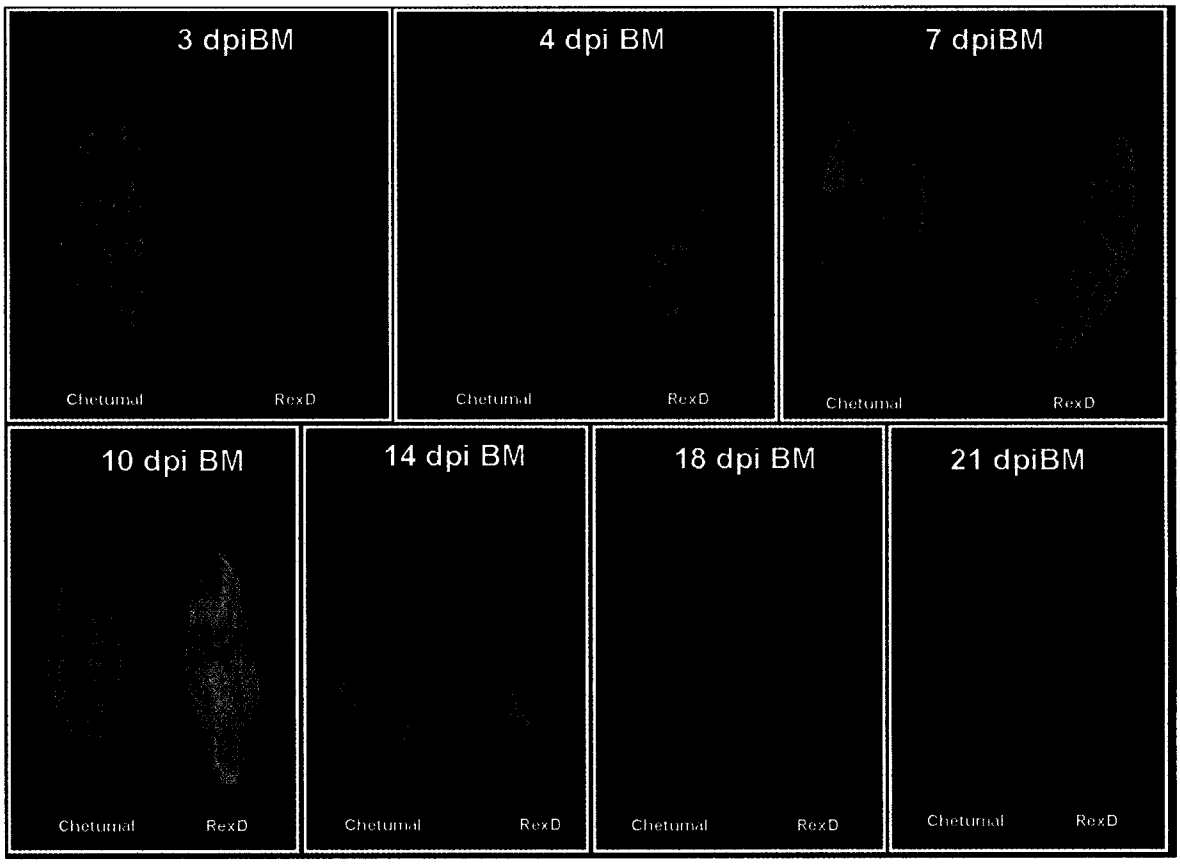


Figure 2.3.1. Time course of DENV-2 Jam 1409 midgut infection in *Aedes aegypti*. Chetumal and Rex-D mosquitoes orally challenged with virus were examined. Midguts from the different time points after the oral challenge with DENV-2 Jam 1409 were assayed by IFA with 3H5 monoclonal antibody. The figures show presence and pattern of viral antigen (green) during infection. Midguts presented are composites from several individual pictures (3-7). The magnification was 200X.

In both strains infection spread laterally, frequently infecting the entire midgut by 7-10 days postinfection. The observed differences in midgut infection between Rex D and Chetumal mosquitoes included a shift in time and foci number. Significant

variability in foci number and viral antigen distribution existed at each time point (Figure 2.3.2) for both strains.

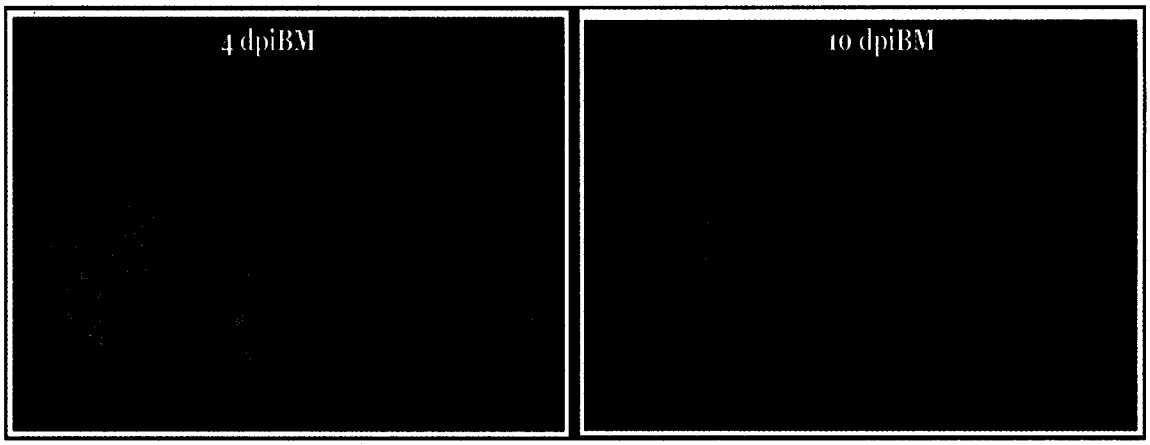


Figure 2.3.2. Representative midguts from Chetumal mosquitoes assayed by IFA at two different time points, 4 (A-C) and 10 dpiBM (D-F). There is variability in virus antigen density and the number of foci present in midguts at each time point.

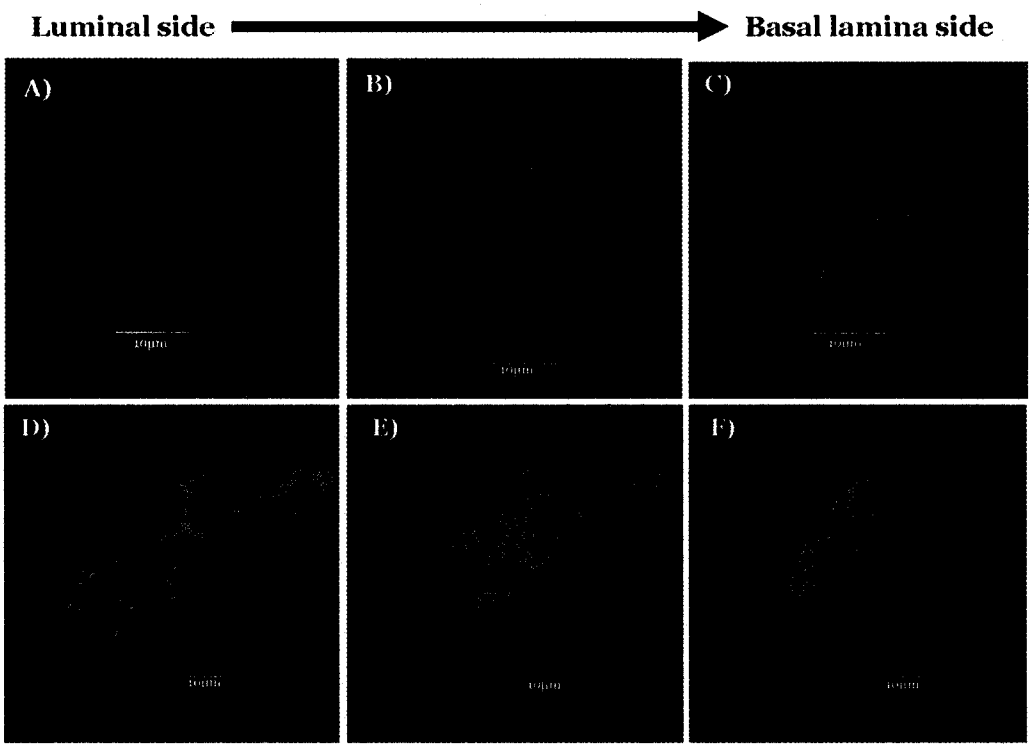


Figure 2.3.3. DENV-2 antigen accumulates in the basal lamina side of the midgut epithelium. Confocal microscope images of DENV-2 infection foci in Chetumal mosquito midgut epithelial cells at 3 (A-C) and 5 dpiBM (D-F). Images taken from the luminal towards the basal lamina side of the midgut revealed basolateral concentration of viral antigen (C and F). Green: viral antigen revealed by a goat antimouse secondary antibody conjugated to Alexa®488 (Molecular Probes Inc.) fluorochrome. Blue: Midgut epithelial cell nuclei stained with TOPRO-3 (Molecular Probes Inc.).

In infected midgut epithelial cells of both mosquito populations, viral antigen accumulated in the basal lamina side of the cytoplasmic membrane, as observed by confocal microscopy (Figure 2.3.3). Viral infection occasionally occurred in the anterior midgut and rarely in posterior midgut (about 9 dpiBM), and cardia (9-12 dpiBM) (data not shown).

Interestingly, starting at day 10 and continuing through 21 dpiBM, the intensity of viral antigen fluorescence in midguts declined (Figure 2.3.1). This could result from virus clearance by mosquito innate immune response, changes in virus transport precluding E protein recognition, or by rearrangements of the E protein hindering detection by 3H5 antibody. To examine the last two possibilities, a polyclonal anti-dengue antibody against the E protein was utilized to detect virus in 21 dpiBM midguts. Although more background staining occurred with the polyclonal antibody despite extensive washes, the same decay in DENV-2 antigen detection was also noticeable (Figure 2.3.4). Thus, the decline in virus antigen in the midguts did not result from E protein transport out of cells or rearrangement impeding E recognition by 3H5.

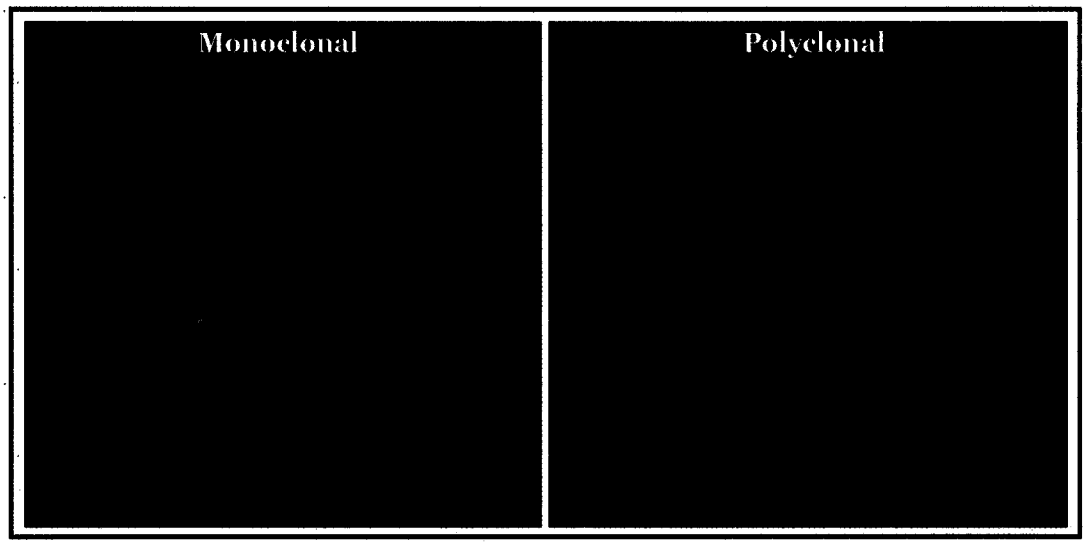


Figure 2.3.4. DENV-2 antigen decreases in midgut over time. Two different antibodies were used to detect antigen. Chetumal mosquito midguts (21 dpiBM) were assayed by IFA using monoclonal mouse anti-DENV-2 antibody and a human polyclonal anti-DENV-2 antibody. The amount of viral antigen reduces dramatically from the amount detected between the 7 and 10 dpiBM).

The musculature surrounding the midgut did not contain DENV-2 antigen at any examined time points (Figure 2.3.5). Although viral antigen decreased in midguts, salivary glands and nervous system became increasingly infected (see Table 2.3.2).

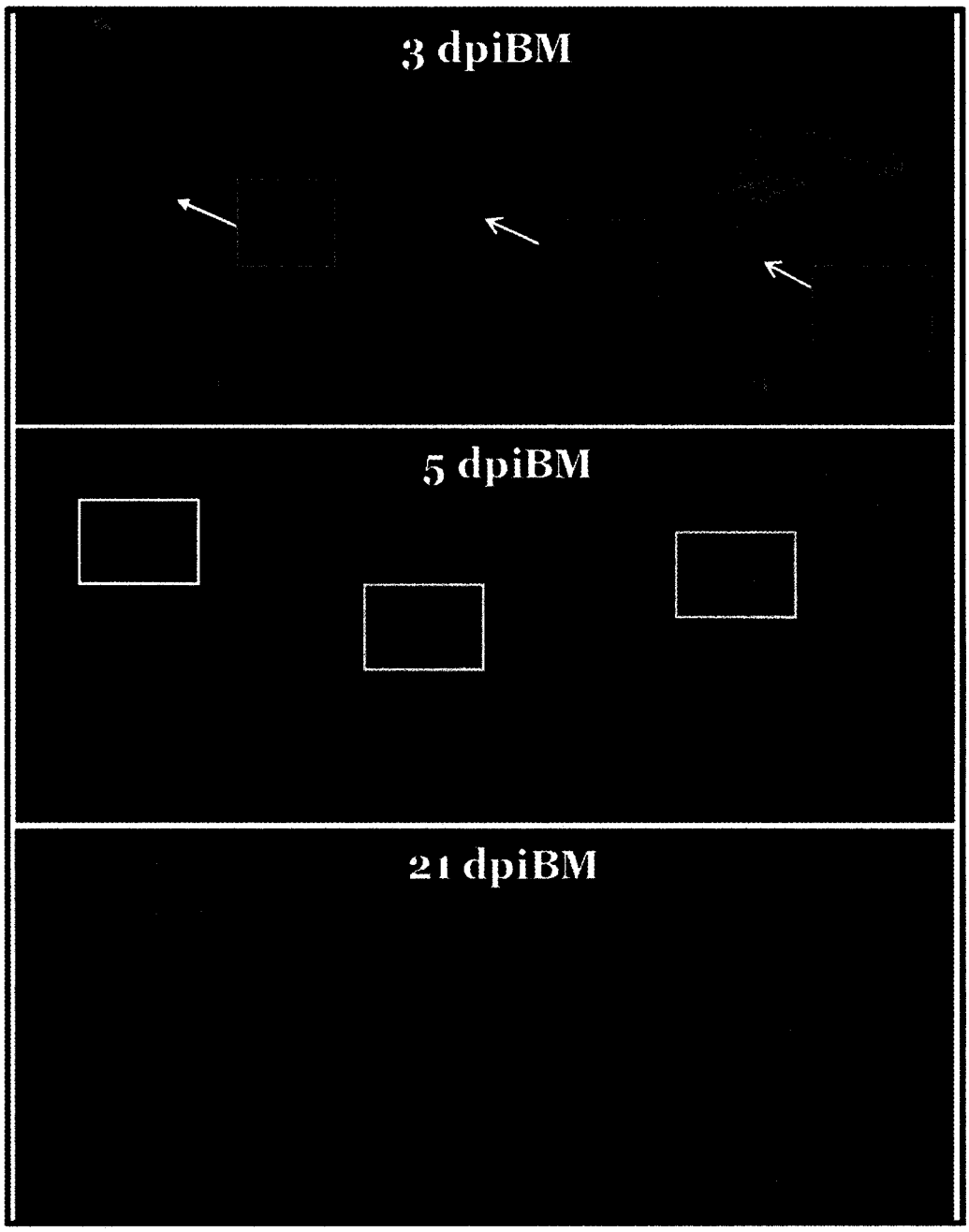


Figure 2.3.5. DENV-2 infection of midguts at three different time points. DENV-2 viral antigen (green) staining used 3H5 mouse antidengue monoclonal antibody and a goat antimouse secondary antibody conjugated to Alexa®488 (Molecular Probes Inc.). Muscle actin was stained with Phalloidin Alexa®546 conjugate (Molecular Probes Inc.). Muscle did not become infected with DENV-2. Red arrows show uninfected muscle (grid-like pattern). Midguts were photographed at 200X magnification and composites made using Photoshop.

2.3.3. Quantitative analysis of DENV-2 in mosquito midguts

To evaluate whether the decrease in viral antigen resulted from clearance of the virus infection, viral RNA and viral infectious particles were quantified in midguts from Chetumal mosquitoes overtime (Figure 2.3.6). Quantitative real time RT-PCR was used to determine the copy number of viral RNA in midguts and end point titrations on C6/36 cells were used to quantify viral infectious doses in the same midguts.

Surprisingly, the viral RNA copy number did not change significantly in midguts at day 14 and 21 after an infectious bloodmeal (Figure 2.3.6A). However, the virus titers at the same time points and in paired midguts declined significantly ($P<0.001$) (Figure 2.3.6B).

Thus, viral genome clearance did not correlate with the decay of DENV-2 levels in the mosquito midgut. Likely nutritional factors, self-limiting or infection containment mechanisms restrict virus transcription and replication in the midgut epithelia.

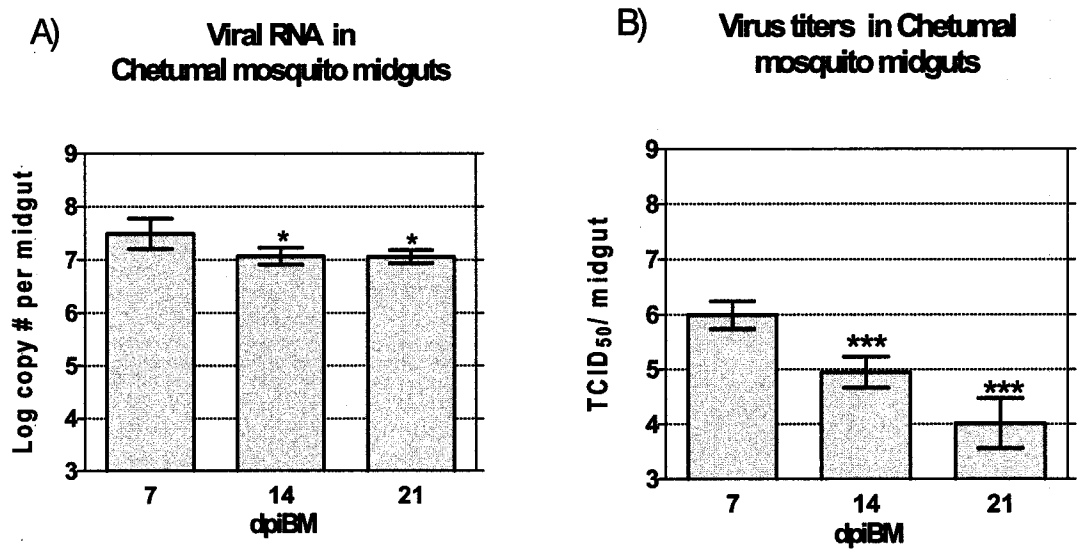


Figure 2.3.6. Quantification of DENV-2 in midguts of orally infected Chetumal mosquitoes. **A)** Quantitative real time RT-PCR to detect viral RNA exhibited indicated differences for 7 vs 14 and for 7 vs 21, while no significant differences existed between 14 and 21 dpiBM n=15. **B)** End point titrations for viral infectious titers showed dramatic differences for all comparisons, n=12. Bars indicate mean ± 95% CI. (*) $P<0.01$, (***) $P<0.001$ by Tukey's multiple comparison test. Bloodmeal contained $1.7 \pm 0.7 \times 10^7$ PFU/ml.

2.3.4. Effect of a second bloodmeal on DENV-2 infected midguts

As previously shown the amount of viral antigen and the virus titer in the midguts decayed significantly between 14 and 21 dpiBM, whereas the viral RNA levels did not (Figure 2.3.6). The constant levels of viral RNA detected in midguts by real time qPCR suggested that virus was inactive but not cleared.

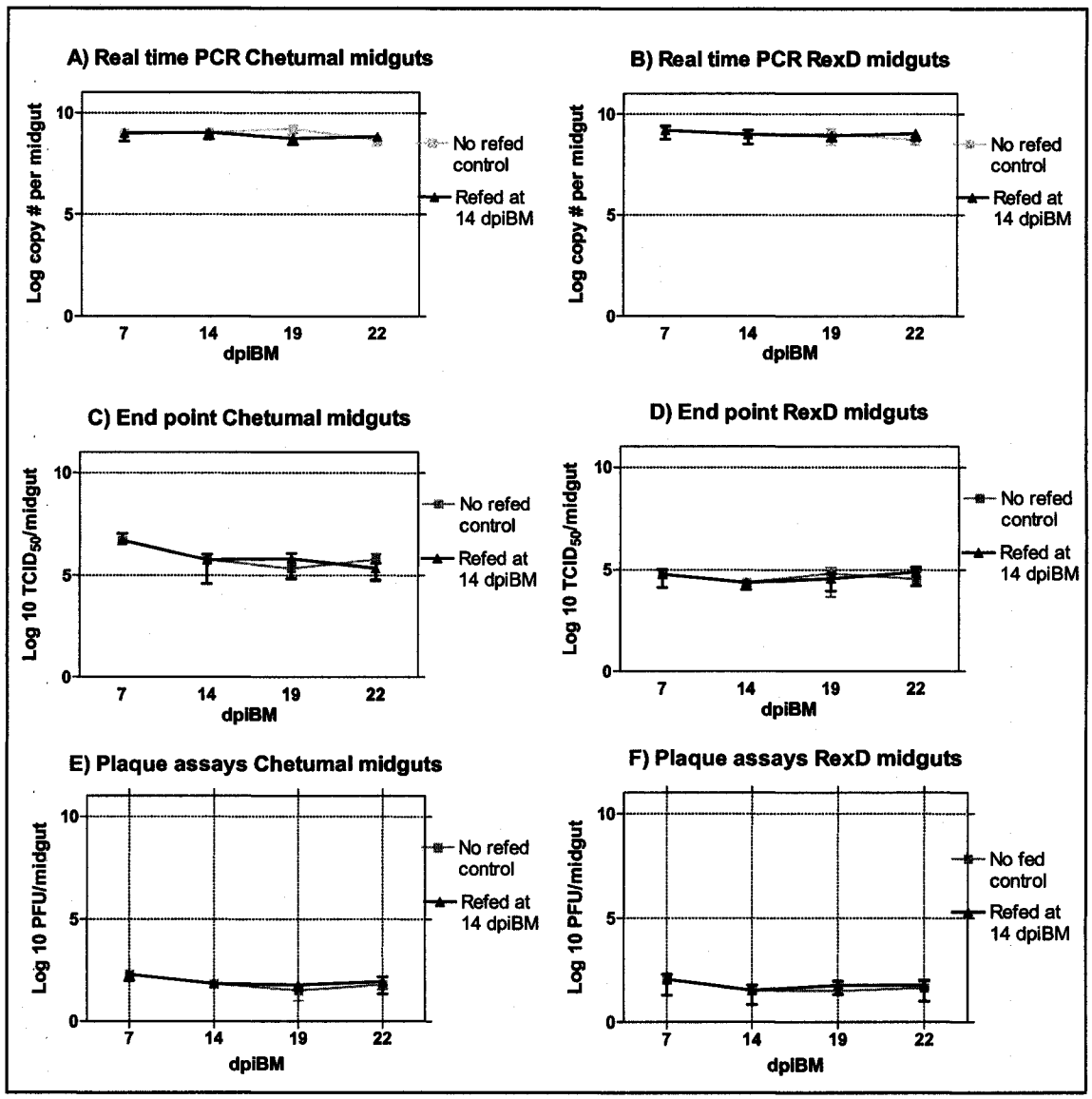


Figure 2.3.7. Quantification of DENV-2 viral RNA and infectious virus titers in Chetumal and RexD midguts after a second bloodmeal. A second bloodmeal 14 days after the initial challenge with DENV-2 Jam 1409. Ten paired midguts were processed by real time quantitative PCR (A and B), end point titration (C and D) and plaque assays(E and F). The dissemination rates in these groups at 14 dpiBM were 88% for Chetumal and 72% for RexD. Bloodmeal used for the initial challenge contained $4x10^7$ PFU/ml. Each point represents mean ± 95%CI.

To determine if a nutritional component conditioned this, DENV-2 challenged mosquitoes were offered a second uninfectious bloodmeal 14 days after the initial one. DENV-2 genomes were quantified and infectious virus titrated in midguts at predetermined days after the second bloodmeal. The results indicated that a second bloodmeal did not affect the infectious virus titers or viral RNA copy number in the midguts (Figure 2.3.7).

2.3.5. DENV-2 dissemination and the mosquito tracheal system

In mosquitoes, the oxygen uptake occurs through the tracheal system. The oxygen enters into their bodies through spiracles at the body wall, then the longitudinal tracheal trunk, eventually diffusing throughout a complex, branching network of tracheal tubes that subdivide into smaller diameter tubes, called tracheoles. Tracheoles are 0.2 to 1 μm in diameter, fluid-filled, and reach every part of the body (Appendix 7.3). To prevent collapse under pressure, a thin, reinforcing cuticle (the taenidia) winds spirally through the membranous wall. Insect cells consume the stored oxygen into the tracheoles using apparently water to regulate gas diffusion (317). The CO_2 passively diffuses through the cuticle.

Analysis of disseminated infection revealed DENV-2 antigen in portions of what looked to be part of tracheal system in 35 ± 5% of Chetumal mosquitoes. The earliest detection time was 2 dpiBM and decreased between 7 to 10 dpiBM. (Figure 2.3.8).The viral antigen did not localize throughout all the tracheal system; rather antigen was confined to small sections of trachea. Infected trachea showed a characteristic pattern of viral antigen distribution as observed in Figure 2.3.8. There was a significant correlation between early virus dissemination and the presence of viral antigen in trachea at 3 dpiBM ($P<0.001$), chi square=16.27 with df=1. Thus, examination of DENV-2 dissemination out of the midgut in Chetumal mosquitoes revealed that portions of trachea containing viral antigen correlated with dissemination. The tracheal system

contain virus antigen early in the infection and thus may play a role in early viral dissemination.

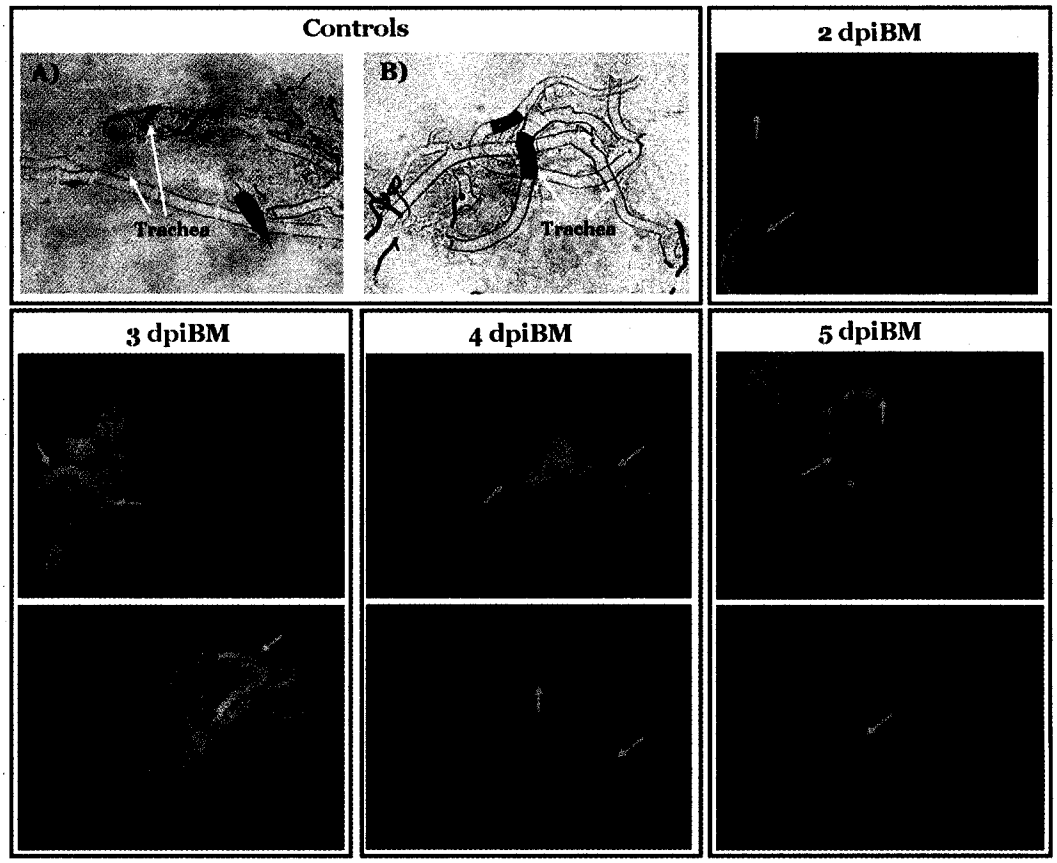


Figure 2.3.8. Presence of DENV-2 antigen in the tracheal system correlates with early dissemination in Chetumal mosquitoes. DENV-2 antigen presence was confirmed in mosquito presumed trachea at 2, 3, 4, and 5 dpiBM. Portions of the trachea (A-C) under light microscope are shown. Green arrows point to infected trachea. Pictures were taken using a magnification of 200X.

2.3.6. DENV-2 secondary target organs and tissue tropisms

The organ-tissue tropisms of DENV-2 Jam 1409 were determined overtime by examining tissues from 30 mosquitoes exposed to an infectious bloodmeal in two independent experiments. Viral antigen was detected in fat body, from abdomen, thorax or head, consistently throughout the time course. Thus, fat body seems to be a major site for DENV-2 replication in the vector.

DENV-2 dissemination to abdominal fat body or thoracic fat body started at 2 and 3 days after the infectious bloodmeal. Other organs and infected tissues included:

trachea, malphigian tubules, hemocytes, oesophagus, ommatidia of the compound eye, nerve tissue, anterior midgut and salivary glands (Figures 2.3.9).

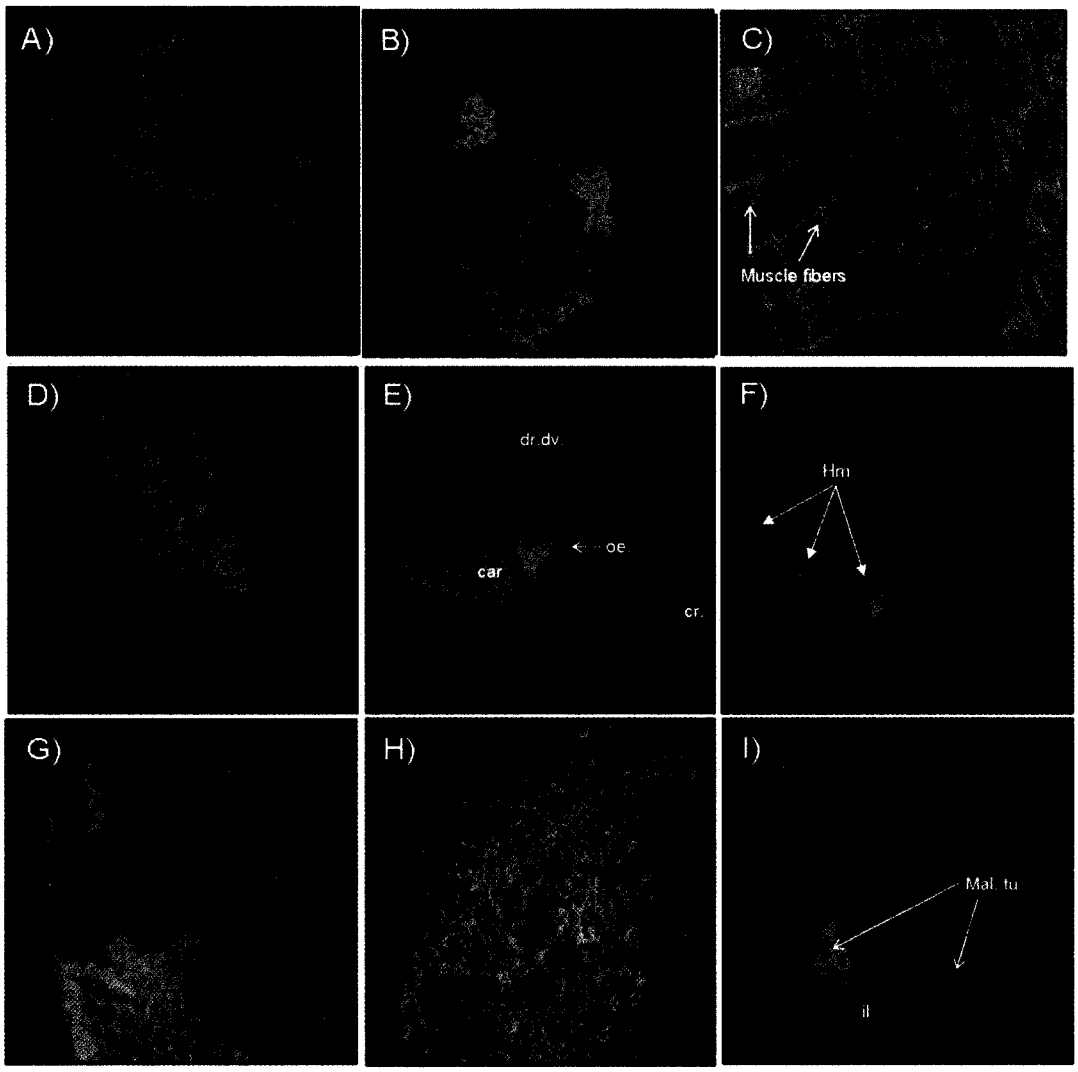


Figure 2.3.9. DENV-2 secondary target organs and tropisms in *Aedes aegypti*. DENV-2 viral antigen distribution was revealed by IFA (green for FITC or Alexa 488). A) DENV-2 antigen in fat body at 2dpiBM in the mosquito abdomen. B) DENV-2 in salivary glands (14 dpiBM). C) DENV-2 infecting epithelial cells but not muscle (red phalloidin-Alexa®546). D) Anterior midgut at 5dpiBM. E) Oesophagus at 7dpiBM, dr.dv. diverticulum, car: cardia, oe: oesophagus, cr: crop. F) Hemocytes in thorax at 10dpiBM. G) Ommatidia of the compound eye at 12dpiBM. H) Nerve cells at 14dpiBM. I) Malphigian tubules (Mal. Tu.) at 16 dpiBM, il: ileum. Original magnification was 200X, but pictures were cropped in Adobe Photoshop to improve presentation.

DENV-2 Jam 1409 displayed a high tropism for tissue of the central nervous system and salivary glands (Figure 2.3.10 and 2.3.11). Oesophagus, cardia, and the hindgut rarely contained viral antigen. The general kinetics of DENV-2 Jam1409

infection and replication in Chetumal mosquito is shown in Table 2.3.2. Viral antigen in the midgut decay starting at 10 to 14 dpiBM. In summary, DENV-2 exhibits multiple tropisms for mosquito organs and tissues including: midgut, fat body, salivary glands, nerve cells, ommatidia, hemocytes, and malphigian tubules, but not for muscle.

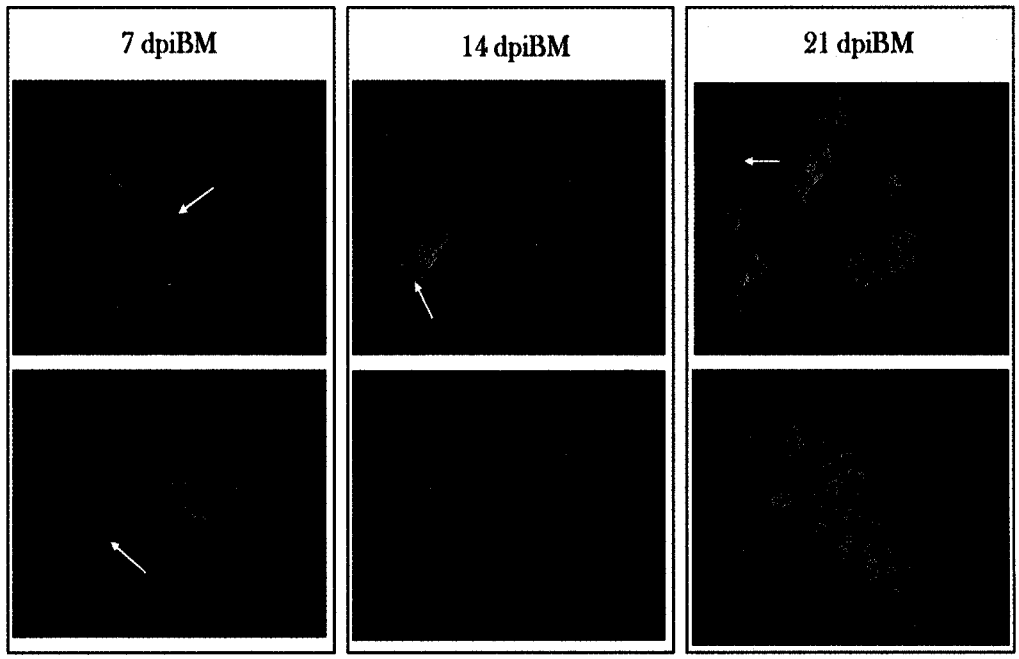


Figure 2.3.10. DENV-2 tropisms for head tissues in *Aedes aegypti*. Heads from Chetumal mosquitoes from different time points analyzed by IFA using 3H5 monoclonal antibody (green). Dengue virus antigen (in green) clearly accumulates in head tissues overtime. Arrows point to ommatidia of the compound eye. Magnification= 100X.

2.3.7. Characteristics of salivary gland infection by DENV-2 Jam1409

Each salivary gland consists of three lobes attached to a common salivary duct. Each lobe comprises a secretory epithelium with a basal and apical surface. The basal ends of the epithelial cells form the outside surface of the glands bound to the basal lamina. Female salivary glands differentiate into two lateral and one medial lobes, which produce distinctive secretions (135). Analysis of salivary glands from Chetumal and Rex-D *Aedes aegypti* after oral challenge with DENV-2 Jam 1409 showed slight differences in the initial infection. Four days after an infectious bloodmeal, DENV-2 was detected in 36±6% of Chetumal mosquito salivary glands (Figure 2.3.11A). Frequently but not

exclusively the infection starts at the distal lateral lobes, spreads to the proximal section of the lateral lobes and involves the entire organ later.

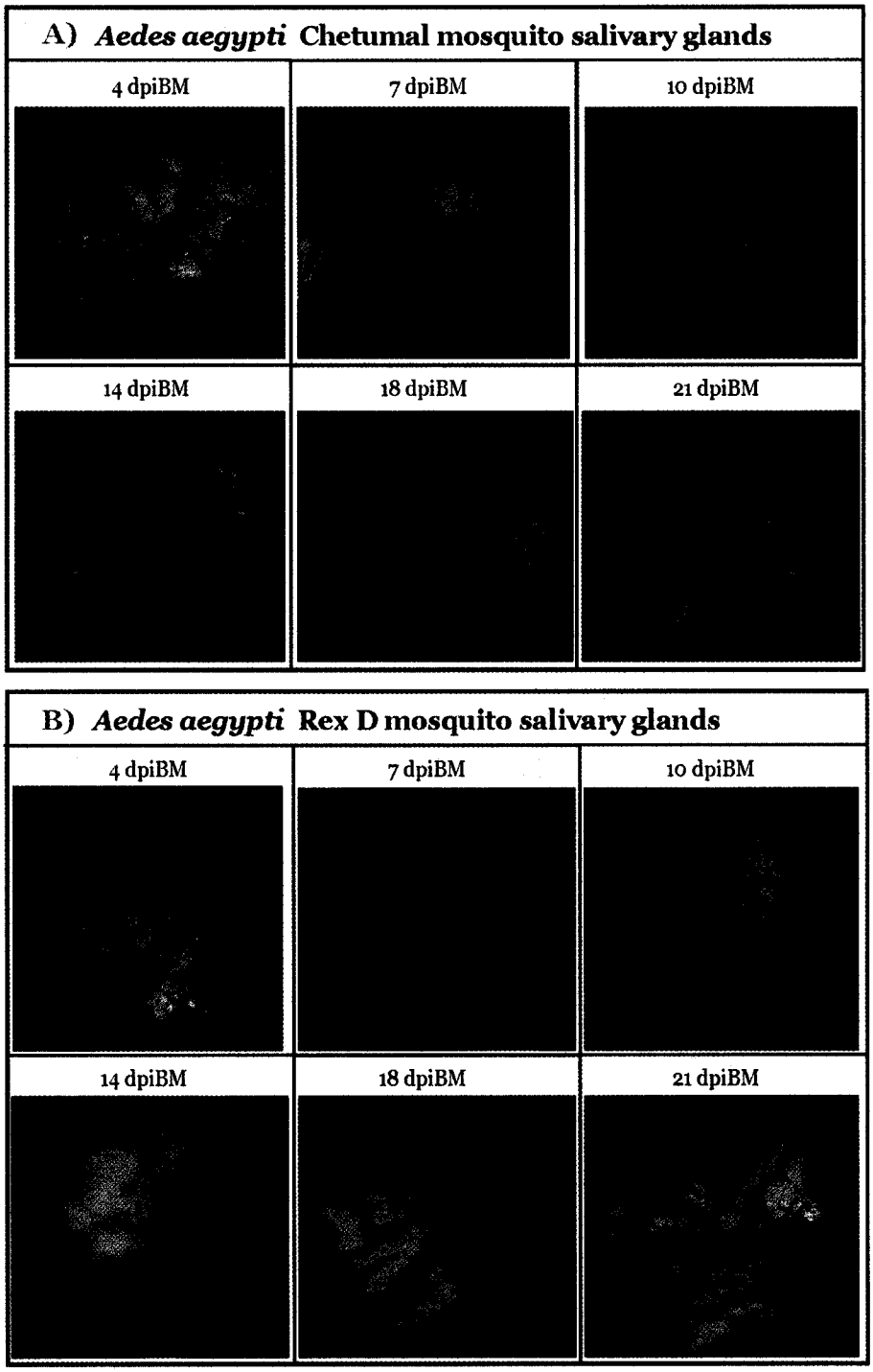


Figure 2.3.11. DENV-2 infection of Chetumal (A) and RexD (B) salivary glands. Salivary glands were dissected and placed on a vectabond pretreated slide and fixed. Tissue was assayed by IFA using 3H5 monoclonal antibody to detect DENV-2. Magnification was 200X (Pictures for RexD mosquitoes kindly provided by Dr. Irma Sánchez-Vargas).

In contrast, at the same time the virus was localized mostly in the fat body surrounding the salivary glands in Rex D mosquitoes (Figure 2.3.11B). Then the infection in both populations progressed, often involving the entire salivary gland.

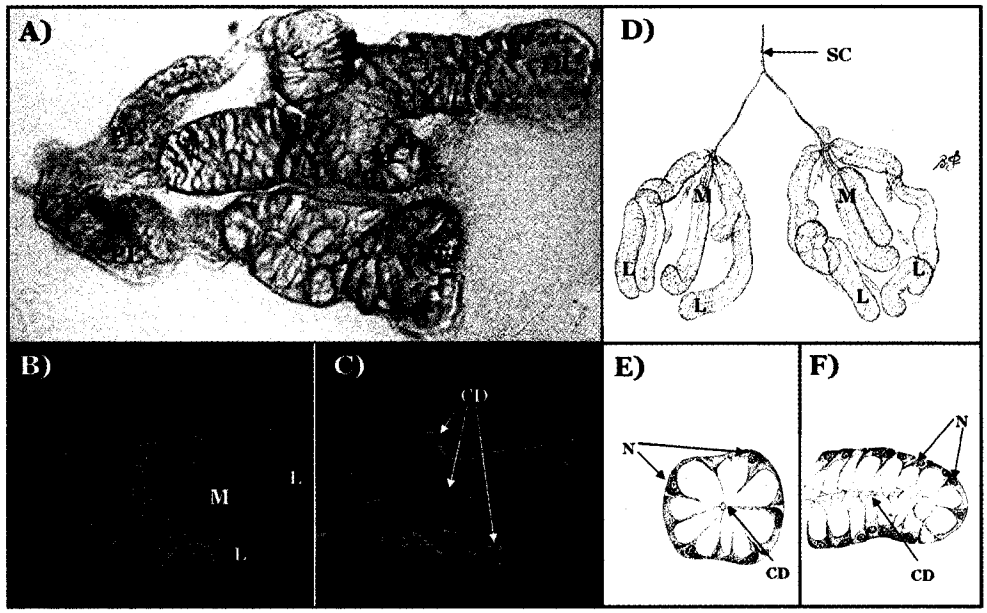


Figure 2.3.12. Mosquito salivary gland structure and organization. A) *Aedes aegypti* salivary gland using light microscopy (no staining), showing the medial lobe (M) and the proximal (PL) and distal lateral lobes (L and DL) Magnification was 300X. B) Salivary glands were counterstained with 0.001% Evans Blue. C) Salivary gland using UV microscopy shows the central duct (CD); D-F) Detailed drawings of salivary glands: complete (D), cross-sectioned (E), and longitudinally sectioned (F) respectively, showing cell nuclei (N) and central duct (CD) (138).

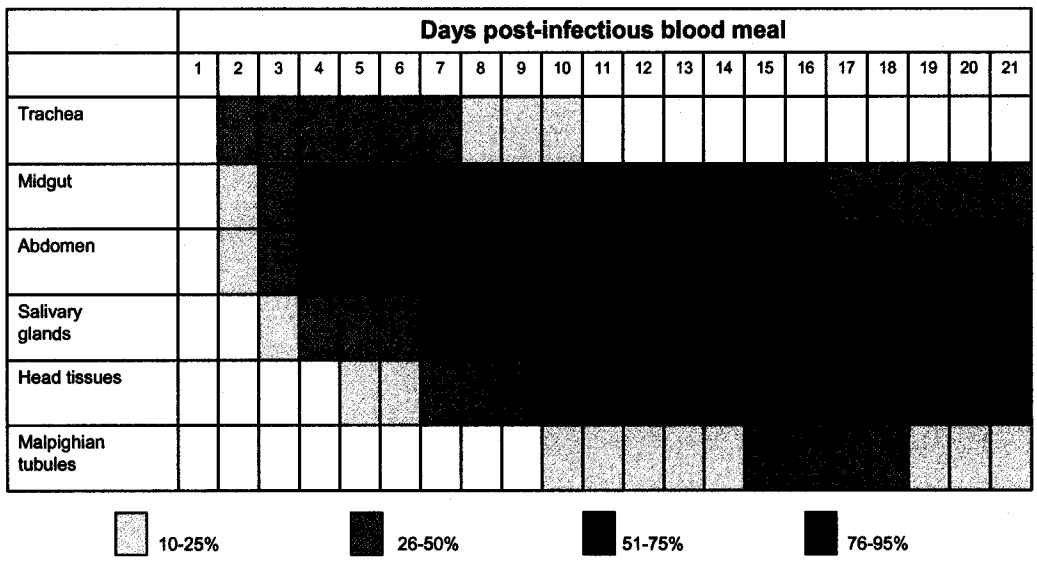


Table 2.3.2. Kinetics of replication and tissue tropisms of DENV-2 Jam 1409 in Chetumal *Aedes aegypti* mosquitoes. Intensity of infection in the respective organ or tissue is shown by the color scale. In abdomen fat body is the major site of virus replication.

Unlike other tissues such as midgut epithelia cells, trachea, and malphigian tubules that were only transiently infected. Virus antigen concentration in salivary glands increased and was retained overtime, indicating an important tropism for this tissue. To be transmitted the virus must reach the saliva and be expelled by the central duct in further blood feedings (Figure 2.3.12).

2.3.8. Effect of virus dose on dissemination in mosquito

There is evidence that infection of the *Ae. aegypti* mosquitoes midgut by DENV is dose dependent (18). Experiments were conducted to investigate the effect of virus dose on dissemination to salivary glands and head tissues in *Ae. aegypti*. To minimize midgut barriers effects on dissemination, D2S3 mosquitoes were used, since they exhibit minimum barriers to productive DENV-2 infection.

The viremia titers of DENV-2 in human blood vary from 10^2 to 10^7 PFU/ml (266). For each experiment 3 serial 10-fold dilutions of the same virus preparation containing 10^5, 10^6 and 10^7 PFU/ml titers of DENV-2 Jam 1409 in infectious bloodmeals were used to infect D2S3 mosquitoes. Dissemination to salivary glands and head tissues at 14 dpiBM were analyzed (Figure 2.3.13).

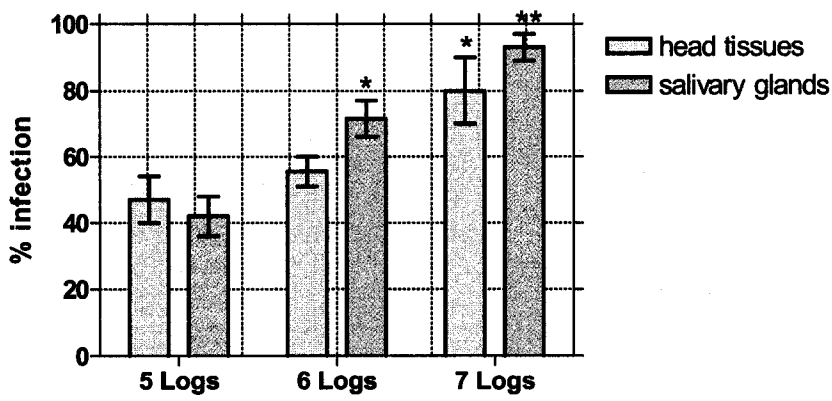


Figure 2.3.13. Dissemination of DENV-2 Jam 1409 in D2S3 mosquitoes. Female mosquitoes (40-50) from two independent experiments were assayed to determine by IFA dissemination rates to heads and salivary glands at 14 dpiBM. Virus titers in the bloodmeals were $1.25\pm0.25x10^5$ PFU/ml (5 Logs), $1.10\pm0.45x10^6$ PFU/ml (6 Logs) and $1.75\pm0.65x10^7$ PFU/ml (7 Logs). Error bars indicate standard error of the mean (SEM). *P<0.05 **P<0.01 by t unpaired test.

In D2S3 mosquitoes challenged with a bloodmeal containing 5 logs PFU/ml of virus, 47% ± 7% of the mosquito head tissues and 42% ± 6% of salivary glands became infected. In mosquitoes challenged with 6 logs PFU/ml, virus disseminated to 55% ± 5% of mosquito head tissues and 71% ± 5% of salivary glands.

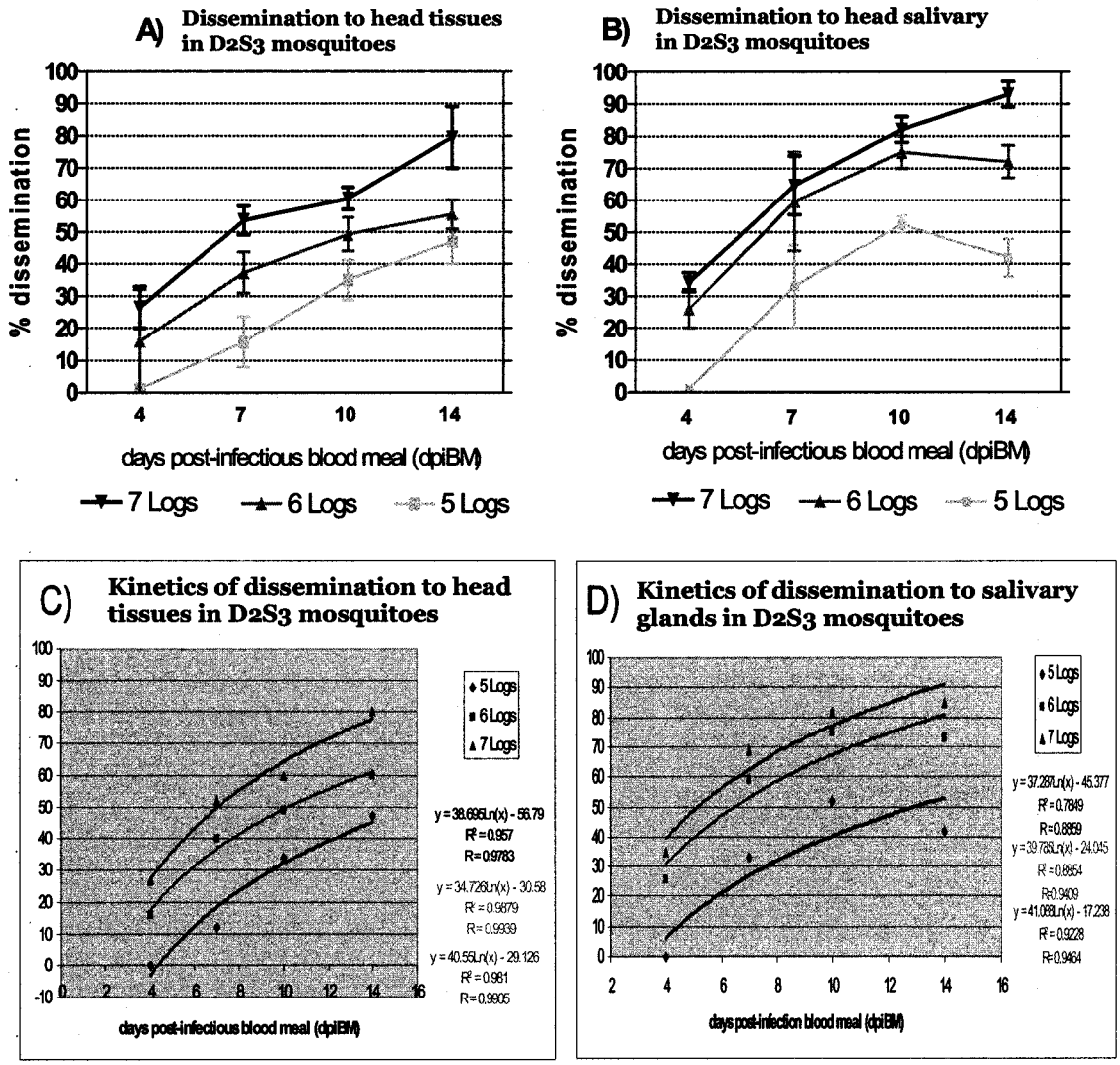


Figure 2.3.14. Kinetics of dissemination to head tissues and salivary glands for DENV-2 is dose dependent in D2S3 mosquitoes. Individuals collected at 4, 7 and 10 dpiBM and at 14 dpiBM from two different and independent experiments. Bars indicate mean values and SEM (Standard error of the mean) n=30-50. A and B represent the values obtained for the data, while C and D are the correlation curves for the data obtained in Excel of Microsoft.

In mosquitoes challenged with an infectious bloodmeal containing 7 logs PFU/ml, 80% ± 10% of head tissues and 93% ± 4% of salivary glands became infected. A

direct correlation between the virus dose and infection of both salivary glands and head tissues was obvious at 14 dpiBM. An exponential equation ($y=Ax^n$, where x represents virus dose and y dissemination rates) best fit the data provided by the three virus doses tested. Thus, the kinetics of dissemination to head tissues and salivary glands were also dose dependent (Figure 2.3.14). Interestingly, the salivary gland infection rates were more pronounced. Curve fitting and regression analysis was performed on the dissemination data. A logarithmic equation ($y=A(Lnx)-B$); where y means dissemination rates and x time postinfection best fit the data in both salivary gland and head tissues kinetics of infection.

2.3.9. Quantification of DENV-2 titers in individual mosquitoes

To establish the kinetics of viral replication, 5 to 10 individual infected Chetumal mosquitoes were assayed by plaque assay from 2, 3, 4, 5, 7, 10, 14, 18, and 21 dpiBM (Figure 2.3.15).

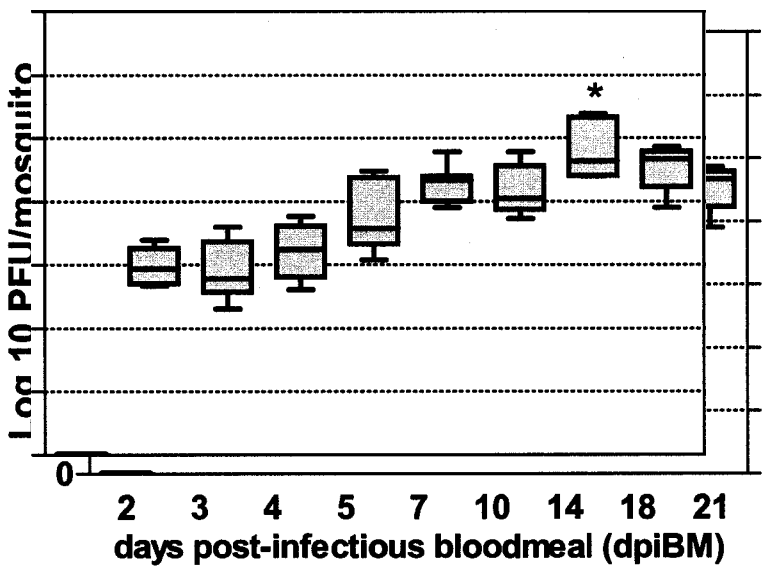


Figure 2.3.15. DENV-2 titers in orally infected whole mosquitoes. Individual Chetumal mosquitoes were titrated by plaque assays. Mosquitoes were assayed at 3, 4, 7, 10, 14, 18 and 21 days after the oral challenge with an infectious bloodmeal. Each point in the graph presents mean and range of the viral titers of n=10 mosquitoes for each time point. The infectious bloodmeals contained 1.7 ± 0.7 x 10^7 PFU/ml. P values: *P<0.05 and **P<0.001, comparing to the 3 dpiBM. Each point shows the mean of titers and range value distribution.

Mosquitoes at 24 hours were not tested because infectious virus in the undigested bloodmeal would still be present. At 2 dpiBM, infectious virus was undetectable in the 10 examined mosquitoes; virus was presumably in an eclipse phase or the sensitivity of the assay failed to detect low titers at this time point.

Infectious virus was first detectable at 3 dpiBM, when mosquitoes contained an average of 10^3 PFU/mosquito. Viral titers continued to increase until day 18 when titers reached about 10^5 PFU/mosquito. Two declines in overall titers occurred: one at 14 dpiBM and the other at 21 dpiBM (Figure 2.3.16). As previously shown virus tropism in mosquito tissues varied during this time frame (Results shown in section 2.3.6).

Finally, virus titers were compared between the Chetumal and the highly susceptible D2S3 mosquitoes at 4, 7, 10 and 14 dpiBM and some important differences were detected (Figure 2.3.16).

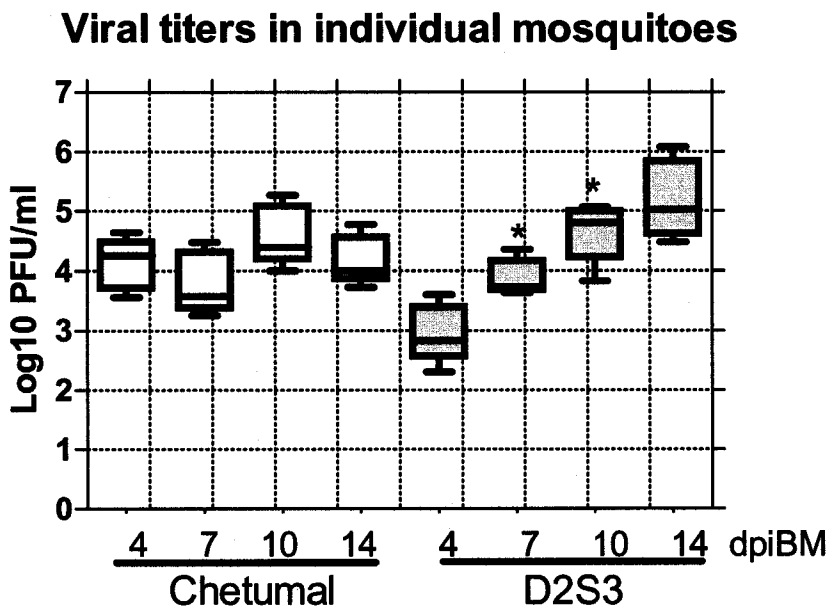


Figure 2.3.16. Comparison of kinetics of DENV-2 replication in orally infected mosquitoes. Titers were higher in Chetumal mosquitoes at 4dpiBM that in the highly susceptible D2S3 mosquitoes. Individual mosquitoes were titrated by plaque assays. Mosquitoes were assayed at 4, 7, 10, and 14 days after the oral challenge with an infectious bloodmeal. Each point in the graph presents mean and standard deviation (SD) viral titers of n=5-10 mosquitoes for each time point. The infectious bloodmeals contained 1.7 ± 0.7 x 10^7 PFU/ml. *P<0.05 by Tukey's multiple comparison test.

Although viral titers were higher at 14 dpiBM in D2S3, DENV-2 infected Chetumal mosquitoes exhibited the high titers since early times (4 dpiBM). Overall titers in Chetumal mosquitoes were high and they did not increase dramatically over time (3.5 to 5 Logs PFU/mosquito).

However, viral titers in D2S3 mosquitoes increased significantly over time. Virus titers started with about 2.5 to 3.5 logs PFU/mosquito at 4 dpiBM and finished with 4.5 to 6 logs PFU/mosquito (Figure 2.3.16). The two very different kinetics of viral titers suggest that different dissemination mechanism in the vector.

2.4. Discussion

After DENV ingestion in a bloodmeal, the virus must infect, replicate, and disseminate through the mosquito body to infect target organs, and be transmitted from salivary glands to continue the transmission cycle. DENV infection of midguts from different mosquito populations showed no major differences in patterns or cell-type tropisms. In RexD mosquitoes frequently the infection started in the posterior region of the midgut, while in Chetumal foci of infection where more randomly distributed through the midgut. The initial infection involved individual cells, evolved to foci spreading laterally to neighboring cells, reached a peak between 7 and 10 dpiBM involving frequently the entire organ, and began to decline (Figure 2.3.2, 2.3.4., and Table 2.3.2). Frequently viral antigen accumulated on the epithelial cell surface in a beehive-like conformation (Figures 2.3.3 and 2.3.5).

DENV-2 transcription, translation and replication are clearly modulated in the vector. The amount of viral antigen and the number of infectious viral particles in the midgut declined over time (Figure 2.3.1 and 2.3.4), while viral RNA numbers remained fairly constant at two of the three examined time points (Figure 2.3.6). DENV-2 replication in the mosquito midgut may be restricted by antiviral response, post-

transcriptional or post-translational repression, self-limiting events, or nutritional factors. Previous experiments demonstrated that before the establishment of the initial DENV infection, the virus remained undetectable in the midgut if nutrients from bloodmeal digestion were not available (197). In the present experiments DENV infection in the midgut became established, peaked, and started to decay before the second bloodmeal took place. Therefore, virus had been exposed in these experiments to more conditions in the mosquito including the immune response that could have participated in limiting the infection.

Unlike LACV (39), DENV virus replication in infected mosquito midgut was not dependent on host cell biosynthetic status. Availability of nutrients in the midgut cells after a second bloodmeal did not increase the virus titers in the midgut, despite presence of viral RNA (Figures 2.3.1, 2.3.4 and 2.3.6). The establishment of persistent infection by DENV has been demonstrated in multiple cell types (161). Recent reports indicate that flavivirus-related sequences can be detected in DNA form in *Aedes* mosquitoes and cell lines, indicating potential integration by a novel mechanism (54). Unique mechanisms must be conditioning flavivirus persistence in their vectors. Determinants of replication restriction/persistence of flaviviruses in their vectors remain to be determined.

The mechanism(s) by which DENV disseminates from the midgut are not well understood. This study suggests that the mosquito tracheal system may be associated with DENV early dissemination (Figure 2.3.8 and Table 2.3.2). Tracheal system acts as conduit for dissemination of different viruses in insects. Notable examples are *Autographa californica* (alfalfa looper) M nuclear polyhedrosis virus (AcMNPV) infecting *Trichoplusia ni* (cabage looper), nucleopolyhedrovirus infecting *Bombyx mori* (silk worm), and Sindbis virus in *Aedes albopictus* (32, 75). The tracheal system is also suspected to be the conduit for midgut escape for Venezuelan equine encephalitis virus (VEEV) in *Ochlerotatus taeniorhynchus* (242). Further, WNV virus-like particles (VLP)

localize focally in tracheal cells after intrathroracic inoculation of *Culex pipiens quinquefasciatus* (260). A recombinant baculovirus of *Anticarsia gemmalis* (velvetbean caterpillar) multicapsid NPV localizes in midgut cells, midgut-associated trachea and hemocytes as early as 3 hour post-infection in the insect larvae (272). To disseminate through the insect body using trachea may represent significant advantages, since this system reaches almost every cell in the insect at tracheole level. Cells of many types are entered by tracheoles, including salivary glands in Muscidae (Diptera) and silk glands of *Bombyx sp* (316). Thus, viruses that infect insects could exploit arthropod physiology to disseminate in ways that are not well understood.

This is the first report indicating that DENV may also use the mosquito tracheal system as conduit to transverse the midgut. The accumulation of virus antigen in portions of what seems the tracheal trunk was documented (Figure 2.3.6). Because trachea did not become infected in DENV-3 intrathoracically injected mosquitoes (178), this suggests that trachea infection may be related only to events occurring during oral infection. How the virus gets from the midgut into the tracheal system remains unclear, but viruses appear to use it to disseminate throughout the vector.

Dissemination rates to head tissues have been used to reveal mosquito susceptibility to various arboviruses (141, 157, 245-247). Interestingly, dissemination rates to head tissues diverged somewhat from those to salivary glands, indicating that salivary gland tissue may be more susceptible to infection by DENV-2 Jam1409 than the head tissues (Figure 2.3.13 and 2.3.14). However, this may be an artifact due to the nature of the assayed material. Whereas head tissues are squashed onto slide and some of the tissue lost, viral antigen in dissected salivary glands is more readily scored since virus is contained in the entire organ fixed to the slide. In any event salivary glands constitute a better indicator of virus transmission potential than head tissues.

The extrinsic incubation period for DENV is between 7-14 days, and is affected by conditions such as temperature, humidity and viral dose (24, 104, 309). Once salivary gland infection occurs and virus reaches saliva (or salivary ducts), mosquitoes are capable of transmitting the virus. In this study, a significant percentage of salivary glands in newly colonized field-relevant Chetumal mosquitoes became infected shortly after an infectious bloodmeal (4 dpiBM) in a dose dependent-manner (Figure 2.3.11 and Table 2.3.2). In *Aedes albopictus* DENV transmission occurs by 10 dpiBM (108). Due to the lack of reliable laboratory animal model for dengue, it is likely the sensitivity of assays used in the past failed to detect small but transmissible amounts of virus at earlier times than the reported one.

DENV-2 displayed major tropisms for salivary glands (2.3.11) and head tissues (Figure 2.3.10). Virus accumulation in these organs could alter normal function. Mosquito salivary glands include two lateral and one medial lobe secreting different enzymes. The proximal region of the lateral lobes secretes enzymes involved in sugar feeding such as amylases and α 1-4 glycosidase (135). The proteins produced by the medial and the distal lateral lobes participate in hematophagy. The secreted products include apyrases, esterase, anticoagulants and vasodilator substances (5, 13, 135, 270). Virus-associated pathological changes in salivary glands and other organs occur for Sindbis virus infection of *Aedes albopictus* and WNV infection of *Culex pipiens quinquefasciatus* (33, 91).

DENV infected mosquitoes need longer probing and feeding times and have an increased secretion of enzymes such as esterase (140, 223). To complete a single gonadotropic cycle, *Aedes aegypti* mosquitoes require from 2 to 3 bloodmeals, feeding with a frequency of 0.63 to 0.76 times a day (261). Thus, DENV infection may perturb

mosquito feeding behavior which could have important epidemiological consequences in virus transmission.

Unlike many other arboviruses, DENV displayed no tropism for midgut-associated or flight muscles (Figure 2.3.5 and 2.3.9). RVFV infects both skeletal and visceral muscles (241). Sindbis virus replicates in visceral muscles of *Aedes albopictus* (33). WNV infects the contiguous muscles of the posterior and anterior midgut in *Culex* mosquitoes (90). The reasons for mosquito muscle refractoriness to DENV are unknown. Rift Valley virus and Sindbis alphaviruses, and DENV-3 exhibit important tropism for cardia tissue (172, 178); while DENV-2 was hardly found in this tissue.

Finally, differences in susceptibility were found between laboratory-reared (D2S3, RexD) and newly colonized field-relevant (Chetumal) mosquitoes. Organs such as midguts and salivary glands in newly colonized Chetumal mosquitoes become infected earlier and about the same rates as the DENV-2 highly susceptible D2S3 laboratory mosquito strain, while progression of infection in RexD mosquito was slower and at lower rates (Figure 2.3.1, 2.3.7 and 2.3.11). Additionally, Chetumal infected mosquitoes exhibited the highest viral titers in whole mosquitoes at early times (4dpiBM) (2.3.16). Thus, long-colonized RexD mosquitoes might be less susceptible than Chetumal mosquitoes and provide different time frames for transmission of some DENV strains and as consequence the epidemic potential. Clearly, it is important to study field-relevant mosquitoes that better reflect the characteristics and impact of the virus-vector interactions in nature.

Chapter III

Correlation of DENV-2 clinical severity in humans and virogenesis in *Aedes aegypti* mosquitoes.

3.1. Introduction

Dengue is an arthropod-borne viral disease of major public health importance. Dengue virus infections are a serious cause of morbidity and mortality in many tropical and subtropical areas in the world. In the Americas, increased transmission of multiple dengue virus serotypes followed the expanded distribution of the mosquito vector and resulted in hyperendemicity in many countries. Most of these countries subsequently developed a continuing cycle of dengue and DHF epidemics at three to five year intervals, with epidemics becoming progressively more severe (84, 111). Unlike Southeast Asia, in the Western hemisphere DHF appears in both children and adults (111).

Secondary infection caused by a different dengue virus serotype is an important risk factor associated with severe forms of the disease (116, 199). There is a positive correlation between disease severity and viremia titers (297). In endemic areas, DENV-2 is more frequently associated with DHF (213). More virulent DENV-2 genotypes relate to severe disease (46). The introduction of the Southeast Asian genotypes of DENV-2 in Latin America coincided with appearance of DHF in the Western Hemisphere (184, 236-238, 297).

Phylogenetic analysis suggests virulent genotypes have displaced those of lower virulence. Genotype replacement has occurred in many countries in the Americas including Mexico (184, 235). The Southeast Asian genotypes not only cause more severe disease outcomes but may also be able to outcompete indigenous American genotypes (7, 50). DENV-2 infection and dissemination rates in *Aedes aegypti* infected with Southeast Asian genotypes are about 3-fold higher than those infected with American genotypes (7). Studies relating infecting DENV genotype and replication efficiency in the mosquito have been addressed only recently (6, 7, 50). The identification of genetic determinants associated with the efficiency of virus replication in both DENV hosts will provide a

better assessment and interpretation of the epidemiology that different DENV-2 genotypes exhibit.

The experiments presented in this chapter examined the correlation between clinical severity of DENV-2 in humans and virogenesis in mosquito and explored differences in sequence in two virus genomic regions (E and 3'UTR). The overall hypothesis was that *some DENV-2 genetic determinants that condition high virulence in humans may also enable more efficient replication and transmission potential by the mosquito vector*. In this study, four DENV-2 Yucatan strains, which originated from cases of different clinical severities, were characterized for their virogenesis in mosquitoes. Two geographically distant *Ae. aegypti* mosquito populations from Mexico were examined in this study. One mosquito population originated in Chetumal in the Yucatan Peninsula, where the studied viruses were isolated (coindigenous), the other originated in Loreto in the Baja California region, Mexico (non coindigenous). Mosquitoes were challenged with the respective DENV-2 Yucatan viruses and dissemination rates as well as viral titers were determined.

Additionally comparisons between DENV-2 American/Asian (Yucatan strains) and American strains included: dissemination rates in Chetumal mosquitoes and comparison of E protein gene and 3'UTR sequences. The E glycoprotein and the 5'- and 3' untranslated regions contain determinants that may influence the efficiency of DENV infection and replication (3, 44, 48, 148, 225, 226, 325). The virus fitness or efficiency to replicate could vary in vertebrate and invertebrate hosts. Potential genetic determinants associated with DHF in DENV have been previously located in the E gene and the 5' and 3' UTR of DENV genomes (249).

These studies revealed a correlation between clinical severity and virogenesis in Chetumal (coindigenous) mosquitoes, but not in Loreto (non coindigenous) mosquitoes. Differences between the American/Asian and American DENV-2 genotypes were

identified in the 3'UTR and in E protein sequences. The potential significance of these results is discussed.

3.2. Materials and Methods

3.2.1. DENV-2 strains

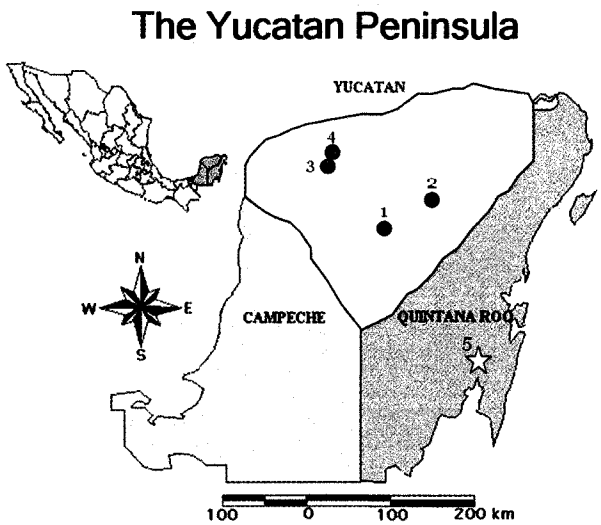
This study included six different DENV-2 strains. Five of these strains clustered into the American/Asian genotype and one of them into the American genotype (Table 2.3.1). All of these strains had a low passage history (as indicates in table 3.2.1) except for the prototype strain DENV-2 Jam 1409, which was high passage. The DENV-2 Yucatan viruses were isolated between 2001 and 2002 from anonymous patients that developed different clinical severity. Viruses were low passage to prevent potential sequence and fitness artifacts due to high passage in a cell line.

DENV-2 Genotype	DENV-2 strain ID	Date of blood sample	Case ID	Clinical case	Passage History
American	QR0094 (Yuc3315)	1994		DFHM	Patient→ 3 (C636)
American/ Asian (Southeast Asian)	Yuc11936	08/ 2001	"less severe"	DFHM	Patient → C636 → BHK → C636
	Yuc12914	09/ 2001		DF	Patient → C636 → BHK → C636
	Yuc14757	09/ 2002	"more severe"	DHFII	Patient → C636 → BHK → C636
	Yuc14497	10/ 2002		DHFIII	Patient → C636 → BHK → C636
	Jam1409 hp	1983		DF	Patient→n(LLC-MK2)→ n(C636)

Table 3.2.1. DENV-2 strains used in this study. The strain ID, date of blood sampling, clinical type and passage history are presented. The genotype was determined based on the prM-E junction sequence.

These viruses were kindly provided by the laboratory of arbovirology from the "Centro de Investigaciones Regionales Dr. Hideyo Noguchi" in the Universidad Autonoma de Yucatan (UADY). The Yucatan Peninsula, which is hyperendemic for dengue, is located in Southeastern Mexico. The sites where viruses originated are shown in Figure 3.2.1.

The rationale to use closely related DENV-2 strains was that sequence changes associated with phenotype changes were more likely to be identified in closely related strains. Temporal and spatial distance causes sequence changes that reflect the different evolutionary lineages of the respective viral genomes could mask genetic determinants associated to specific phenotypes (e.g. disease severity).



1. DENV-2 Yuc11936. Santa Elena. 08/2001.
2. DENV-2 Yuc12914. Tekax. 09/2001.
3. DENV-2 Yuc14497. Merida. 10/2002.
4. DENV-2 Yuc14757. Merida. 09/2002.
5. DENV-2 QRo094

Figure 3.2.1. Map showing the localities where the studied viral strains were isolated.

3.2.2. The mosquito populations

In order to characterize the virogenesis in mosquito of the DENV-2 Yucatan viruses, two mosquito populations from geographically distant localities and different susceptibilities were used. The rationale was to separate effects of virus virulence from those of potential geographic or vector competence- associations. In addition to the Chetumal mosquito population described in detail in Chapter II, *Ae. aegypti* mosquitoes from Loreto were included in this study. These recently colonized (F-3 to F-6) mosquito populations were collected from: 1) Loreto, the Baja California Region in northwest México; 2) Chetumal, the Yucatan Peninsula in Southeast México (Figure 3.2.2.).

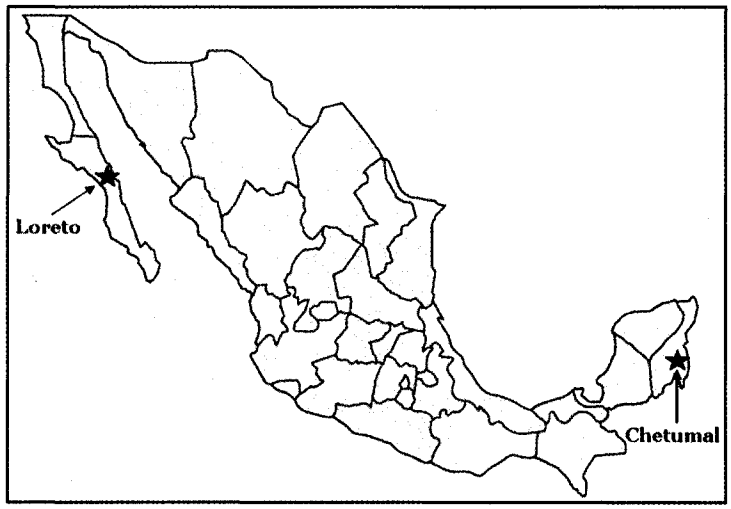


Figure 3.2.2. Origin of mosquito populations in Mexico.

3.2.3. Mosquito oral challenges with DENV-2 and dissections

Protocols for oral infection of mosquitoes and tissue processing for virus infection assays were detailed in sections 2.2.4 and 2.2.5.

3.2.4. Indirect Immunofluorescence and virus titration assays

Protocols for IFA and virus titrations were described in detail in sections 2.2.6 and 2.2.7.

3.2.5. Virus RNA extractions

Viral RNA was isolated from stocks using the QIAamp viral RNA minikit from QIAGEN. Briefly, 560 µl of buffer AVL containing carrier RNA was added to 140 µl of virus suspension, followed by pulse-vortexing for 15 seconds, incubation at room temperature for 10 minutes, and brief microcentrifugation. Then addition of 560 µl of ethanol 100% was followed by pulse vortexing for 15 sec and microcentrifugation. QIAamp spin columns loaded with the total mix were centrifuged at 6000 rpm x g (8000 rpm) for 1 minute. The remaining liquid in the column was collected into a clean tube and the centrifugation step was repeated, then 500 µl of buffer AW1 were added and the columns centrifuged at 6000xg. Next, 500µl of AW2 buffer followed by centrifugation at

14,000 rpm (20,000xg) for 3 minutes were performed to wash the RNA in the column. The RNA eluted into a clean tube with 60 µl of AVE buffer, equilibrated at room temperature, and centrifuged at 6000xg (8000rpm) for 1 minute to obtain viral RNA, which was stored at -70°C until cDNA synthesis.

3.2.6. cDNA synthesis

The first strand of cDNA synthesis used SuperScript™ III reversetranscriptase (RT) from Invitrogen (Carlsbad, CA) and primers D2-2479R (5'ARATCCCGCTG CCACATTTT3') and D2-10723R (5'AGAACCTGTTGATTCAA CAGCACCA3'). Briefly, an RNA-primer mix containing 5µl of RNA solution was combined with 1µl of 10mM dNTPs mix, 1µl of specific primer (50µM), and 2µl (or to 10µl) of DEPC-treated water. Following incubation at 65°C for 5 minutes, the mixture was placed in ice for 1 minute and 10 µl of cDNA synthesis mixture were added to each RNA-primer reaction. The cDNA synthesis mixture included 2µl of 10X RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 100mM DTT, 1µl of RNaseOUT™(40U/µl) and 1µl of SuperScript™III-RT(200 U/µl). The reactions were then incubated for 50 minutes at 50°C, followed by 5 minutes at 85°C, and chilled on ice.

3.2.7. PCR products for sequencing

Two PCR products were used as the templates for the sequencing reactions. To sequence the E protein a PCR product of 1,659 bp was obtained using the primers D2-820F (5'TTGAGACATCCAGGCTTCACCATA3') and D2-2479R (5'ARATCCCGCTGC CACATTTT3'). To sequence the 3' UTR, a PCR product of 1,043 bp was obtained with the primers D2-9680F (5'CACAAGTGCCTTTCTGTTCACACC3') and D2-10723R (5'AGAACC TGTTGATTCAACAGCACCA3'). Each PCR reaction included: 3µl of cDNA template, 5µl of 10XPCR buffer, 3µl of dNTPs(2.5mM), 3µl of primer1 (50 µM), 3µl of primer2 (50 µM), 3.7µl of DMSO, 7µl of 5MBetaine, 25.3µl of sterile water, and 1U of TaKaRa ExTaq®DNApolymerase. The PCR conditions were: 1 minute at 94°C, followed by 40 cycles of: 10s denaturation at 94°C, 30s annealing at 60°C and extension for 3minutes at

68°C. Then, final extension at 68°C for 7 minutes and 4°C until purification. QIAquick PCR purification Kit from Qiagen was used to clean the PCR products.

3.2.8. E protein gene sequencing

The rationale to study the E protein was supported by the evidence that specific virulence phenotypes associated to amino acid changes in this protein (34, 102, 166, 211). These phenotypes include: neutralization escape mutants (180), genotype identity differentiating between American and Asian (228), and changes in fusogenic activity (109, 195). Additionally, this gene better documents the geographical origin of the viruses (214).

In DENV-2, neurovirulence associated amino acid changes include E-71 (Glu-Asp), E-126 (Glu-Lys), and E-390 (Asp- His) (34, 102, 251). Generation of neutralization escape mutants occurs by changes in amino acids such as E-69 (Thr-Ile), E-71 (Glu-Asp), E-112 (Ser-Gly), and E-124 (Ile-Asn), and E-124 (180). Genotype identity seems to reside in mutations such as E-390 (Asn-Asp), which seem critical in the transition from the Asian to American genotype (228). The resistance to low pH has been associated with a change in E-153(Asn-Asp) (110). Some changes in fusogenic activity involve changes in E-6 (Ile-Met) or E-134 (Asn-Ser) (110). Some of the mutations in domain I and II of the E protein that alter the fusion pH threshold can affect virulence as well (195).

Primer	Sequence	Length
D2-1146F	5' ATGAAGAGCAGGACAAAAGGTT 3'	22 nt
D2-1233F	5' AGGATGGGGAAATGGATGTGGAT 3'	23 nt
D2-2018F	5' AAGATAGCCCAGTCAACATAGAAG 3'	24 nt
D2-1245R	5' ATTTCCCCATCCTCTGTCTACCAT 3'	24 nt
D2-1656R	5' TTCTTCGCGTGGGGATTTTTG 3'	21 nt
D2-2076R	5' TTCARTTGTCCCGGCTCTACTACTCCT 3'	27 nt
D2-2461R	5' ARATCCCGCTGCCACATTT 3'	19 nt

Table 3.2.2. Primers used to sequence E protein gene of DENV-2.

Thus, multiple studies have shown that some remarkable differences in virulence result from minor amino acid changes in the viral glycoprotein E, which is associated with receptor-binding, entry and fusogenic activity of DENV. Assembly of the entire E gene (1485 bp) required 7 different primers (Table 3.2.2 and Figure 3.2.3).

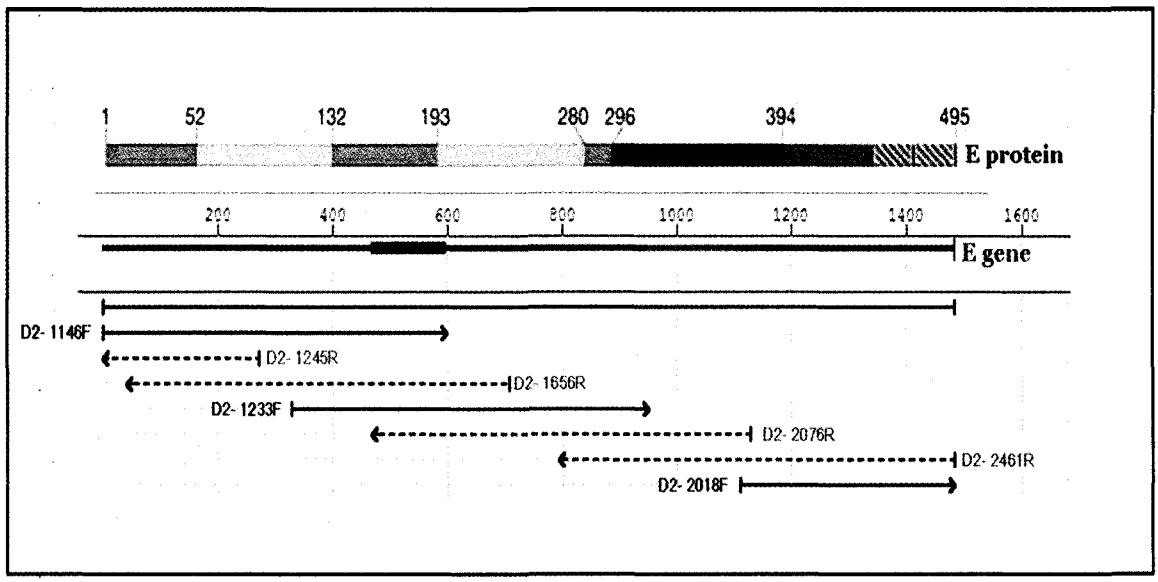


Figure 3.2.3. Assembly map of E gene of DENV-2. The primers used are noted.

3.2.9. 3' UTR sequencing

The 3' SL structure in the 3' UTR relates to the host range (190) and its conserved motifs engage in cyclization of the viral genome that promotes efficient virus translation and replication (3, 44, 48, 148, 225, 226, 325). The 3' UTR is also associated with differences between the Asian and American DENV-2 (171).

Primer	Sequence	Length
D2-10146F	5' GGCAAAGAACATCCAAACAGC 3'	21 nt
D2-10304F	5' GTCAGGTCGGATTAAGCCATAGTA 3'	24 nt
D2-10522R	5' CCCGTTGTTGCTGCGATTTGTA 3'	22 nt
D2-10723R	5' AGAACCTGTTGATTCAACAGCACCATTCCA 3'	31 nt

Table 3.2.3. Primers used to sequence 3' UTR region of DENV-2.

Sequences were assembled using SeqMan program from Lasergene (DNASTAR Inc.). Assembly of the 3'UTR sequence (454 nt) requiered 4 primers: D2-10146F, D2-10304F, D2-10522R and D2-10723R (Table 3.2.3 and Figure 3.2.4).

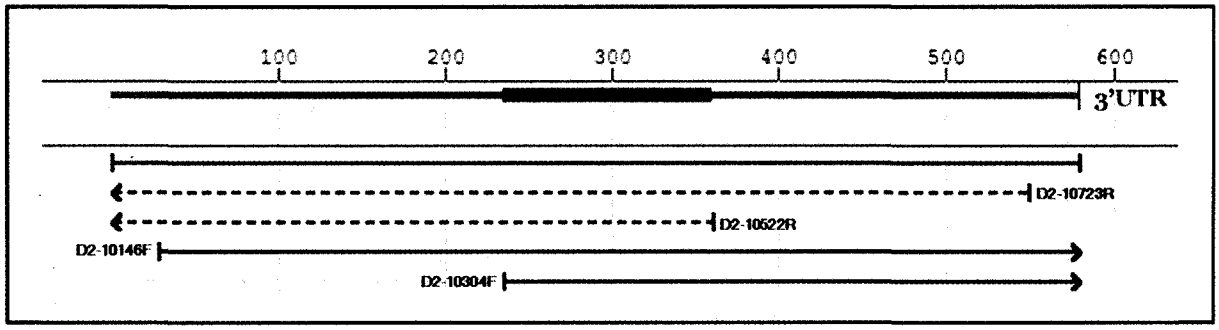


Figure 3.2.4. Assembly map of 3'UTR sequences. Primers used are noted.

3.2.9. Sequence comparisons

Sequence data were assembled for each strain into a contig corresponding to either the E gene or the 3'UTR sequence. Nucleotide data were translated using the ExPaSy translational tool and aligned using ClustalW program on the DeCypher supercomputer (http://decypher. stanford.edu/decypher/cgi-bin/ docfilter?file=/decipher/userindex.html).

3.3. Results

3.3.1. Dissemination of DENV-2 Jam 1409 in studied mosquito populations.

To provide a control and reference for the further experiments conducted with the Yucatan viruses, the susceptibility of Loreto and Chetumal mosquitoes to DENV-2 Jam 1409 was established. Susceptibility was measured as the ability of the virus to disseminate to salivary glands and head tissues. Virus titers were also determined in the mosquitoes at 4, 7, 10 and 14 days after the oral challenge with DENV-2 Jam1409 (Table 3.3.1).

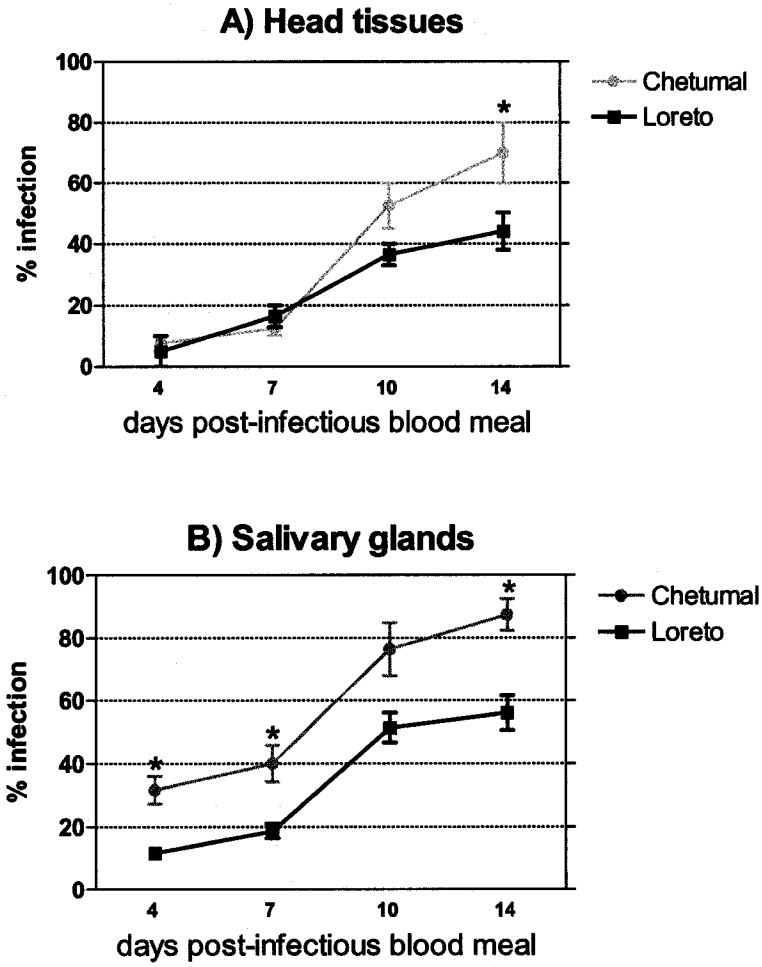


Figure 3.3.1. DENV-2 Jam 1409 dissemination to head tissues and salivary glands in Chetumal and Loreto mosquitoes. Thirty to forty mosquitoes for each time point were processed in three different and independent experiments. *P<0.05 by unpaired t test. Error bars show mean values and SEM (Standard error of the mean).

Results indicated *Aedes aegypti* Chetumal mosquitoes were significantly more permissive to disseminated infection than Loreto mosquitoes. At 14 days post-infectious bloodmeal (dpiBM), DENV-2 antigen was detectable in 70%±10% of Chetumal mosquito head tissues, but only in 44% ± 6% of Loreto head tissues. For salivary glands, DENV-2 antigen was detected in 87.5%±8.5% of Chetumal mosquitoes, but only 54%±9% of Loreto mosquitoes. Thus, the overall dissemination rates to the salivary glands were consistently greater than those to head tissues at every examined time point in both mosquito populations. Infection rates of head tissues were clearly different ($P<0.01$) between Chetumal and Loreto mosquitoes at 14 dpiBM (Figure 3.3.1A). Salivary glands infection rates between Chetumal and Loreto mosquitoes were significantly different ($P<0.01$) at 4, 7, and 14 dpiBM (Figure 3.3.1B).

To establish whether different virus titers were obtained after oral challenge, individual mosquitoes from the two mosquito populations were titrated by plaque assay at 4, 7, 10, and 14 dpiBM.

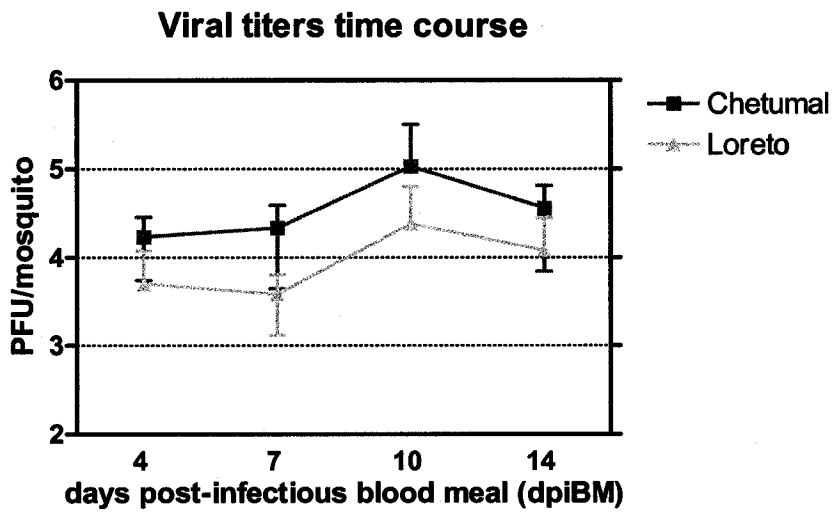


Figure 3.3.2. Replication of DENV-2 Jam 1409 in Chetumal and Loreto mosquitoes. 10 were titrated from each group at the different time points. Viral titers were determined by plaque assays. Both Loreto and Chetumal mosquitoes ingested bloodmeals containing $1.7\pm0.7\times10^7$ PFU/ml of DENV-2 Jam 1409. Bars show standard deviation (SD).

At each time point virus titers in Chetumal mosquitoes were greater than those in Loreto mosquitoes. However, the differences were not statistically significant by ANOVA or t test (Figure 3.3.2). Overall, these two populations of mosquitoes (Loreto and Chetumal) seem to differ in their susceptibility to DENV-2. Thus they provided a comparative system to test the virogenesis of the DENV-2 viruses.

3.3.2. Characterization of DENV-2 Yucatan viruses in cell cultures.

The characteristics and replication levels of DENV-2 Yucatan strains in cell cultures were examined. Parameters measured included: the cytopathic effect caused, the virus titers yielded in mammalian and insect cells, and the plaque morphology induced.

The viral titers for each one of the Yucatan viral strains in C6/36 cell cultures were determined at 12 dpi (days post-infection) in multiple experiments. All viruses, including the control strains DENV-2 Jam 1409 and DENV-2 QR0094 (American genotype), produced 7.3-8.5 log 10 PFU/ml (Figure 3.3.3). The titer differences observed were not statistically significant by either ANOVA analysis or t test.

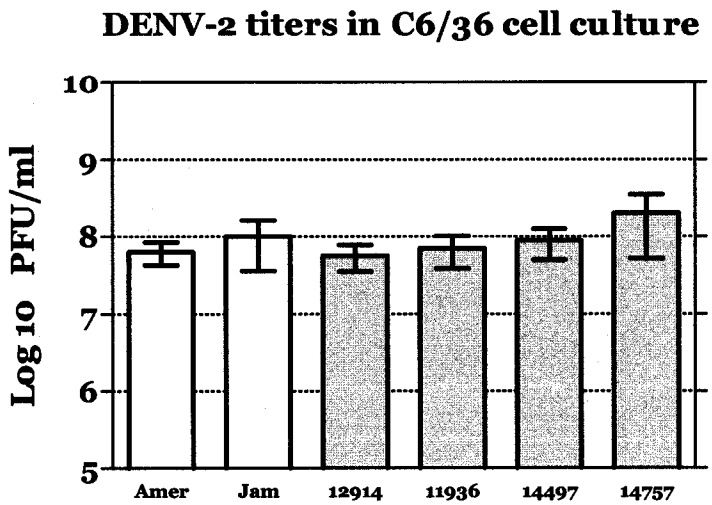


Figure 3.3.3. Viral titers of DENV-2 Yucatan viruses in C6/36 cell cultures. An MOI of 0.001 was used to infect confluent cell culture monolayers in T-25 culture flasks. No statistically significant differences were detected at 12 dpi from different experiments. Bars represent mean value from 5-6 experiments and SEM (Standard error of the mean).

Analysis of the plaque morphology produced by the original stocks of DENV-2 Yucatan viruses revealed important differences. Plaques of DENV-2 Yucatan strains in LLC-MK2 cells were similar in size or larger than those induced by the high passage reference strain DENV-2 Jam 1409. These plaques were much larger than the ones produced by DENV-2 QRoo94 (American genotype), which were the smallest. DENV-2 Jam 1409 plaques were between 1.5 to 2.5 mm in diameter. The DENV-2 Yucatan strains yielded plaques from 1 to 4 mm in diameter, and the DENV-2 QRoo94 strain from the American genotype produced minuscule plaques between 0.4 to 1 mm (Figure 3.3.4).

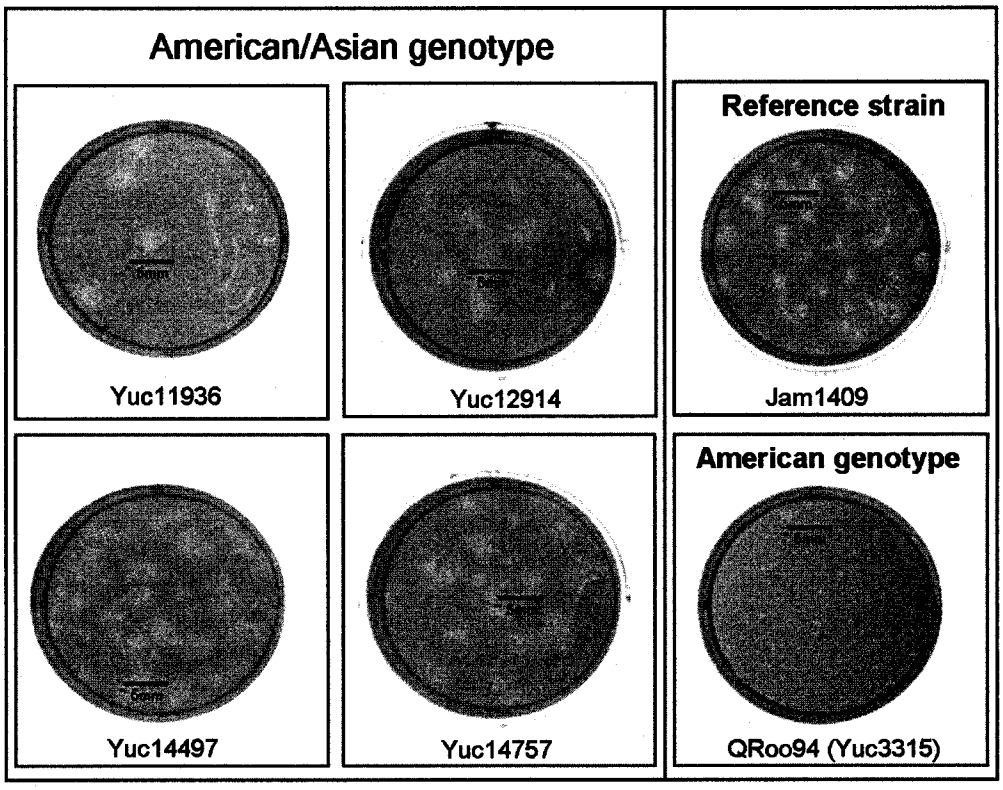


Figure 3.3.4. Plaque morphology of the different DENV-2 strains studied. Monolayers of LLC-MK2 cells grown on 6-well plates until confluency were infected with different dilutions of the virus and processed at 12 dpi.

The cytopathic effect caused by the various DENV-2 Yucatan strains on infected C6/36 cell cultures was compared with other DENV-2 genotypes. All the Yucatan viral strains caused syncytia to some degree in C6/36 cells between 10 and 11 dpi when infected at MOIs of 0.001 or 0.01 (Figure 3.3.5).. Neither the reference strain DENV-2

Jam 1409 nor the American (DENV-2 QR0094) genotype caused these effects at this specific time point (10 dpi).

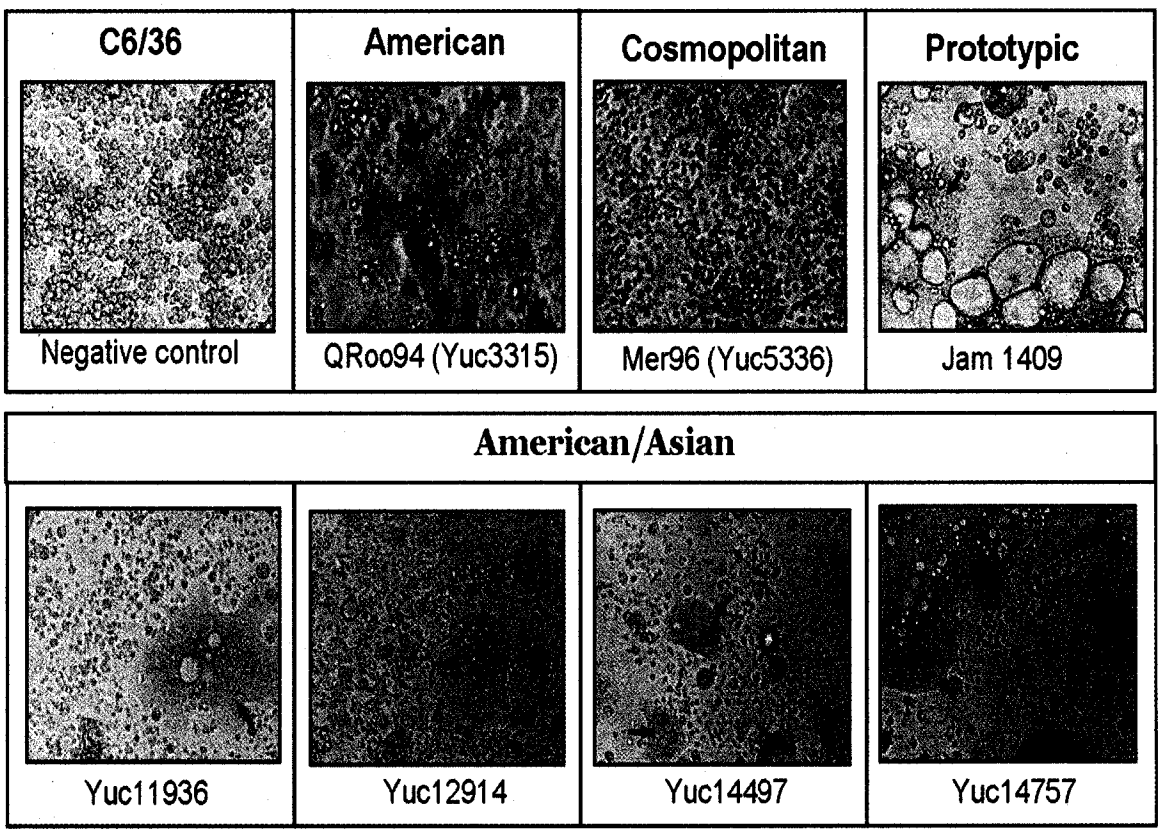


Figure 3.3.5. Cytopathic effect caused by the different DENV-2 Yucatan strains and other DENV-2 genotypes on infected C6/36 cell monolayers. Cytopathic effect was examined in cell monolayers infected at MOIs of 0.01 and 0.001. Cytopathic effect was examined and documented at 10 days post- infection. Cell cultures shown were infected at MOI of 0.001 using L-15 2%FBS medium at pH=7.2. Arrows show syncytia.

In order to determine the efficiency of replication of the viruses, invertebrate and vertebrate cells were infected. The ratio of viral titers in insect cells (C6/36) and mammalian cells (LLC-MK2) was used as a measure of the fitness of viruses using the original virus stocks. DENV alternates between and effectively replicates in insect and mammalian cells. Therefore, the titer ratio may reflect the virus ability to replicate in one or the other cell type. Each viral titer in C6/36 cell culture was divided by the titers produced in LLC-MK2 cells to obtain the ratio (Table 3.3.2).

DENV-2 Strain	Disease severity	End points Titers in C6/36 cells ($TCID_{50}$/ml)	Plaque assays Titers in LLC-MK2 cells (PFU/ml)	Ratio C6/36 / LLC-MK2
Yuc 11936	DFHM	5.6 x10^6	2.0 x10^5	28.0
Yuc 12914	DF	5.6 x10^6	8.0 x10^5	7.0
Yuc 14497	DHFIII	5.6 x10^6	6.6 x10^5	8.5
Yuc 14757	DHFII	3.2 x10^6	3.3 x10^5	9.7
Jam 1409 hp*	DF	1.0 x10^9	2.5 x10^8	4.0
Jam 1409 lp**	DF	5.6 x10^5	5.3 x10^6	0.1

Table 3.3.1. Ratio of viral titers obtained with DENV-2 Yucatan viruses in two cell types. Insect (C6/36) and mammalian (LLC-MK2) cells. * hp: high passaged virus **lp: low passaged virus.

The DENV-2 Yucatan strains exhibited larger ratios (7.0 to 28.0) than the prototype DENV-2 Jam 1409 high (4.0) and low passage (0.1) strains (Table 3.3.2). Overall virus titers of the Yucatan strains in both cell types were 3 logs (10^5 and 10^6) lower that those produced by DENV-2 Jam 1409 (10^8 and 10^9). Nevertheless, the normalized values showed that in all instances the Yucatan strains produced 7-28 fold higher titers in insect than in mammalian cells. DENV-2 Jam 1409 high passage produced only 4 fold higher titer in insect than in mammalian cells, whereas DENV-2 Jam 1409 low passage strain replicated relatively better in mammalian than in insect cells. The differences between the DENV-2 Jam1409 high passage "hp" and low passage "lp", may be a result of the laboratory history passage of these viruses.

3.3.3. Characterization of the DENV-2 Yucatan viruses infection of mosquitoes.

To investigate the correlation between the clinical severity and virogenesis in mosquitoes, DENV-2 Yucatan strains were fed to the Chetumal (coindigenous) and Loreto mosquitoes (non coindigenous). The experiments permitted start examinating the effect of the mosquito geographic origin, differences in vector competence, and potential coadaptational or coevolutionary events between vector and virus. However

further studies involving virus collected from other geographical region and feed to the Chetumal mosquitoes will be necessary. Parameters used to evaluate virogenesis in mosquitoes included: dissemination rates to salivary glands and head tissues, viral titers and anatomy of virus infection in organs such as salivary glands and midguts.

Dissemination rates to head tissues and salivary glands as well as viral titers in whole mosquitoes were determined 14 days after the infectious bloodmeals. Dissemination rates to salivary glands and head tissues obtained for Chetumal and Loreto from the two independent experiments are shown in the Table 3.3.3.

		Chetumal mosquitoes		Loreto mosquitoes	
DENV-2 ID	**Clinical Severity**	Salivary glands (% infection)	Heads (% infection)	Salivary glands (% infection)	Heads (% infection)
Yuc11936	DFHM	37.0±3.0	45.0±5.0	45.0±5.0	40.5±5.5
Yuc12914	DF	49.5±5.5	47.0±3.0	48.0±6.0	67.5±3.5
Yuc14497	DHFIII	48.0±3.0	67.0±3.5	62.0±2.0	77.0±6.0
Yuc14757	DHFII	71.0±5.0	76.0±3.5	49.0±4.0	57.5±7.5

Table 3.3.2. Dissemination rates to mosquito salivary glands and heads by the DENV-2 Yucatan viruses. Each column provides Mean±SEM from 2 independent experiments.

Dissemination rates of the DENV-2 Yucatan viruses in Chetumal mosquitoes (coindigenous) revealed significant correlations between virulence in humans and virogenesis in mosquitoes (Figure 3.3.6A). Dissemination rates to both salivary glands and head tissues in Chetumal mosquitoes were directly correlated with the clinical outcome (less or more severe disease) in humans (chi square test applied to data indicated a $P<0.0001$ with 3 df). In head tissues, the dissemination rates differed significantly by t test ($P<0.05$) between the "less severe" group (11936/12914) and "more severe" (14497/14757) viruses. However, analysis of dissemination to salivary glands showed significant differences only between 11936 (less severe) and 14757 (more severe) Yucatan strains ($P<0.05$). Thus, virulence in humans exhibited significant correlation with dissemination rates to the head tissues of coindigenous Chetumal mosquitoes.

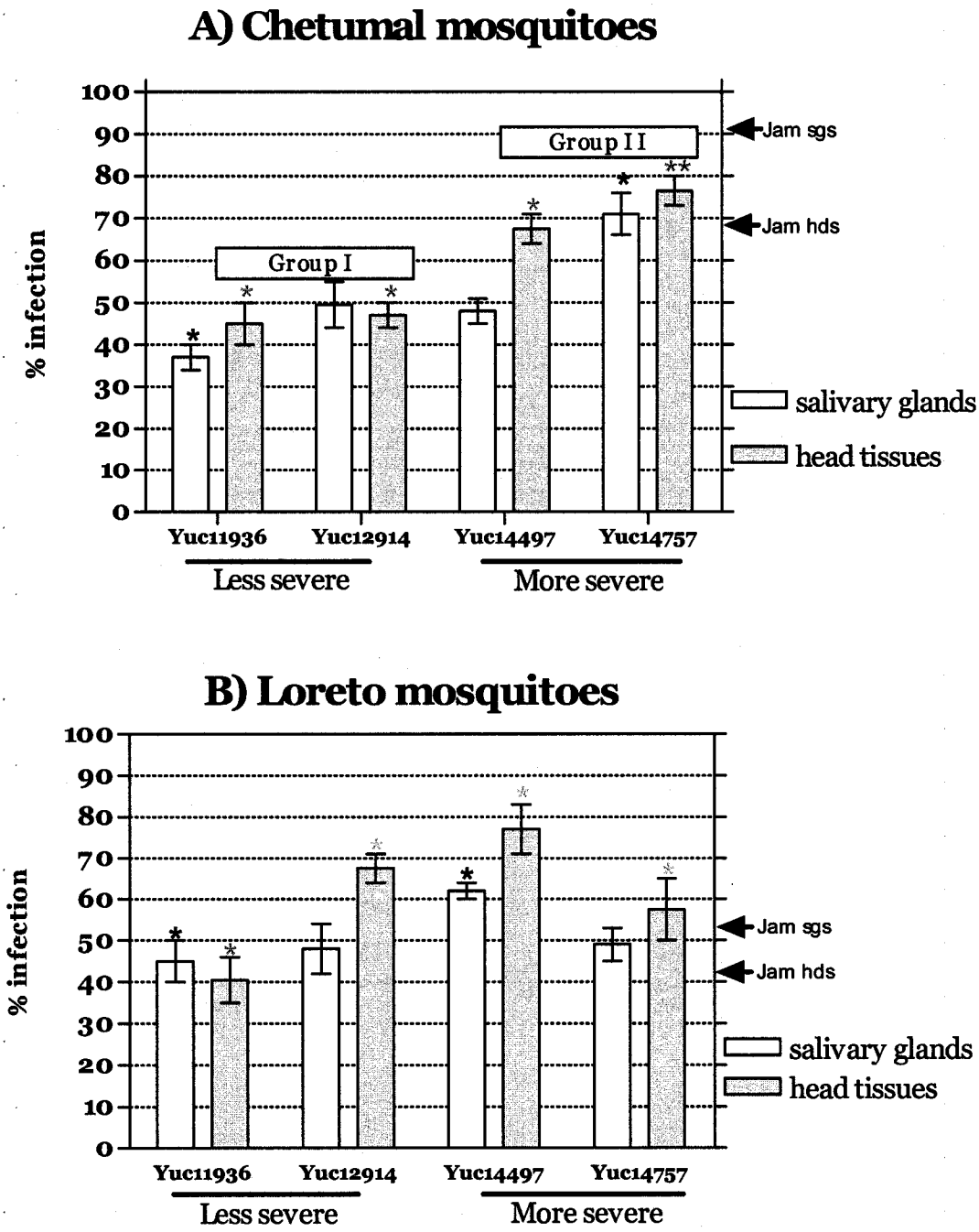


Figure 3.3.6. DENV-2 dissemination rates in Chetumal and Loreto mosquitoes 14 days after challenge with infectious bloodmeals. 30 mosquitoes from each group were examined from two independent experiments. The viral titers in the assayed bloodmeals were: Yuc11936= 1.0±0.2x10^7 PFU/ml; Yuc12914= 1.4±0.4x10^7 PFU/ml; Yuc14497= 1.6±0.8x10^7 PFU/ml; Yuc14757= 1.7±0.9x10^7 PFU/ml. Bars represent Mean±SEM from two independent experiments. Dissemination values for DENV-2 Jam1409 are used as reference. *P<0.05 and ** P<0.01 when comparing by t test. The chi square test applied to data indicated a P<0.0001 with 3 df, when group I and II in Chetumal mosquitoes were compared.

Loreto mosquitoes showed higher dissemination rates for two of the Yucatan strains (Yuc12914 and Yuc14497) than for DENV-2 Jam1409, but these differences did not consistently correlate with clinical severity. These mosquitoes were significantly less susceptible to DENV-2 Jam1409 than the Chetumal mosquitoes (as shown in section 3.2.1), but interestingly were as or more susceptible to some of the Yucatan viruses as the Chetumal mosquitoes (Figure 3.3.6B).

A trend associating dissemination in Loreto mosquitoes with the clinical outcome (less or more severe disease) in humans was revealed for some Yucatan viruses. Significant differences in dissemination rates to head tissues occurred for Yuc11936, Yuc12914 and Yuc14497 ($P<0.05$ by t unpaired test). Dissemination rates to salivary glands differed significantly only between Yuc11936 and Yuc14497 ($P<0.05$, unpaired t test) (Figure 3.3.6B). Thus, the correlation between clinical severity and dissemination rates to both salivary glands and head tissues in Loreto mosquitoes was consistent only for the Yuc11936 and Yuc14497 strains but not for the other Yucatan viruses.

The characteristics of salivary gland viral infection caused by DENV-2 Yucatan strains was determined in Chetumal and Loreto mosquitoes (Figure 3.3.7). Salivary glands from 5 to 8 individuals from each different mosquito population and DENV-2 strain were dissected at 14 dpiBM and assayed by IFA. Salivary glands from highly susceptible DS3 mosquitoes were used as a control. For DENV-2 Jam 1409, salivary gland infection typically started at the lateral distal lobes and spread overtime to involve sometimes the entire organ (as previously described in Chapter II). Infection of the salivary glands by the DENV-2 Yucatan strains occurred mostly in the distal part of the salivary glands, although some variation in amount of virus antigen was observed among individual mosquitoes (Figure 3.3.7).

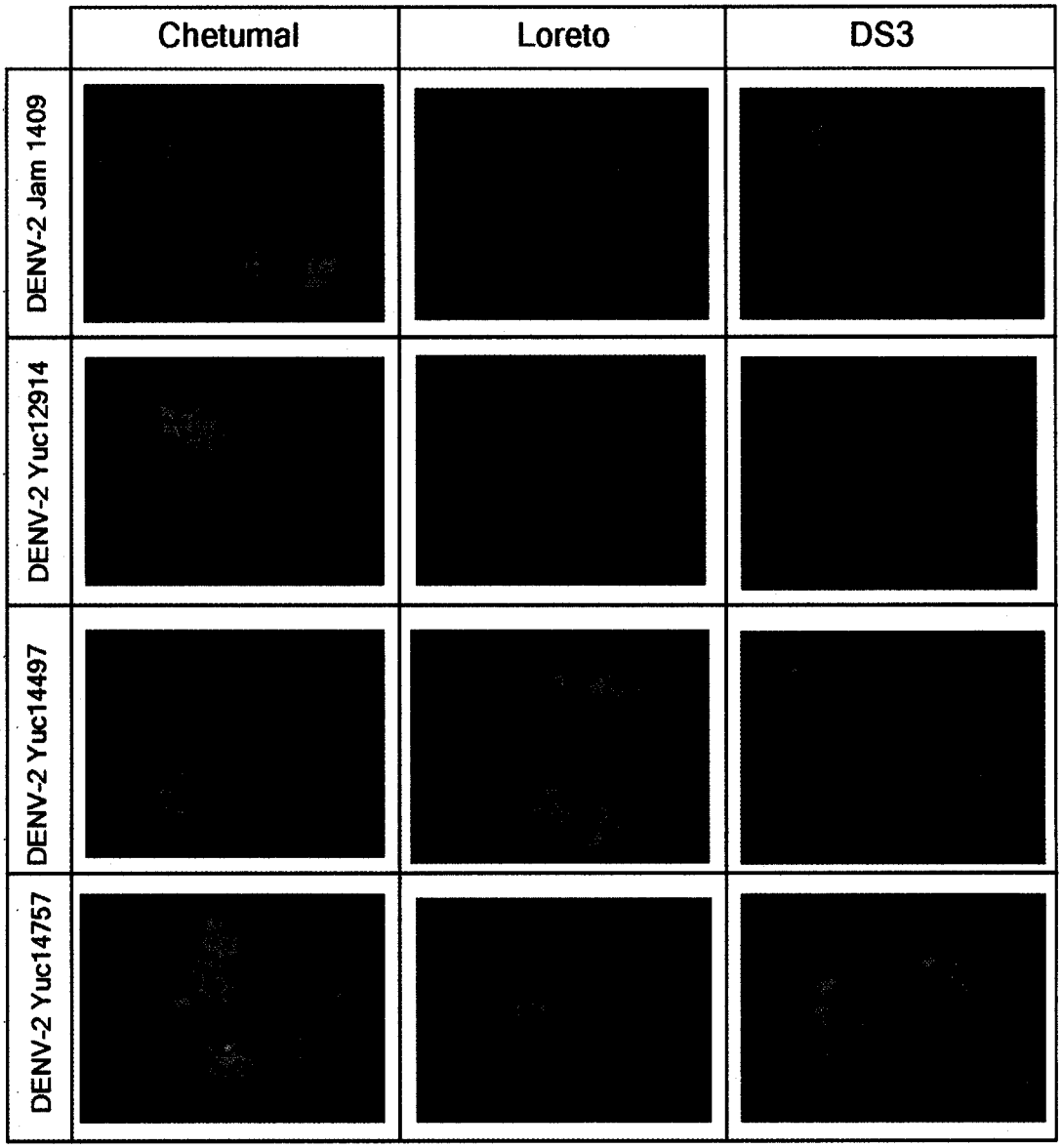


Figure 3.3.7. Anatomy of salivary gland infection by the DENV-2 Yucatan viruses. Salivary glands from 5-8 blood fed mosquitoes were dissected at 14 dpi, fixed and assayed by indirect immunofluorescence using an anti-dengue monoclonal (3H5) antibody. DS3 mosquitoes were used as positive control due to its high susceptibility to DENV-2 infection. The infection pattern as previously observed frequently but not exclusively started at the lateral distal lobes. Bloodmeals contained the following viral titers: Yuc11936= 1.0±0.2x10^7 PFU/ml; Yuc12914= 1.4±0.4x10^7 PFU/ml; Yuc14497= 1.6±0.8x10^7 PFU/ml; Yuc14757= 1.7±0.9x10^7 PFU/ml. Pictures were taken to 200X magnification and cropped to improve presentation.

More virus antigen was observed in Chetumal and D2S3 mosquitoes infected with the "more severe" strains (Yuc14497/Yuc14757) than those infected with "less severe" strains (Yuc12914). DENV-2 Yuc14497 was the only virus that consistently infected equally well the three tested mosquito strains (Chetumal, Loreto, and DS3).

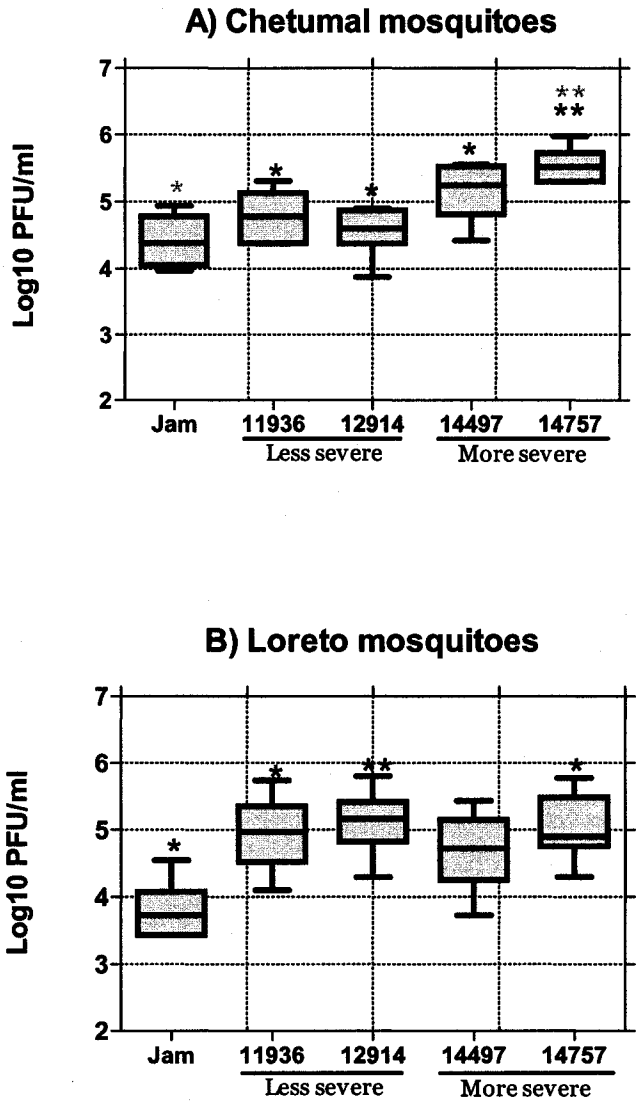


Figure 3.3.8. Viral titers produced by DENV-2 Yucatan viruses are correlated to disease severity in Chetumal but not in Loreto mosquitoes. For the Chetumal infected mosquitoes, 7-10 infected individual mosquitoes were titrated by plaque assays. For Loreto, 10-20 individuals were titrated individually. Viral titers in bloodmeals were: Yuc11936= 1.0±0.2x10^7 PFU/ml; Yuc12914= 1.4±0.4x10^7 PFU/ml; Yuc14497= 1.6±0.8x10^7 PFU/ml; Yuc14757= 1.7±0.9x10^7 PFU/ml; DENV-2 Jam1409= 0.4±0.5x10^7 PFU/ml. *P<0.05 and **P<0.001 by Tukey's multiple comparison test.

Virus titers produced by the DENV-2 Yucatan strains in mosquitoes were also compared to establish correlations with clinical severity. Significant differences in viral titers positively correlating with disease severity were observed in Chetumal, but not in Loreto mosquitoes (Figure 3.3.8). In Chetumal mosquitoes, the highest viral titers were produced by Yuc14757 (~5.5 to 6.0) and the lowest titers by Yuc12914 (~3.8 to 4.8).

Virus titers produced by DENV-2 Yucatan strains in Loreto mosquitoes did not differ significantly, although they were consistently higher (~4.3 to 5.5) than the ones yielded by DENV-2 Jam1409 (~3.4 to 4.2) (Figure 3.3.8). Chetumal mosquitoes infected by viruses from "more severe" outcomes exhibited significantly higher (5.3-5.5) mean titers than those infected by viruses from "less severe" outcomes (4.5-4.8).

Characterization of DENV-2 Yucatan strains in Chetumal mosquitoes revealed significant differences in midgut infection early after the infectious bloodmeal. There were distinctive differences when mosquitoes were fed on bloodmeals containing the same virus titers (Figure 3.3.9). Typically at 3 dpiBM, a strikingly larger number of infection foci (~10-fold) were observed in midguts of DENV-2 Yuc14757-infected than in DENV-2 Jam1409-infected mosquitoes.

Figure 3.3.9. Midguts of two Chetumal mosquitoes blood fed on either DENV-2 Jam 1409 or DENV-2 Yuc14757 at 3 dpiBM. Bloodmeals contained 4 x10^7 PFU/ml of either DENV-2 Jam 1409 or DENV-2. Figures are composites of multiple pictures taken to a 200X magnification.

These experiments uncovered an important correlation between disease severity and virogenesis in coindigenous mosquitoes. Surprisingly these relationships were not apparent in distant unrelated Loreto mosquitoes. Whether this is due to geographic or genetic factors conditioning DENV susceptibility in the vector remain to be determined.

3.3.4. Chetumal mosquitoes infected by two different DENV-2 genotypes.

To investigate the influence of virus genotype on vector infection, the midgut infection and dissemination rates caused by American/Asian (Yuc12914 and Yuc14757 strains) and American (QR0094) genotype viruses were examined (Figure 3.3.10). This analysis was performed in mosquitoes (Chetumal) and viruses originating from same geographic region, the Yucatan Peninsula. This enabled better inferences about the field significance of these findings. Infections of midguts and dissemination rates to salivary glands and head tissues were used as the measure of virus ability to infect and replicate in mosquitoes.

Equivalent virus titers in bloodmeals of each examined virus were fed to mosquitoes. Then midguts were dissected at 4 and 7 dpiBM and salivary glands and head tissues at 14 dpiBM to be assayed by IFA and to document infection rates. Despite equivalent midgut infection rates, significant differences ($P<0.01$ by ANOVA one way comparison test) in dissemination rates to the salivary glands and heads were revealed. The viruses from the American/Asian genotypes (Yuc12914/Yuc14757) were far more infective than the virus from the American (QR0094) genotype.

DENV-2 QR0094 virus (American genotype) exhibited equivalent patterns and midgut infection rates at 4 and 7 dpiBM, but dissemination rates to either salivary glands or head tissues were low (Figure 3.2.10). The DENV-2 Yucatan viruses (American/Asian genotype) exhibited dissemination rates to head tissues of 50-80% and to salivary glands of 60-80%. The American genotype (QR0094) showed only ~3% dissemination to head tissues and ~19% dissemination to salivary glands. The analyzed American/Asian (Yuc12914 and Yuc14757) and American (QR0094) viruses exhibited dramatic differences in their ability to infect and replicate in Chetumal mosquitoes.

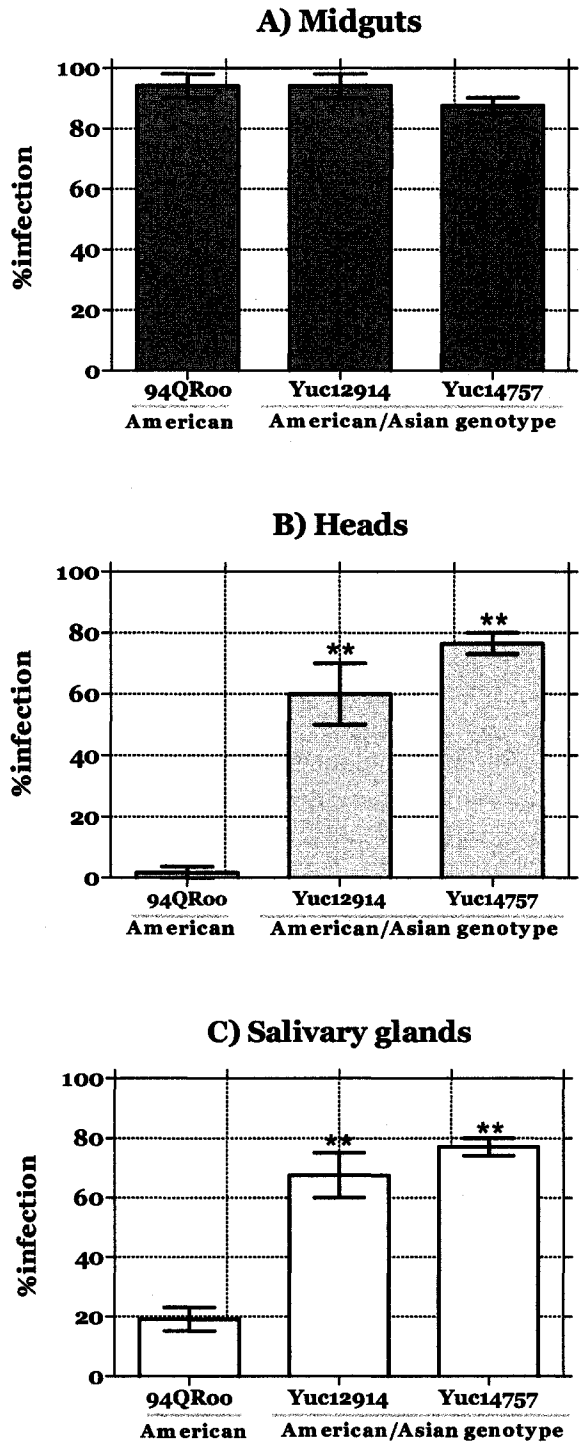


Figure 3.3.10. Comparison of dissemination rates between the Yucatan strains and an American genotype virus. Midgut infection rates were evaluated at 4 and 7 dpiBM in 20 individuals (A). Then dissemination rates to heads (B) and salivary glands (C) were established examining DENV-2 in duplicate experiments. Bars represent mean± SEM, n=30. ** P<0.01 by ANOVA one-way test. Virus titers in bloodmeals were: $2x10^7$ PFU/ml for QRoo94; $7x10^7$ PFU/ml for Yuc12914; and $2.1x10^7$ PFU/ml for Yuc14757.

To begin investigating genotypic differences that might underlay the phenotypic differences observed between these two genotypes, the E protein gene and 3' UTR for DENV-2QR0094 and DENV-2 Yucatan viruses were sequenced and compared.

3.3.5. Analysis of the E glycoprotein gene sequence of viruses from the Yucatan Peninsula.

The E viral protein is a major component of DENV envelope and is a determinant of virus tropisms. Remarkable differences in virulence may result from minor changes in viral proteins (34, 71, 168, 180, 188, 193). Amino acid changes in E protein and in the 5'- and 3'-terminal nucleotide regions affect virus entry and replication efficiency, respectively, and affect virulence (50, 171). Hence, virulence can correlate with viral sequences that affect both tropisms and replication efficiency. The entire E protein genes of DENV-2 QR0094 (American genotype), the DENV-2 Yucatan viruses (American/Asian genotype), and the DENV-2 Jam1409 (also American/Asian genotype) were sequenced using a set of seven primers for the target sequence. The nucleotide identity in each position of the gene was obtained and confirmed in both directions (three times). A total of 148 nucleotide substitutions (Appendix 7.7) were observed while comparing the different E genes, however most of these resulted in synonymous substitutions. Conservative and non conservative amino acid substitutions in the sequence were located in the multiple alignment analysis (Figure 3.3.11).

All the changes were mapped on the putative E protein structure (130), using either QR0094 or Jam1409 as the backbone sequence (Figures 3.3.12 and 3.3.13). Fourteen amino acid changes were found in total (Table 3.3.4), four of which were non conservative changes (E-46, 131, 340 and 390). Considering the E protein by domains (196), of the fourteen changes observed; 4 occurred in domain I, 6 in domain II, and 4 in domain III. Three of the observed changes have been previously reported, these are: E-6, which is associated with a mutant that induces fusion at elevated pH (110); E-71 that

relates to escape neutralization mutants (180); and E-390, which is located in the motif that may govern binding to mosquito host cell and associate also with genotype identity (228). In the E protein, domain I contains the molecular hinge region involved in the conformational changes that take place at low pH. Domain II contains the fusion peptide and is involved in the dimerization process. Finally, domain III is likely involved in virus attachment to its receptor and contains the carboxy-terminal Ig-like section. There are two potentially glycosylated asparagines (Asn) on each DENV E subunit: Asn-153 on domain I and Asn-67 on domain II (195). Domain III undergoes the most significant displacement in the dimer-to-trimer transition; this transition is irreversible, since trimers are a structurally more stable conformation for the E protein than dimers (196). Only one amino acid change was distinctive between "less severe" (Yuc11936/Yuc12914) and "more severe" (Yuc14497/Yuc14757) viruses, a non conservative change in E-46 (T to I) located in domain I.

	American Genotype	**American/Asian genotype**				
aa changes	QR0094	Yucatan "less severe" (Yuc11936/12914)	Yucatan "more severe" (Yuc14497/14757)	Jam1409	Domain	Previous Reference
E-6	I	I	I	M	I	(59, 110, 222)
E-46*	I	T	I/T	I	I	
E-55	S	T	T	T	II	
E-71	D	E	E	E	II	(168, 180)
E-81	T	S	S	S	II	
E-91	V	I	I	L	II	
E-129	I	V	V	V	II	
E-131*	Q	Q	Q	L	II	
E-139	V	I	I	I	I	
E-162	V	I	I	I	I	
E-340*	M	T	T	M	I	
E-390*	D	N	N	N	III	(228)
E-484	I	V	V	V	III	
E-491	V	A	A	A	III	

Table 3.3.3. Amino acid changes comparing American and American/Asian genotypes examined. * non conservative changes.

Figure 3.3.11. Sequence alignment of the E proteins from all the DENV-2 Yucatan strains compared to the DENV-2 Jam 1409 (American/Asian genotype) and QRoo94 (American genotype). The domains of this protein are indicated as well. ✦ Amino acid substitution sites. * nonconservative amino acid substitutions.

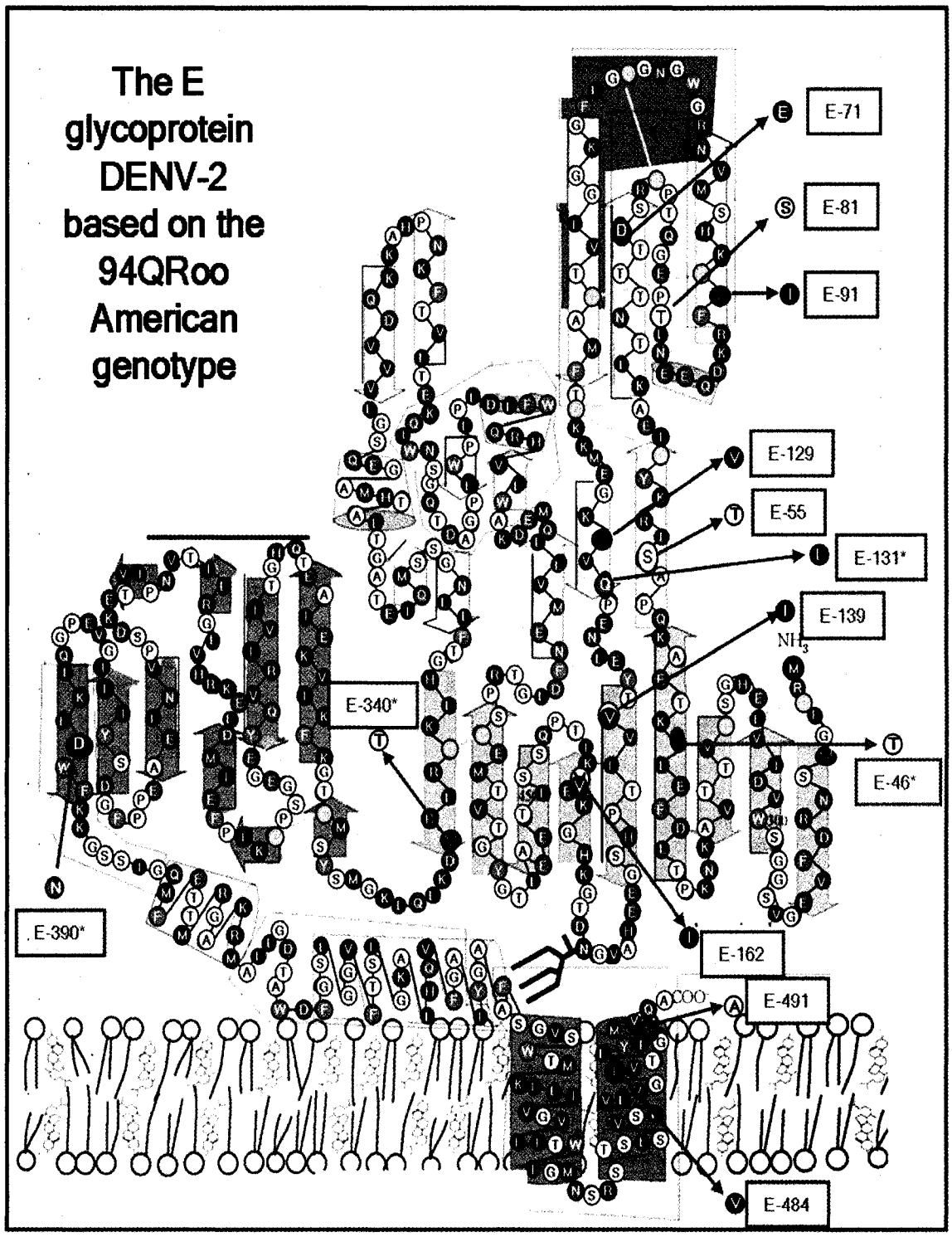


Figure 3.3.12. Putative E protein conformation in the trimer structure and location of amino acid changes. The backbone corresponds to the DENV-2 QRoo94 American genotype. All the amino acid substitutions found between the DENV-2 QRoo94 (American genotype) and Yucatan strains (American/Asian genotype) are shown. *non conservative changes. (Structure based on Hrobowski *et al.*, 2005).

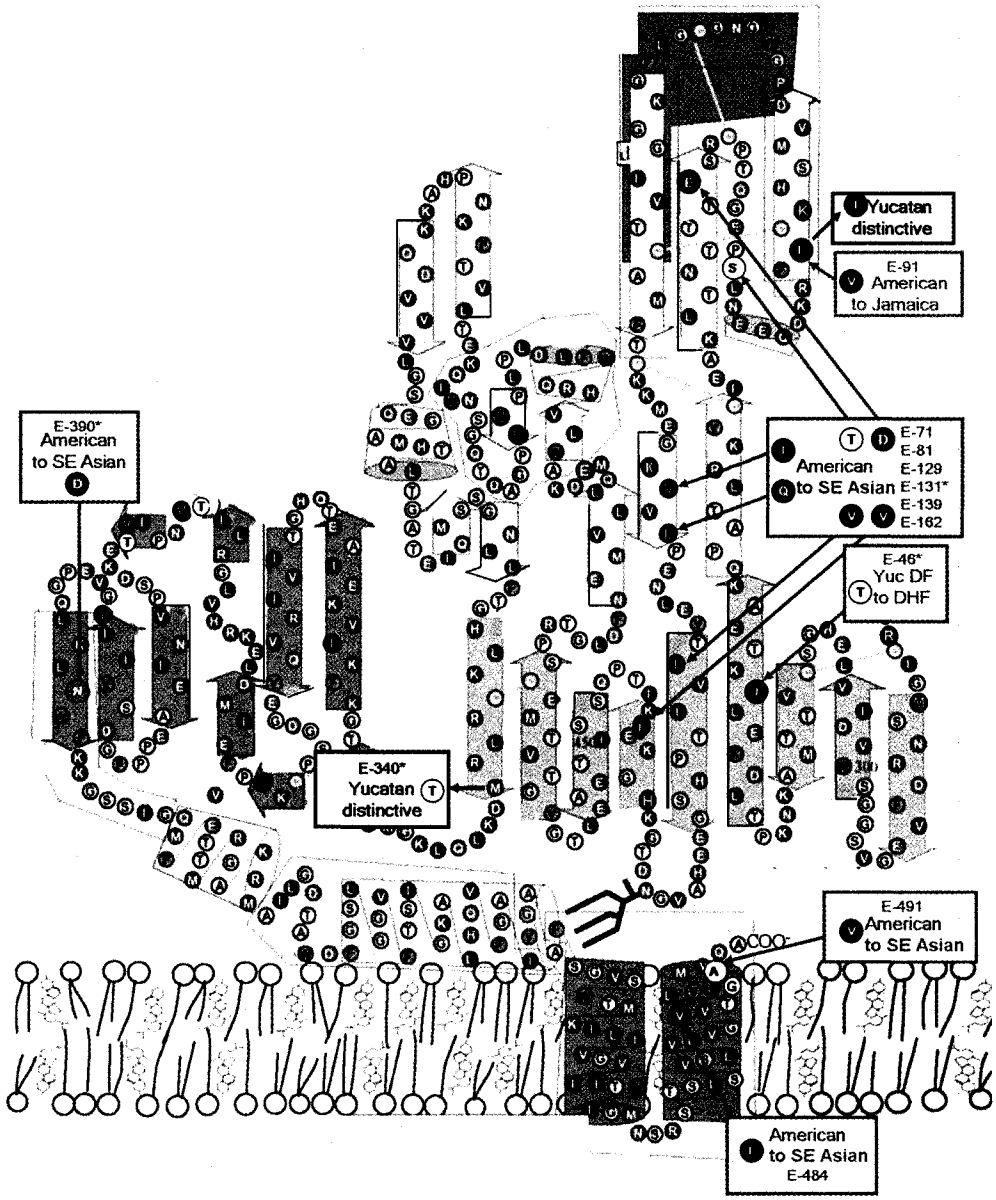


Figure 3.3.13. Putative E protein structure (130) and overall amino acid substitutions. Backbone corresponds to DENV-2 Jam1409. Showing all the amino acid substitutions and their significance. *non conservative changes.

3.3.6. Analysis of the 3'UTR sequence of DENV-2 viruses from the Yucatan Peninsula.

Comparison of the 3' UTR nucleotide sequence of studied viruses revealed multiple differences between the Yucatan strains (American/Asian genotype) and the coindigenous American genotype (QR0094). These differences included deletions, insertions and nucleotide changes affecting critical functional structures for the genome

cyclization (Figure 3.3.14 and 3.3.15). The 3' UTR comprises the last 454 nt, 10269 to 10723 nt of the genome) of the DENV genome and includes conserved motifs important for viral replication. Important motifs include for example: the cyclization domains (CS and RCS2) and the 3'SL (SLA and SLB).

Only one nucleotide change ($3'UTR_{103}$) was distinctive between the "less severe" (Yuc11936/Yuc12914) and "more severe" (Yuc14497/Yuc14757) viruses. However, multiple and profound changes were found between American (QR0094) and American/Asian genotype (Yucatan and Jam1409 strains) viruses. All these changes are listed in the Table 3.3.5. The most important changes included two nucleotide transversions (nt 299 and 413) in CS2 and 3'SL motifs, and insertion of two adenines right upstream of the CS1 motif (nt 350), and a deletion in nt 267.

	American Genotype	**American/Asian genotype**			
Position of Substitutions	QR0094	"Less severe" (Yuc11936/12914)	"More severe" (Yuc14497/14757)	Jam1409	Motif (44)
28	C	T	T/C	C	
60	G	A	A	A	
106		G	A	A	
113-123	CTTTTGA	TCACAAAA	TCACAAAA	TCACAAAA	
137	C	T	T	T	
167	A	G	G	G	
180	T	C	C	C	
181	C	T	T	T	
253	T	A	A	A	
255	T	A	A/G	A	
257	G	A	A	A	
267	-	A	A	A	
271	T	C	C	C	
299	G	A	A	A	CS2
338	G	A	A	A	
340	C	C	T/C	C	
350	AAA	A	A	A	CS1
413	G	A	A	A	3'SL (SLA)

Table 3.3.4. Differences in the 3'UTR found between American and American/Asian genotype viruses from the Yucatan Peninsula.

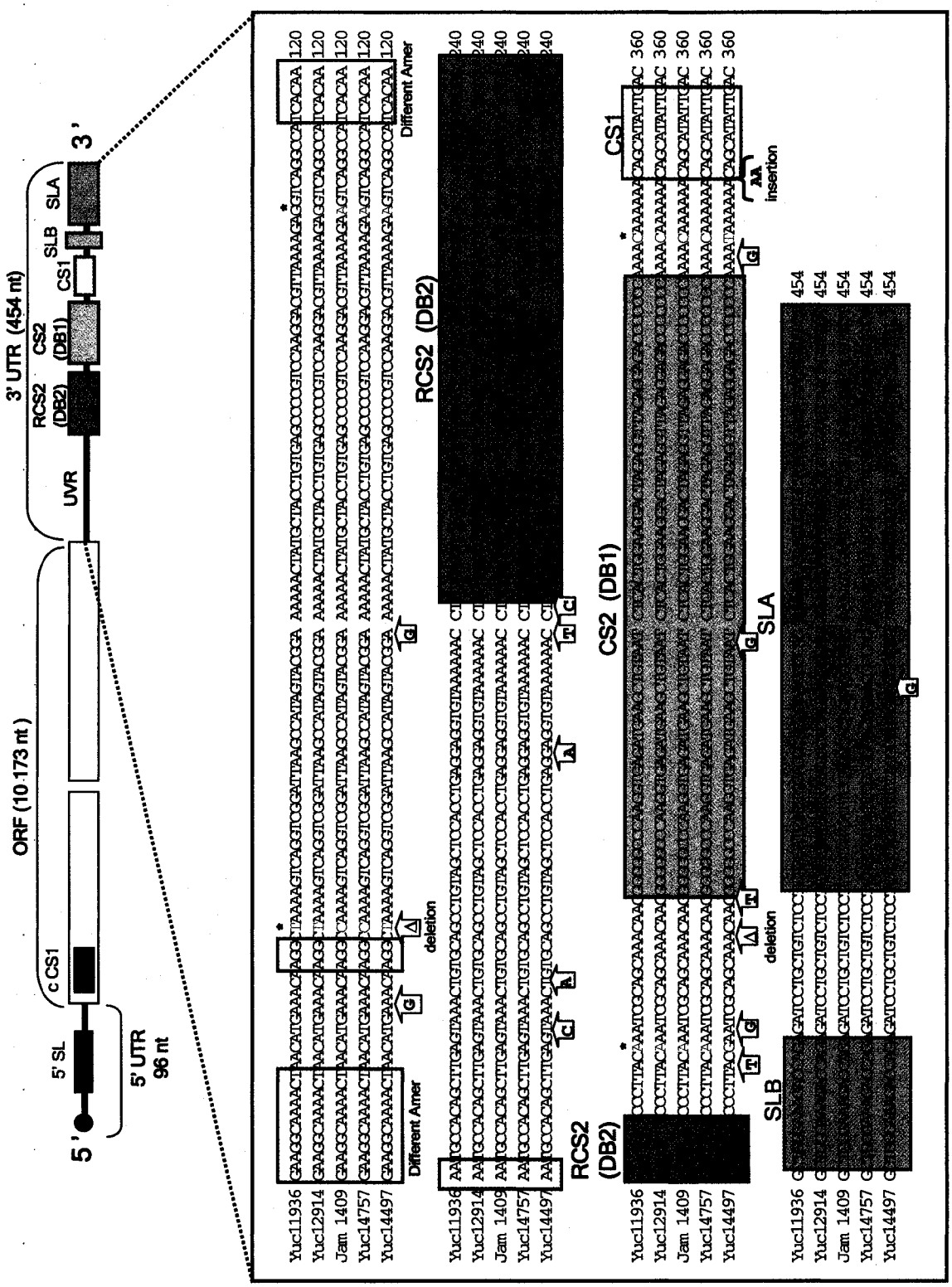


Figure 3.3.14. The 3'UTR sequences for American/Asian (Yucatan strains) and American genotype (QRoo94) viruses. Changes included: deletions of the AAA in position 115-120 and another A in position 269. An insertion at 348 position of a dinucleotide AA, as well as punctual changes indicated by arrows. Functional crucial motifs appear highlighted (Also see Figure 3.3.15).

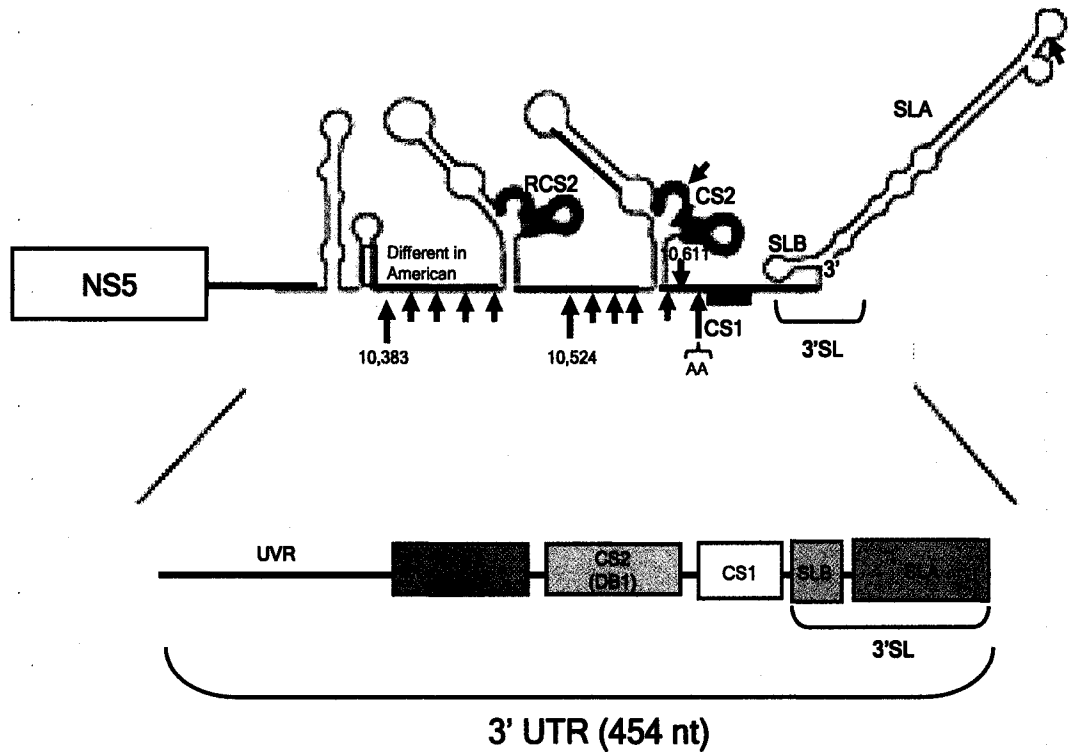


Figure 3.3.15. Putative structure of the conserved motifs in the 3'UTR of DENV-2. Differences among the Yucatan strains are noted in blue, while differences between the Yucatan viruses (American/Asian) and the QR0094 (American) genotypes are in red. (Diagram based on Alvarez *et al.*, 2005; Chiu *et al.*, 2005).

3.3.7. The phylogenetic relationship among the studied viruses

The phylogenetic relationship among the studied viruses was examined. Dendrograms obtained from ClustalW for the analyzed regions were used to determine associations among these viruses (Figure 3.3.16). The Yucatan viruses clustered in two groups related to disease severity, when the E protein gene sequence was used for the analysis (Figure 3.3.16A). However, in the 3' phylogenetic analysis Yucatan viruses that caused "more severe" disease segregated differently (Figure 3.3.16B). Further studies including sequences from other isolates that help to establish the actual phylogenetic distances will be necessary to clarify the relationships and origins from these viruses.

Clearly, the American genotype (DENV-2 QR0094) was the most distantly related of these viruses, when analyzing either E protein gene sequence or 3'UTR.

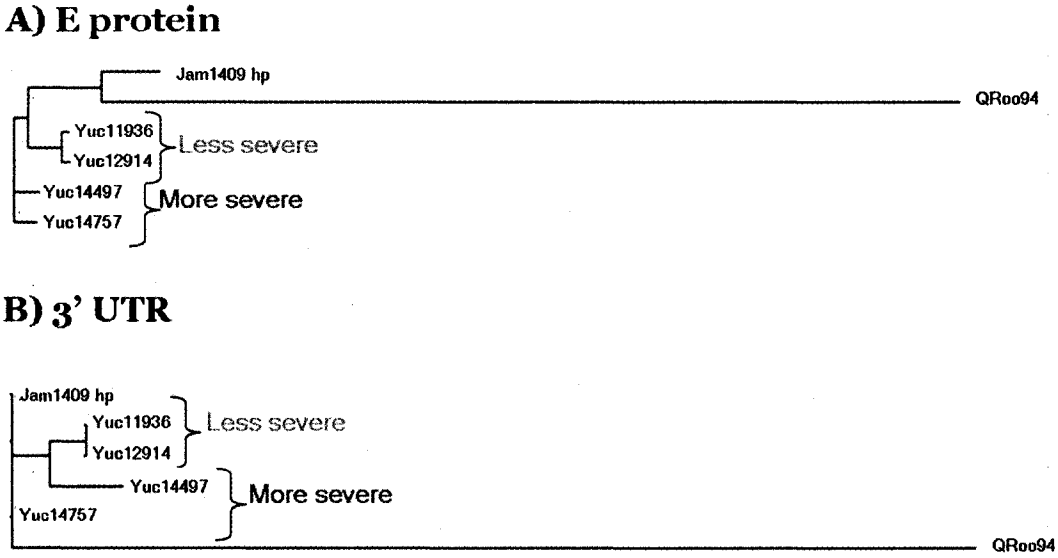


Figure 3.3.16. Relationship among studied viruses analyzed by E protein gene sequence (A) and 3'UTR sequence (B). Dendrograms were obtained with the Clustal W program. Line distance do not correspond to phylogenetic distances.

3.4. Discussion

Multiple studies have tried to determine the relationship between disease severity and epidemiological potential of dengue viruses. Presumably, viruses with high epidemiological impact are both more efficiently replicated and transmitted from one host to the other (235). Production of higher viral titers in key tissues may be a critical to assure this event. Higher dengue viremia titers correlates with disease severity in humans (53, 297, 323). Here, the results showed significant correlation between disease severity and virogenesis in Chetumal mosquitoes (Figures 3.3.6-3.3.9). Viruses were isolated from clinical cases of different severity in a hyperendemic area of dengue (The Yucatan Peninsula, Mexico). Data suggest that viruses from more severe cases (Yuc14497/Yuc14757) replicate more efficiently in mosquitoes (Figure 3.3.6 and 3.3.8). The analyzed viruses not only exhibited higher dissemination rates, but also higher virus titers in mosquitoes (Figure 3.3.8).

During epidemics, high levels of virogenesis in mosquito vector might be an important determinant to spread the infection to humans. Thus a virus that is able to

infect mosquitoes more efficiently will have a better chance of being transmitted and a higher epidemic impact as consequence. Previous analysis of breeding structure of mosquitoes from Mexico proved genetic homogeneity in all mosquito collections from the Yucatan Peninsula (96). Additionally, they exhibited high susceptibility to oral infection by DENV-2 Jam1409 (18).

The case severity for the Yucatan strains (American/Asian genotype) correlated better with virogenesis in coindigenous Chetumal mosquitoes than in the geographically unrelated Loreto mosquitoes (Table 3.3.3; Figure 3.3.6 and3.3.8). In the Yucatan Peninsula, where Chetumal mosquitoes originated, dengue is hyperendemic. Whereas Loreto mosquitoes originated from the Baja California region where dengue is not a major public health problem. Thus, Loreto mosquitoes originated in a distant and unrelated place from where the DENV-2 Yucatan strains were isolated (Figure 3.2.2). Loreto mosquitoes were not highly susceptible to DENV-2 Jam 1409 infection (Figures 3.3.1 and 3.3.2), but they exhibited higher susceptibility to some of the Yucatan strains (Figure 3.3.6 and3.3.8). Results indicated that dissemination to head tissues in coindigenous mosquitoes exhibited the strongest correlation with diseases severity (Figure 3.3.6).

Although more studies are necessary to understand mechanisms involved, occurrence of coadaptational events or virulent variant selection between DENV and vectors in endemic areas could explain this trend. Future studies addressing potential coadaptational events in the virus-vector relationship might provide better understanding of the epidemiology and virulence of dengue.

Virus genetic determinants that condition replication efficiency in host cells may be common to both mosquitoes and humans. The DENV-2 Yucatan viruses produced even higher overall viral titers in mosquitoes than the mosquito cell-adapted DENV-2 Jam1409 (Figure 3.3.8). Arboviruses such as DENV constitute an example of obligate

host alternacy in nature; they must successfully replicate in both humans and mosquitoes to maintain their transmission cycle. Hence viruses that replicate efficiently and achieve high viral titers in both hosts facilitate their transmission and increase their epidemic impact. In this regard, viruses from the Southeast Asian genotype (DHF cases) replicate better in both mosquitoes and human dendritic cells than viruses from the American genotype (DF cases) (50).

The DENV-2 Southeast Asian genotype is divided into three subgroups: the Asian 1, Asian 2 and America/Asian genotypes (127, 294). Viruses from the Asian genotypes seem to disseminate better in laboratory mosquitoes that American genotype viruses, while they replicate at similar rates in mosquito cell cultures (6, 7). However, there are not many reports for the American/Asian genotypes. The significant increase in the number of DHF cases occurring between 2001 and 2002 in the Yucatan Peninsula was certainly associated with the more frequent isolation of America/Asian genotype viruses (184).

Chetumal mosquito midguts were infected with either American (QR0094) or American/Asian (Yucatan strains) genotypes from the Yucatan Peninsula showed equivalent rates (Figure 3.3.10) and patterns. However the American genotype virus (QR0094) poorly disseminated out of the midgut. There were numerous amino acid changes in the E protein between the DENV-2 American and American/Asian genotype viruses from the Yucatan Peninsula (Figures 3.3.11-3.3.13). These changes involved the three protein domains, but the largest number of changes occurred in domain II, which contains the fusion peptide. Only one of these changes was non conservative (E-131) (Table 3.3.4). Most of the 12 amino acid substitutions detected between the DENV-2 American and American/Asian genotypes were conservative. Since the majority of the 148 nucleotide changes observed encoded synonymous amino acid changes (Figure 3.3.13 and Appendix 7.7 and 7.8), this reflects perhaps the functional importance and

constraints for the E protein. Many of the amino acid substitutions were observed in regions involving β-sheets and some changes occured in the transmembrane region. In Class I fusion proteins, this domain mediates the transition from hemifusion to full fusion (73). However, for Class II fusion proteins, such as the E protein of dengue, the function is unknown.

The 5' and 3'-UTRs are the main determinants of translational efficiency (64). In addition, the 3' UTR increases mRNA stability and participates in virus replication and the 3' SL located in this region is critical for virus infectivity (44, 325). There were differences in the 3' UTR region of DENV-2 American and Asian genotypes, and the upstream 300 nucleotides of this region contain perhaps determinants of disease severity (171). Results showed significant and profound differences in the 3' UTR between the American (QR0094) and American/Asian (Yucatan viruses and Jam1409) genotypes isolated from the Yucatan Peninsula (Figures 3.3.14 and 3.3.15). Replication ability of DENV-2 members of the American genotype have been previously addressed (228).

Interestingly, differences in the 3'UTR of the QR0094 virus (American genotype) compared to the Yucatan strains (American/Asian) involved nucleotide substitutions in structures known to be critical for efficient virus replication. Dissemination rates of the QR0094 virus (American genotype) were highly inefficient, despite high midgut infection rates. DENV-2 QR0094 disseminated to salivary glands in 16-23% of infected mosquitoes, while DENV-2 Yucatan viruses disseminated in 50-70% of mosquitoes (Figure 3.3.8). Therefore the regions affected in the 3'UTR could represent critical sites to bind factors that promote replication/translation in the cell-types that the virus infects in the mosquito or human hosts. Host cell factor binding to the viral 3' UTR have been previously demonstrated for DENV (56, 83, 322). The inefficiency of American genotype virus to disseminate in mosquitoes may be greatly related to the differences in the 3'

UTR. These changes could affect structures that bind host cell and virus factors and determine virus replication/translation efficiency. If this represents a generic feature of the American genotype viruses, it is likely that more virulent genotypes would outcompete (50) and actively replace (238) the American ones. Thus, the relative inability of American genotype viruses to disseminate in mosquitoes would obviously limit their epidemiological impact.

The effect of these potential genetic determinants on DENV-2 replication efficiency in cells and mosquitoes, could be determined in studies using directed mutagenesis on the DENV-2 infectious clone (222) might aid in establishing their importance. Additionally, some reporter DENV system (66, 126) could be used to evaluate the effect of 3' UTR changes on transcription/translation efficiency. To test the differences in replication efficiency between the American and American/Asian serotypes, competition experiments between the two different genotypes in various cell culture types could be performed.

Chapter IV

Physiological determinants of DENV-2 infection in mosquitoes.

4.1. Introduction

Dengue viruses are maintained in nature by efficiently replicating in two phylogenetic distant organisms, humans and mosquitoes. Disparate physiological conditions exist in these two organisms; for instance: temperature, proteases (presence or absence), pH, cholesterol content, and cell-type differ. The virus overcomes potential restraints and effectively infects, multiplies and is transmitted between mosquitoes and humans.

In humans, DENV binding and entry to blood cells occurs at slightly alkaline conditions of 7.35 to 7.45. However, virus fusion to the host membrane occurs in the endosomal compartment at pH ≤ 6.0. In the mosquito, only the pH of *Ae. aegypti* larval midgut had been reported before this study. In larvae, the pH in the midgut is alkaline: 8.0 in the posterior section and 10-11 in the anterior portion (30, 31, 55). In larvae, the pH is essential to sustain physiological processes and survival (31, 51).

The larval mosquito midgut presents one of the highest pH values known in organisms. Alkalization of this organ involves bicarbonate/carbonate ions, produced by action of carbonic anhydrase. However, mosquito larvae are morphologically and physiologically different from the adult mosquitoes (59). The extent to which this physiological characteristic is preserved in the adult mosquito midgut was unknown. In a whole organism, the conditions prevailing at the cellular surface of tissues and organs may govern virus binding and entry.

In the mosquito, the midgut is the first organ to be in contact with viruses in viremic blood. After an infectious bloodmeal is ingested, viral particles encounter in the mosquito midgut an environment radically different to the human host. The midgut is potentially a hostile environment for pathogens including DENV. Within the midgut, not only pH but also temperature and cell-lipid content change abruptly from the conditions in the human host. Additionally, proteolytic enzymes begin the blood digestion and the

peritrophic matrix isolates the bloodmeal from the midgut epithelium. The virus must successfully navigate the physiological conditions and functions in the mosquito to sustain the transmission cycle. Rapid exit from the midgut may be the most effective preservation strategy for most mosquito-borne pathogens to succeed (14).

In the first hours after the bloodmeal, some differences predominate: a change in pH and the exposure to trypsin-type proteolysis. Importantly, viral particles in blood of viremic patients are present in either infected cells, B cells (133) and monocytes CD14+ (281), or free in plasma. Whether this has some significance in mosquito infection is unknown. A general scheme of the early events occurring in midgut after a bloodmeal is shown in Figure 4.1.1. Understanding the events that promote pathogen attachment, productive infection, and enable them to traverse the midgut may provide novel approaches for transmission intervention (13, 14).

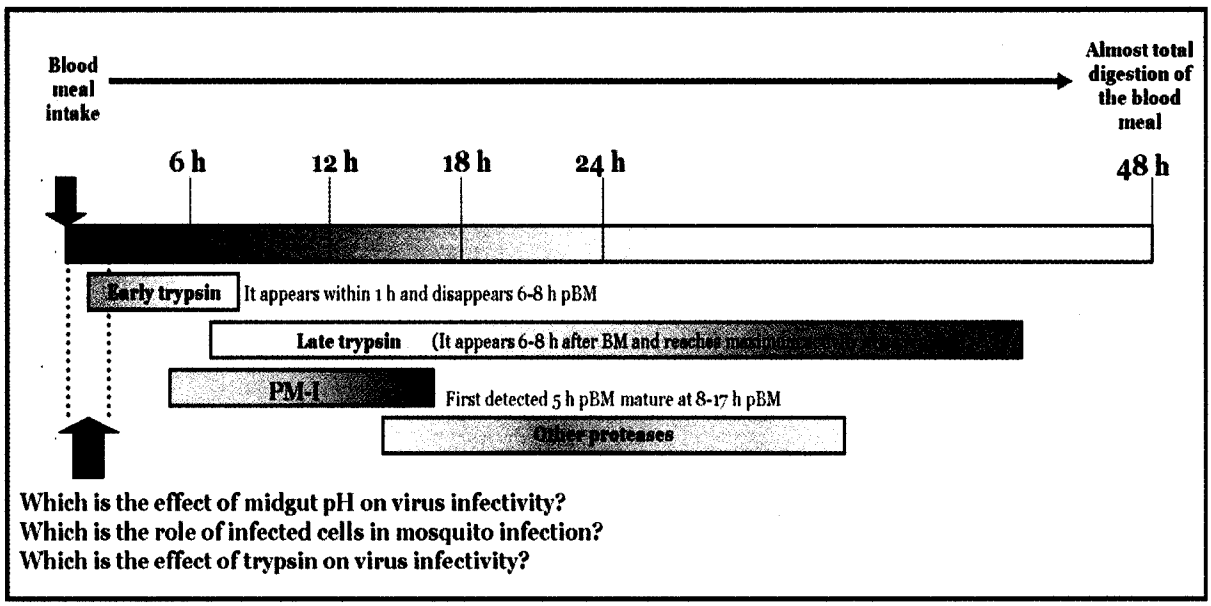


Figure 4.1.1. Major events that occur in the mosquito midgut after ingestion of a bloodmeal. The model shows physiological and anatomical conditions encountered by the virus in the mosquito midgut, after ingestion of the blood. Several questions arise about the bloodmeal processing on virus infectivity.

Studies of DENV in solution show that pH markedly affects viral infectivity. The virus was most stable at pH 9.0 (half-life~10h), and infectivity rapidly declined at pH

values below or above this (268). However, the optimum binding of DENV-2 to LLC-MK2 and C6/36 cell surface occurs at a pH between 5.5 and 6.0 (287). Blood has buffer capability; pH measures *in situ* show the bloodmeal pH increases from 7.40 to 7.52 in *Aedes aegypti* and to 7.58 in *Anopheles stephensi* after blood ingestion (22). To infect, the virus must intimately contact the epithelial cells, which do not directly contact the bloodmeal.

The perithrophic matrix (PM) effectively divides the midgut lumen into an endo- and ecto-peritrophic space, with an intra-PM compartment, effectively modulating digestion (169, 299). It is likely the pH in the ecto-peritrophic space near the midgut epithelial cells remains unaltered, but it is not known. Low pH is required for membrane fusion of most enveloped viruses, which allows the nucleocapsid to be delivered into the cell cytoplasm (43, 86, 121, 122).

In the mosquito midgut extensive proteolysis also takes place after a bloodmeal, when early trypsin and other proteases participate later in the bloodmeal digestion (200, 207, 208, 299). The effect of this activity on DENV infectivity is still unknown, although some effect on infection and dissemination of DENV-2 in *Aedes aegypti* may exist (97, 197). Whether its importance resides directly in a proteolytic cleavage of the viral particle or indirectly on the general nutritional status of the cells is unclear.

Intracellular cleavage by proteolytic enzymes is essential in assembly and maturation of numerous viruses including: alphaviruses, picornaviruses, adeno-viruses and retroviruses (134). In DENV, cleavage of prM viral protein by the host convertase furin occurs during the virus transit through the cell secretory pathway. This cleavage results in rearrangements in the receptor-binding E protein and probably plays a role in virus infectivity.

DENV in blood is mostly present in an estimated portion of 54% and 19%, in cells and plasma respectively (133, 151, 281). Thus most DENV ingested by the mosquitoes is

associated with cells. Nothing is known about how this fraction participates in mosquito infection.

The overall hypothesis of these studies was that *Dengue viruses effectively exploit the mosquito physiological functions to effectively replicate and sustain its transmission cycle.* This investigation was conducted to start clarifying the effect of midgut environment and physiology on virus infectivity. Questions addressed included: What is the pH in the mosquito midgut? Do changes on midgut pH have an effect on virus infectivity? What is the role of cell-associated DENV on vector infection?.

In these studies the pH inside the adult mosquito midgut, the effect of carbonic anhydrase (CA) inhibitors on overall pH, and DENV infectivity *in vitro* and *in vivo* were determined. Finally, the role of cell-associated virus may have in mosquito midgut infection and dissemination was explored.

4.2. Materials and Methods

4.2.1. Virus preparations

Three DENV-2 strains used in the studies included: Jamaica 1409 hp (high passage), Jam 1409 lp (low passage), and Yuc14757 (from a DHF type II case). The viruses' origin has been described in section 3.2.3. C6/36 mosquito cells were propagated and infected as described in section 2.2.2. Bloodmeal composition differed and is described in each experimental section.

4.2.2. Mosquitoes used

Aedes aegypti Chetumal mosquitoes originated from eggs collected from the Yucatan Peninsula in Mexico were used for most of these experiments. The mosquito characteristics and rearing conditions were as in section 2.2.3. For the cell-associated virus experiments, the D2S3 mosquito reference strain was used due to its high susceptibility to DENV-2 infection(15).

4.2.3. Medium and reagents

Leibovitz medium (L-15), 10X nonessential amino acid solution, sodium bicarbonate, and Sf-900 II SFM medium (Gibco Invitrogen Co). Carbonic anhydrase (CA) inhibitors [methazolamide (MTZ) and acetazolamide (ACZ)], adenosine triphosphate, cresol red, phenol red, neutral red, Dulbecco's phosphate buffered saline, 0.25% Trypsin-EDTA, and DMSO were bought from Sigma Aldrich Co. (St. Louis, MO, USA). Finally fetal bovine serum (FBS) was acquired from Atlas Biologicals (Ft. Collins, CO, USA).

4.2.4. Examination of the pH in the mosquito midgut lumen

To determine the pH in adult *Aedes aegypti* mosquito midgut, a battery of four water soluble pH indicators was used including: cresol red, neutral red, phenol read and thymol blue at initial concentration of 1%. Female *Ae. aegypti* mosquitoes fed on a "medium mix" containing 0.02% of pH indicator at 37°C using glass feeders and parafilm membrane as described in section 2.2.4. The mix included 50% MEM 2X (without phenol red), 20% FBS, 10% nonessential amino acids (Gibco), 1 mM ATP, and 0.25% sodium bicarbonate, in PBS at a final pH between 7.2 and 7.4. Mosquitoes fed for 15 min and were chilled down at 4°C for examination. The pH was estimated by comparing the color developed by each pH indicator in the interior of the midgut against a group of pH standards. The standards for each pH indicator used PBS solutions at different pH and indicators at the same concentrations used in the medium mix. Pictures of individual mosquitoes showing indicator color inside their midguts were taken with a Kodak 6.1 Megapixel digital camera using the same illumination source and background for all pictures. Experiments were performed multiple times examining 10 to 20 mosquitoes each time.

4.2.5. Carbonic anhydrase (CA) activity on the overall midgut pH

CA effect on maintenance of pH in the midgut was investigated. Methazolamide (MTZ) and acetazolamide (ACZ) specifically inhibits CA. Thus, Chetumal mosquitoes fed on meals containing pH indicators and CA inhibitors. Meals contained 10^{-3} mmol/L of MTZ and ACZ to evaluate effect on midgut pH (Table 4.2.1). Cresol red was used in following experiments, due to its sensitivity at this pH range. Since, MTZ and ACZ are soluble in dimethyl sulfoxide (DMSO), one of the control groups included the same amount of DMSO in the meal to evaluate background effect. The final pH in the mix was 7.2 to 7.4, adjusting when necessary. Female adult mosquitoes fed on the mixture for 30 min at 37°C using a Parafilm® membrane on a glass feeder. After 20 min, based on an assumed normal feeding time of 2.5–19.5 min (231), mosquitoes were chilled at 4°C for 10 min and midguts were dissected.

	Nutritious mix	**0.02% Cresol red**	**0.001% DMSO**	**10^{-3} mM MTZ**	**10^{-3} mM ACZ**
Control – DMSO	+	+	-	-	-
Control + DMSO	+	+	+	-	-
MTZ + DMSO	+	+	+	+	-
MTZ+ACZ+DMSO	+	+	+	+	+
Acidic control (SfII900 medium)	+	+	-	-	-

Table 4.2.1. Experimental groups tested to examine the effect of CA inhibitor on pH. Studies performed on *Aedes aegypti* female mosquito midguts.

Paired treated and untreated midguts were photographed at room temperature. Cresol red was used for further experiments because of its sensitivity and the midgut pH range. Microscope images were obtained in the Picture Frame v 1.01 software set in the monochromatic mode. The ImageQuant TL software from Amersham-Biosciences® used pictures saved as TIF-8 bit format to analyze and quantify differences.

4.2.6. The pH studies *in vitro*.

To examine the effect of pH on DENV-2 infectivity, C6/36 cell monolayers were infected at three different pHs (6.0, 7.4 and 8.5). The range of pH tested reflected the environments that the virus particle encounters in the transmission cycle. The pH of the endosomal compartment is ≤6.0, the pH of human blood is 7.35-7.45 ; and ≥ 8.5, which is the pH in the mosquito midgut (59). The virus inoculum (at MOI of 0.01) was suspended in 1 ml of L-15 with 2%FBS medium adjusted to each of the selected pHs. Flasks containing C6/36 inoculated monolayers were incubated for 1h with constant rocking at room temperature. After incubation, 4 ml of L-15 with 2%FBS, pH=7.2 were added, and flasks were placed at 28°C for 7 days, when medium was replaced. At day 10, cell monolayers were examined for cytopathic effect and photographed. At day 12, cell virus suspensions were obtained, aliquoted and frozen at -70°C until titrated by plaque assays (as described in section 2.2.7).

4.2.7. CA inhibitors and DENV-2 dissemination in mosquito

To investigate the effect of pH on virus infectivity in mosquitoes, carbonic anhydrase (CA) inhibitors were added to infectious bloodmeals. The carbonic anhydrases in *Aa. aegypti* larvae and adults have been studied (51, 58, 59). Chetumal mosquitoes were fed on DENV-2 infectious bloodmeals containing either DMSO (control) or CA inhibitors (10^{-3} M MTZ /ACZ treated) in DMSO (Table 4.2.2).

	Sheep red blood cells	**DENV-2 –infected cell culture suspension**	**0.001% DMSO**	**10^{-3} M MTZ /ACZ**
Control group	+	+	+	-
Treated group	+	+	+	+

Table 4.2.2. Composition of bloodmeals to determine effect of CA (Carbonic Anhydrase) inhibitors on DENV-2 mosquito infection. Bloodmeals feed to mosquitoes included 50% wash sheep red blood cells, 50% virus suspension and 0.001% DMSO.

Three different DENV-2 viral strains were used: Jam1409hp (high passage), Jam1409lp (low passage) and Yuc14757. Each bloodmeal preparation contained: 2.5 ml

of washed sheep red blood cells, 2.5 ml of virus suspension and 5 µl of MTZ/ACZ (10^{-3} M) or DMSO. Mosquitoes fed on meals placed in glass blood feeders and kept at 37°C. After 45 min, engorged mosquitoes were separated and maintained in an insectary environment until processing. Bloodmeal aliquots were kept at -70°C for further titration by plaque assays.

Twenty to fifty mosquitoes from two different and independent experiments were dissected at 7 and 14 dpiBM, fixed, and assayed by indirect immunofluorescent assay (IFA) (section 2.2.6). Dissemination rates in the treated and untreated control group of mosquitoes were determined for the three different DENV-2 strains.

4.2.8. Midgut infection and dissemination of cell- associated DENV-2.

DENV-2 suspensions obtained from C6/36 infected cell cultures were separated into supernatant (cell-free virus) and pellet (cell-associated virus) portions by centrifugation at 3,000 rpm for 5 min. In a variation of the conditions, the supernatant fraction was passed through a 0.2 µm filter to eliminate any cell contamination. The pellet fraction was then resuspended in L-15 2% FBS medium, and each fraction was mixed with an equal amount of washed sheep red blood cells. The mixture was used to feed D2S3 mosquitoes for 45 min. Engorged mosquitoes were sorted and kept in insectary conditions with a constant source of sugar and water until processing at 4 and 7 days post-infectious bloodmeal (dpiBM). Mosquitoes were assayed by IFA to establish midgut and dissemination rates. Viral titers in the bloodmeals were $2x10^5$ PFU/ml for cell-associated virus (pellet) and 0.22-1.4 $x10^7$ PFU/ml for supernatant.

4.3. Results

4.3.1. The pH in the interior of the adult mosquito midgut.

The pH of the mosquito midgut was established by comparing mosquitoes fed on a battery of water soluble pH indicators. Color in the midgut lumen was compared to a group of standards, which revealed that the pH in the lumen of *Ae. aegypti* mosquito midgut is between 8.5 and 9.5 (Figure 4.3.1).

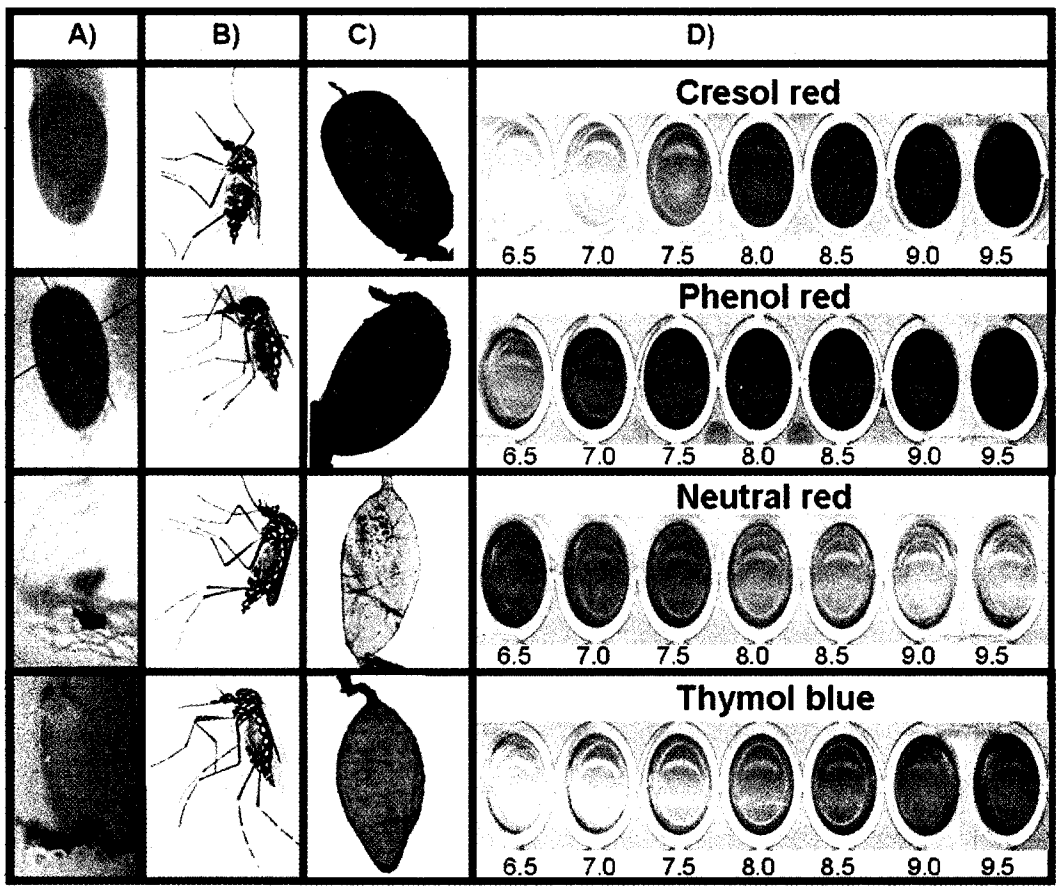


Figure 4.3.1. The pH of the midgut lumen of adult *Aedes aegypti* mosquito. Adult females were fed a "medium mix" (section 4.2.4) containing 0.02% pH indicator. A) Dissected mosquito midgut under a stereoscopic microscope. B) *Aedes aegypti* females immediately after feeding on the pH indicator mixture. C) Dissected midgut of engorged mosquitoes photographed at 10X. D) pH standards.

To prove the consistency of this test, three other species of mosquitoes were examined using the same method. These species were *Aedes triseriatus*, *Anopheles gambiae* and *Culex tarsalis*, all of which are important vectors of human diseases.

Consistently, the midgut pH in these mosquitoes was alkaline, and between 8.0 and 9.5 (Figure 4.4.2).

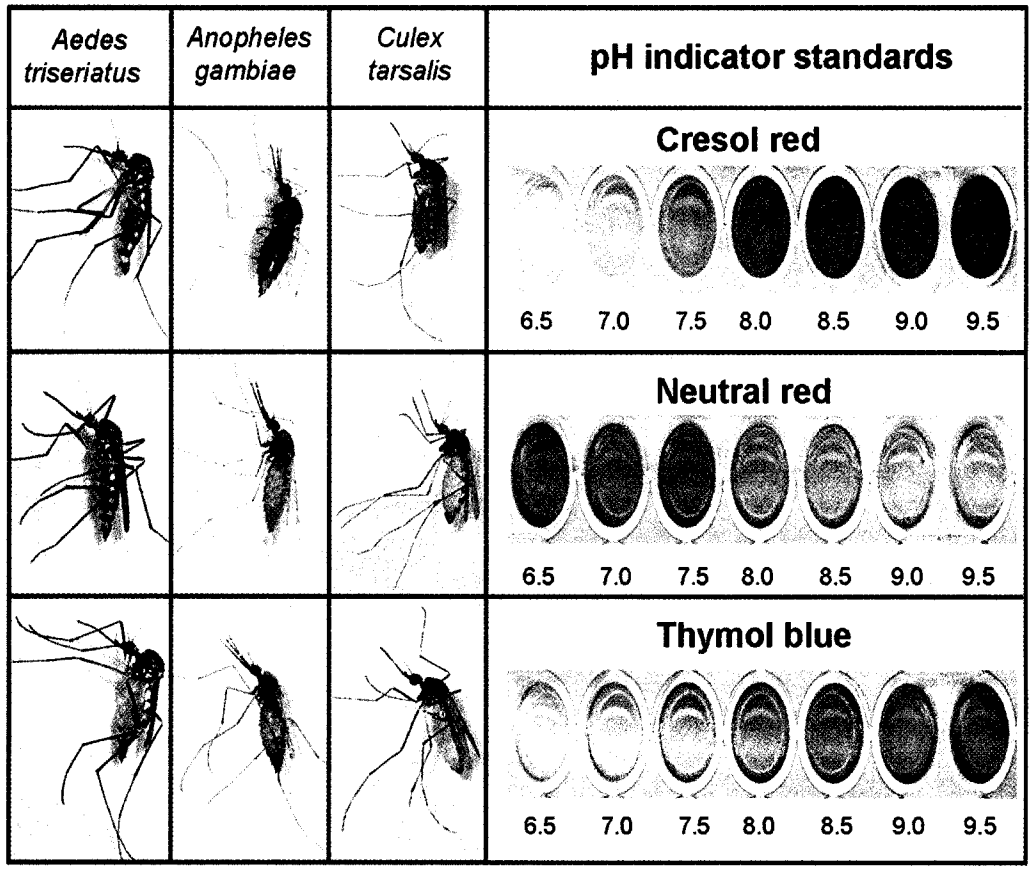


Figure 4.3.2. The pH in the midgut lumen of other adult mosquitoes. *Aedes triseriatus*, *Anopheles gambiae* and *Culex tarsalis* females fed on a mixture containing 0.02% pH indicator. After the feeding mosquitoes cooled down, examined and photographed. The indicators used were cresol red, neutral red and thymol blue.

4.3.2. Effect of carbonic anhydrase inhibitors on the midgut pH.

To determine if carbonic anhydrase (CA) played any major role in pH maintenance in the adult mosquito midgut, mosquitoes were fed on a mix of pH indicator and CA inhibitor and examined. Previous use of the Hansson's histochemical method revealed that CA in *Ae. aegypti* is widely distributed in the adult mosquito midgut. This method induces a black precipitate at the site where CA is active as either cytosolic or membrane-bound form, and *Ae. aegypti* showed extensive darkening in the midgut when this method was applied (Figure 4.3.5C). The ^{18}O isotope exchange

analysis, which is highly sensitive and aided in quantifying soluble cytosolic CA in larval midguts (51), was used in adults. Unlike other mosquitoes, the CA activity in *Ae. aegypti* was demonstrated in both anterior and posterior midgut (data not shown) (59). After only 15 to 20 minutes of CA inhibitors activity on the midgut, the pH in this organ dropped a whole pH unit (to about 7.5-8.0) (Figures 4.3.3 -4.3.4)

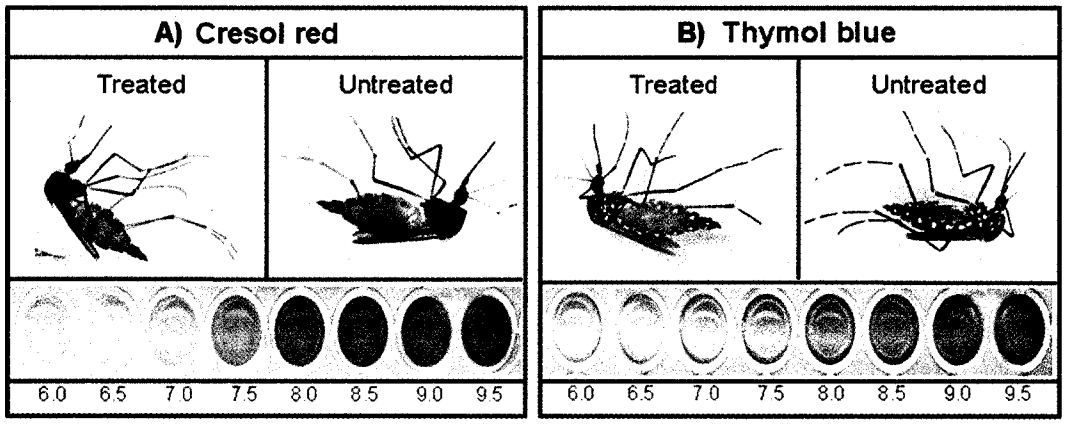


Figure 4.3.3. CA inhibitors decrease the pH inside the midgut in the adult female *Aedes aegypti*. A significant decrease in the pH inside the midgut was observed after treatment using cresol red (A) and thymol blue (B). Mosquitoes ingested a meal containing both pH indicator and CA inhibitors to a concentration of 10^{-3} M.

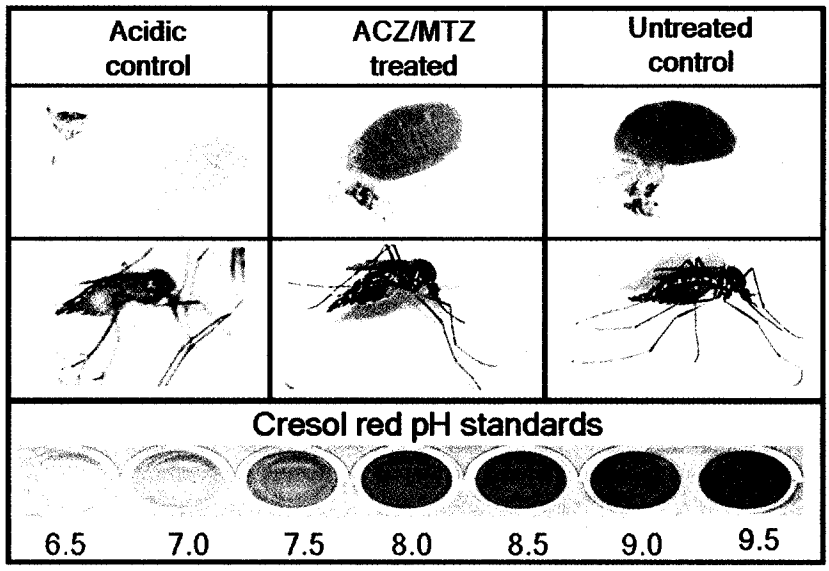


Figure 4.3.4. Carbonic anhydrase conditions the pH inside the midgut of adult *Aedes aegypti* mosquito. Combined treatment of CA specific inhibitors (10^{-3} M MTZ and ACZ) resulted in a significant decrease in the overall pH inside the midgut. A) Midguts and B) whole mosquitoes, photographed using a Kodak digital camera 6.1 Mega pixels of resolution and the same illumination source and background. C) Midguts photographed using an Olympus Microfire™ camera at 10X, under the same light source.

The presence of DMSO (at 0.001%) in the meal had no effect on either the overall pH of the mixture or the pH inside the mosquito midgut. Controls for acidification developed a yellow color in the midgut, indicating a pH ≤ 6.5 (Figure 4.3.4).

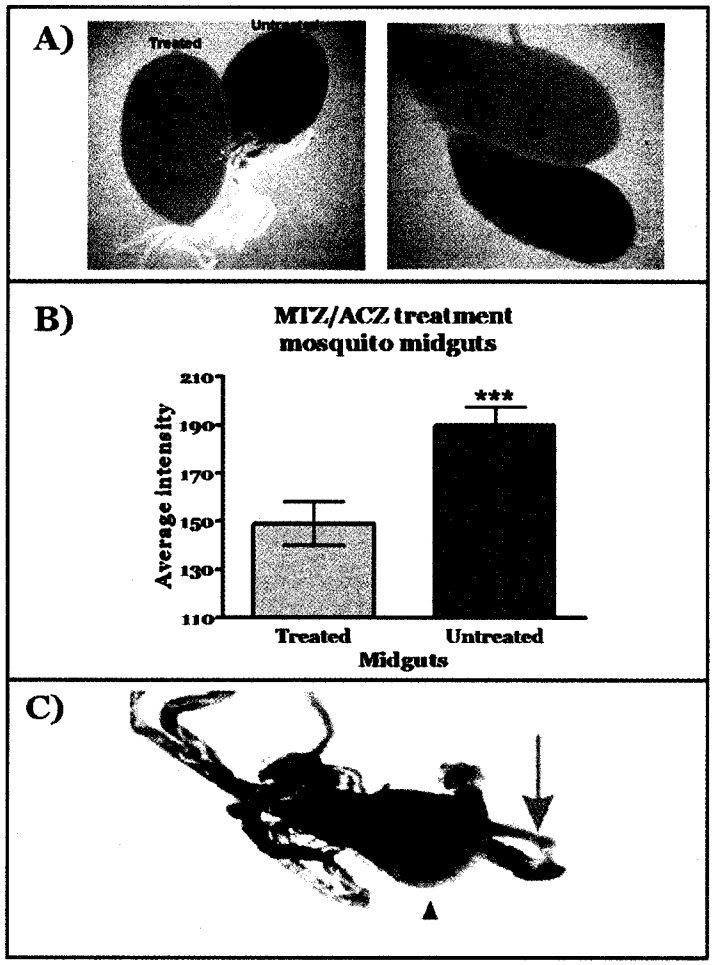


Figure 4.3.5. Analysis of paired ACZ/MTZ treated and untreated midguts. Gray TIF-8 bit format digital images analysis used ImageQuant TL software from Amersham-Biosciences®. Differences between the treated and untreated group were determined in 20 paired specimens (10 treated and 10 untreated). A) Paired pictures of midguts fed on a meal containing cresol red. B) Histogram showing the intensity mean and the 95%CI for each group analyzed (Table 4.3.3). Differences were significant with a P value < 0.0001. C) The distribution of CA in the Aedes aegypti midgut analyzed by Hanson's histological staining. Darkening shows sites of CA activity.

Finally, gray TIF- 8 bit pictures from 10 treated-untreated paired midguts were used for the quantitative analysis of differences in pH. The program analyzes color intensity by quantifying the pixel number. Results indicated a clear and significant difference between the color developed inside treated and untreated midguts. The

significance for this analysis was a P value <0.0001 by unpaired t test (Figure 4.3.5 and Table 4.3.1).

Thus, CA inhibitors had a marked effect on the overall pH of the midgut of *Ae. aegypti* mosquitoes, showing that this enzyme is essential in maintaining the pH in this organ. Additionally this enzyme is active broadly distributed in the midgut of *Ae. aegypti* mosquitoes (59).

	Cresol red MTZ/ACT/DMSO treated midguts		**Cresol red DMSO (untreated group)**	
	Average Intensity	Standard Deviation	Average Intensity	Standard Deviation
Analysis of TIF-8 bit paired images with ImageQuant® TL- Amersham Biosciences® software	166.63	1.42	194.06	1.55
	157.00	2.36	177.34	2.56
	139.58	3.91	179.98	2.29
	153.51	1.92	177.46	2.62
	155.96	6.18	193.19	1.45
	149.22	1.55	199.90	1.91
	127.06	2.47	185.02	1.43
	142.78	1.56	212.34	1.49
	136.56	2.13	185.34	2.05
	163.69	7.59	194.12	4.73

Table 4.3.1. Values obtained from ACZ/MTZ treated and untreated midguts. The ImageQuant TL software from Amersham-Biosciences® was used for the analysis. The mean value and SD for each midgut is shown, paired treated and untreated midgut were analyzed in the same pictures.

4.3.3. Effect of pH on virus infectivity *in vitro*.

The effect of pH on DENV infectivity was tested *in vitro* by infecting C6/36 and measuring the resulting virus titers. The physiologic environments encountered by virus particles passing through the transmission cycle were investigated. The pH considered were: 6.0, approximately the endosomal compartment pH; 7.4, the pH in human blood; 8.5, approximately the pH in the midgut lumen.

First fusogenic activity of DENV-2 (Jam1409hp, Jam1409lp, and Yuc 14757) was measured as syncytia formation. Virus infected C6/36 cell monolayers (MOI of 0.01)

were examined at 10 dpi for cytopathic effect (Figure 4.3.6). All tested strain yielded cytopathic effect including syncytia formation at pH≤6.0 in all the tested strains.

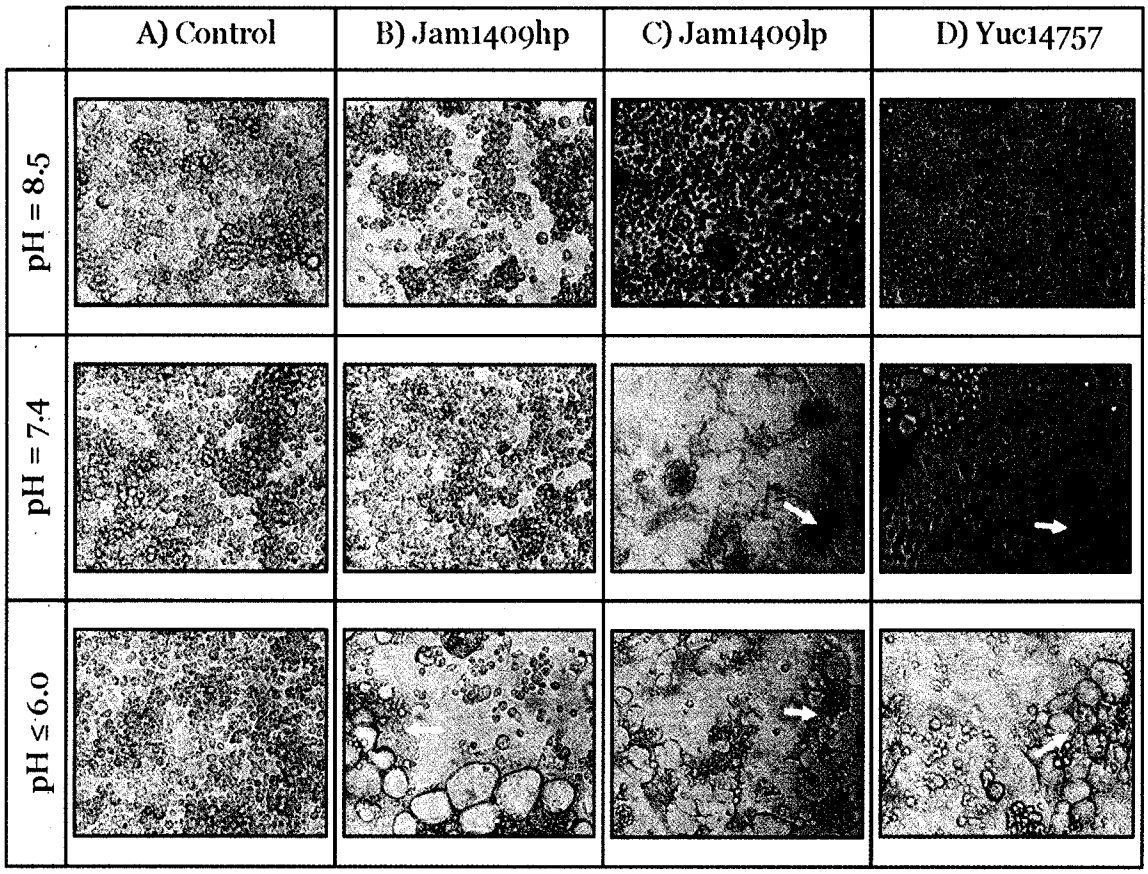


Figure 4.3.6. Cytopathic effect produced by different DENV-2 strains at 10dpi. For each DENV-2 strain, the inoculum used the same virus stock suspended in 1 ml of L15-2%FBS medium at pH of 6.0, 7.4 and 8.5. Flasks containing C6/36 monolayers were inoculated and incubated for 1h with constant rocking at room temperature to allow virus binding and entry. Magnification is 400X. Arrows indicate syncytia in the cell monolayer.

However, at pH 7.4 only the DENV-2Jam1409lp and Yuc14757 exhibited syncytia, which occurs when virus produces cell membrane fusion (polykaryocyte formation). DENV-2 Jam1409hp was able to produce syncytia only at pH=6.0, whereas DENV-2 Jam1409lp and Yuc14757 produced syncytia at pH of 6.0 and 7.4. None of the strains formed syncytia when infection occurred at a pH of 8.5. Other characteristics of the cytopathic effect included extensive vacuole formation. DENV-2 Jam1409lp and

Yuc14757 caused fusion in a pH-independent-manner, while DENV-2 Jam1409hp caused fusion only at low pH (6.0).

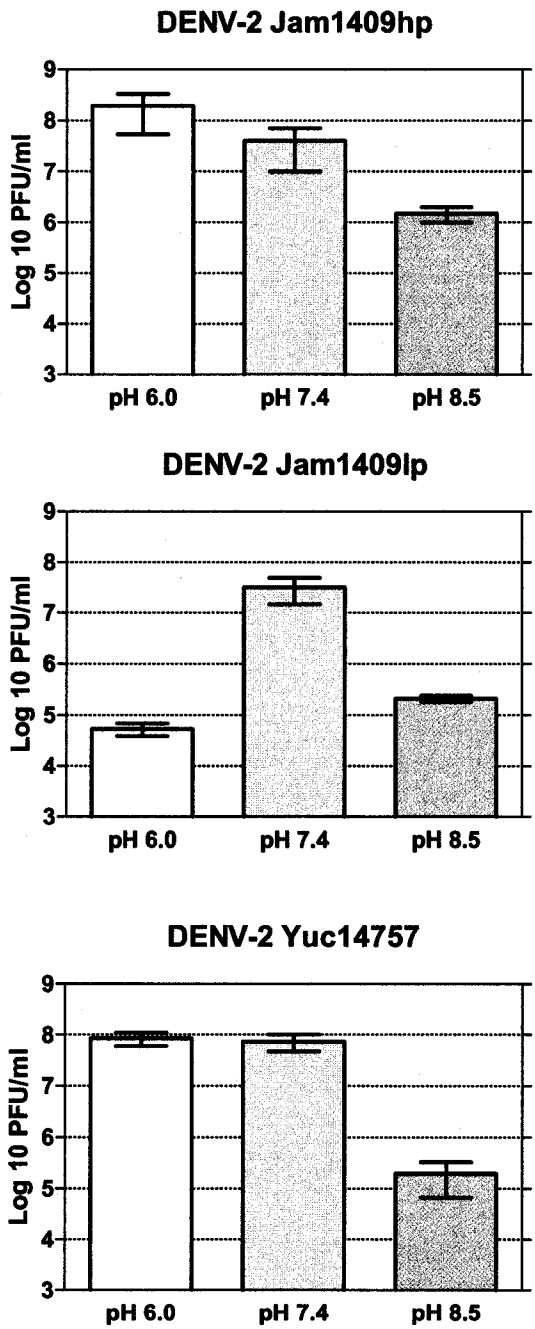


Figure 4.3.7. Infection pH affects virus titer. C6/36 cell monolayers were infected with the respective virus at different pH. Virus suspensions were collected at 7dpi and kept at -70°C until titration. Error bars represent standard error of the mean (N=3). Observed differences were not significantly different by either ANOVA or t test.

To quantify whether virus production was affected by the infection pH, cell suspensions from the monolayers infected at different pH were collected at 7 dpi. Virus suspensions from three different and independent experiments were titrated by plaque assays. Highest viral titer production was at pH 6.0 with DENV-2 Jam1409hp, but at pH 7.4 with DENV-2 Jam1409lp and DENV-2 Yuc14757 (Figure 4.3.7 and Table 4.3.2).

	pH 6.0	pH 7.4	pH 8.5
DENV-2 Jam1409hp	0.1-5.0x10^8 PFU/ml	0.02-1.0x10^8 PFU/ml	1.3-1.7x10^6 PFU/ml
DENV-2 Jam1409lp	3.0-8.0x10^4 PFU/ml	0.13-7.0x10^7 PFU/ml	1.7-2.7x10^5 PFU/ml
DENV-2 Yuc14757	0.6-1.0x10^8 PFU/ml	0.5-5.0x10^8 PFU/ml	0.67-3.3x10^5 PFU/ml

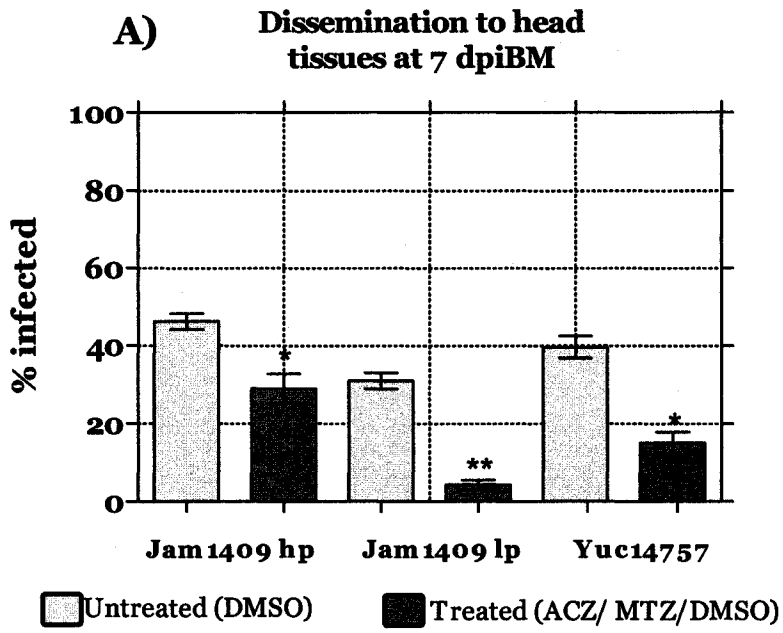
Table 4.3.2. Viral titers yielded by different DENV-2 viral strains infected at different pHs. Virus in suspensions collected at 7 dpi from two independent experiments was quantified.

Despite the consistent trends observed in triplicate experiments of the pH effect *in vitro*, the analyses (ANOVA, t test, and Tukey's multiple comparison test) failed to prove them to be statistically significant. Further analysis increasing the number experiment may provide a better assessment of the significance of such trends.

4.3.4. Effect of pH on DENV-2 mosquito infection

To examine the role of pH on virus infectivity in the mosquito, pH in the midgut was reduced using CA inhibitors (ACZ/MTZ) and dissemination rates were compared to control groups. The CA inhibitors aided in testing the effect of pH on virus infectivity *in vivo*. The effect of these inhibitors on midgut pH was shown in section 2.3.4 (59). Since, CA inhibitors are diluted in DMSO and it has a toxic effect on cells and organisms at some concentrations, the control infectious bloodmeals contained DENV and 0.001% DMSO.

Dissemination to head tissues was examined at day 7 and 14 after the infectious bloodmeal. Significant differences were found between the reduced pH and untreated group at 7 dpiBM ($P<0.05$ and $P<0.01$) (Figure 4.3.8A), but differences were not obvious or statistically significant at 14 dpiBM (Figure 4.3.8B).



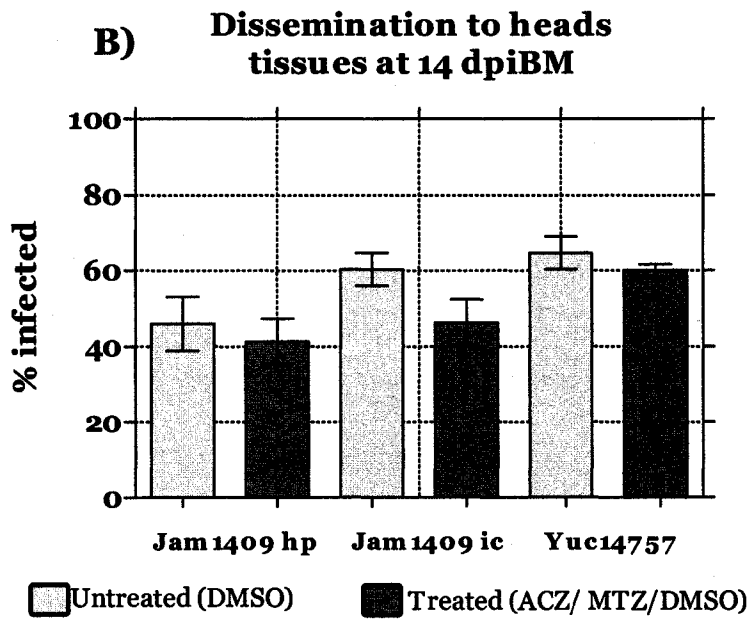


Figure 4.3.8. CA inhibitors may negatively affect DENV-2 dissemination rates to head tissues mosquitoes at 7 dpiBM. At 7 (B) and 14 (A) days after the oral challenge of *Aedes aegypti* mosquitoes with bloodmeals containing DENV-2 and CA inhibitors were examined. Bars represent infection rates ± SEM from three separate experiments, n=20. CA inhibitors markedly affect the overall pH inside the mosquito midgut. Control group included 0.001% DMSO in the bloodmeal, whereas the treated group contained 10^{-3} M ACZ/MTZ and 0.001% DMSO. *P<0.05 and ** P<0.01 by unpaired t test. Viral titers in bloodmeals are shown in Table 4.3.6.

Blood+virus+	DENV-2 Jam104hp	DENV-2 Jam1409lp	DENV-2 Yuc14757
DMSO	0.67 - 1.93x10^7 PFU/ml	1.1-2.3x10^6 PFU/ml	4.6 - 9.5x10^7 PFU/ml
DMSO+ACZ+MTZ (CA inhibitors)	0.67 - 1.47x10^7 PFU/ml	1.3-1.7x10^6 PFU/ml	4.0 - 8.9x10^7 PFU/ml

Table 4.3.3. Viral titers in the bloodmeals used to evaluate the effect of CA inhibitors on DENV-2 mosquito infection. Bloodmeal aliquots were collected once all the components mixed and placed it the glass feeder. After collection, samples were kept at -70 °C.

Dissemination at 7 dpiBM were higher in the control group (normal midgut pH) than in the treated group (lower pH due to CA inhibitors). The negative effect of CA inhibitors (that reduce pH) was observed in the three examined virus strains (DENV-2 Jam1409lp, Jam1409hp and Yuc14757). Reducing the pH in the mosquito midgut (by using CA inhibitors) has a negative effect on early (7dpiBM) DENV-2 dissemination. These results seem to oppose the observations of *in vitro* infections, different conditions and cell types in cell cultures in compatison to mosquitoes may explain this trend. Hence, reducing the pH in the midgut possibly affects initial stages of virus infection in mosquito and dissemination as consequence. This suggests that virus exploits the natural basic pH in the mosquito midgut to efficiently infect and rapidly disseminate out the midgut. However, more detailed studies will be necessary to establish the possible mechanism.

4.3.5. Effect of cell- associated DENV-2 on dissemination in mosquito

In patient's blood, mononuclear cells: CD14+ and B cells (133, 281) are infected by DENV-2. Thus a mosquito feeding on a viremic individual acquires infected cells (cell-associated virus) as well as DENV free in plasma. To explore the role that cell-associated virus may have on productive vector infection, DENV-2 infected cell cultures were separated into cellular (cell-associated virus) and cell-free fractions and bloodmeals were prepared to feed D2S3 mosquitoes. The kinetics of dissemination of DENV-2 Jam1409 to salivary glands (Figure 4.3.9A) and head tissues (Figure 4.3.9B) with both fractions was determined. Surprisingly, at 4 dpiBM viral antigen was found in ~36% of the salivary

glands of mosquitoes ingesting either of the cell-free, cell-associated or the complete virus suspension. Dissemination rates to the heads in mosquitoes that ingested cell-associated virus or cell-free virus preparations differ at 4 and 7 dpiBM, but were similar at 14 dpiBM. This occurred despite the fact that viral titers were 2 Logs lower in cell-associated (cellular) than in cell-free (supernatant) fractions.

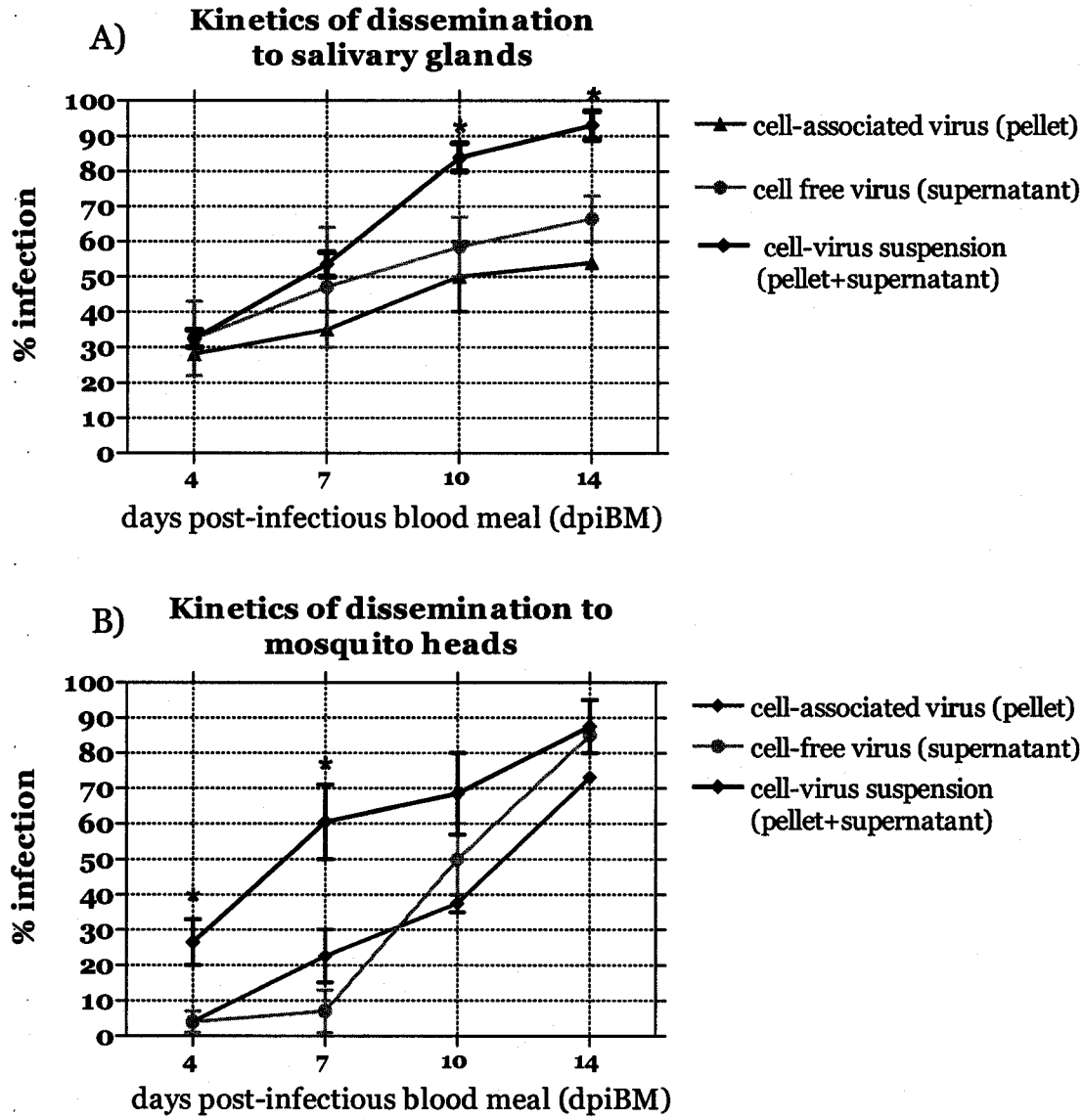


Figure 4.3.9. Dissemination rates of cell-associated and cell-free DENV-2 Jam1409hp in D2S3 mosquitoes. For these experiments infectious bloodmeals containing pellet or supernatant from infected cell cultures were used. Viral titers in the bloodmeal were: 1.7±0.7x10^7 PFU/ml for the complete virus suspension, 1.8 x10^7 PFU/ml for the supernatant and 5.3x10^5 PFU/ml for the pellet. N=20-30 mosquitoes per time point. *P<0.05 by unpaired t test.

To investigate this in more detail, midguts and dissemination rates were analyzed at 4 and 7 dpiBM in mosquito fed on either preparation. In this study the supernatant fraction was filtered to remove cells and ensure a cell-free portion. The dissemination rates and number of mosquitoes examined are indicated in Table 4.3.4 (Figure 4.3.10 and Table 4.3.4).

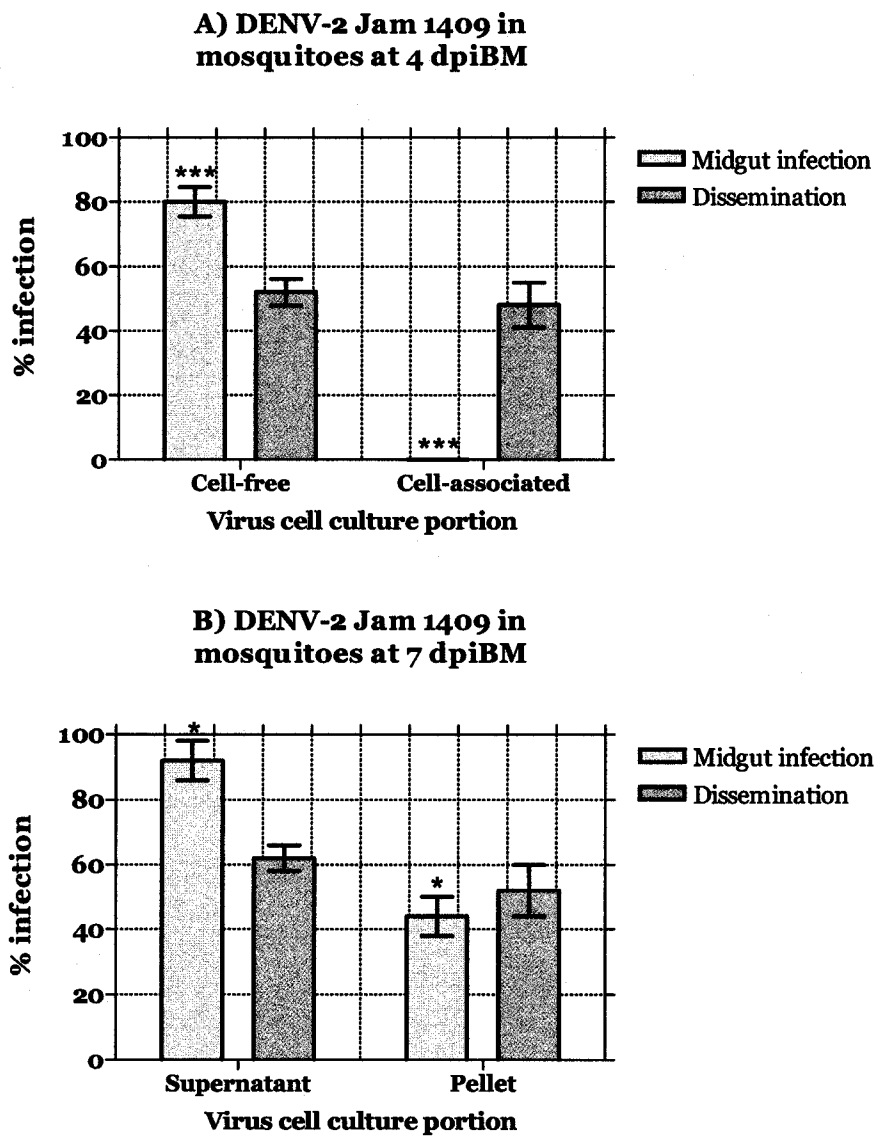


Figure 4.3.10. Midgut infection and dissemination in D2S3 mosquitoes that ingested either DENV-2 Jam1409 cell-free (supernatant) or the cell-associated (pellet) virus. C6/36 cell cultures infected with DENV-2 Jam 1409 were used for these experiments. Dissemination to the abdomen or any other organ or tissue occurred about the same rate in both groups. N=30-50 mosquitoes. Viral titers in the cell-associated virus bloodmeals were 1.0-2.5x10^5 PFU/ml and 0.22-1.4 x10^7 PFU/ml in the cell-free fraction. *P<0.05 *** P<0.001 by unpaired t test.

	Cell-associated virus (Culture pellet)		Cell-free virus (Culture supernatant)	
	Midgut	Dissemination	Midgut	Dissemination
4 dpiBM	0% n= 22	48%±12% n= 44	80%±8% N= 39	54%±6% n= 65
7 dpiBM	44%±6% n= 27	52%±8% n=29	92%±6% N= 30	62%±4% n= 30

Table 4.3.4. Midgut infection and dissemination rates caused by either the cell-associated or cell-free virus fractions. The numbers represent the average of two independent experiments, bloodmeal Viral titers in the bloodmeals were for pellet $2x10^5$ PFU/ml, for supernatant 0.9-1.4 $x10^7$ PFU/ml. Salivary gland infection was established only in one of these experiments at 7 dpiBM and were for pellet 33% and for supernatant 25%.

At 4 dpiBM, midgut infection was detectable only in mosquitoes that ingested the cell-free virus fraction, while dissemination occurred in both fractions (Figure 4.3.10A). Differences between the two fractions in midgut infection were less dramatic but still significant ($P<0.05$) at 7 dpiBM (Figure 4.3.10B). Thus, cells may have an important role in DENV dissemination in the mosquito vector. More detailed examination and evaluation of the biological significance of this finding will be necessary.

4.4. Discussion

The insect midgut contains a single layer of epithelial cells supported by constituents of the basal lamina. On the luminal side, the epithelial cell membranes fold to form the numerous actin-filled microvilli. Microvilli increase the surface area and play an important role in absorption of nutrients. The microvilli are exposed to the lumen and to pathogens ingested in bloodmeals as consequence. Arboviruses encounter changes, such as pH in the midgut environment. The results using pH indicators (Figures 3.3.1 and 3.3.2) suggest the pH inside the adult *Ae. aegypti* mosquito midgut is between 8.5 and 9.5. In the other tested mosquito species, including *Aedes triseriatus*, *Anopheles gambiae* and *Culex tarsalis*, the pH range is between 8.0 and 9.5. Results obtained with

cresol red showed that *Anopheles gambiae* has the most alkaline pH of all examined species (Figure 4.3.2). Possibly different pH environments surround the microvilli and some gradient between anterior and posterior midgut may exist as it occurs in larvae (31). This could explain the discrepancies observed between the data obtained *in vitro* and *in vivo* (Figure 4.3.8 and 4.3.9). The viral titers yielded *in vitro* when infection was carried out at pH of 8.5, were lower than those obtained from cells infected at pH of 7.4. In contrast, the reduction of the pH *in vivo* (midgut) consistently affected virus infectivity and dissemination at 7 dpiBM in mosquitoes (Figure 4.3.8A). The effect on virus infectivity could be either direct on the virus-cell interaction or indirect on other physiological functions that impact DENV dissemination by affecting pH. Data suggest other factors may be important and determine virus infectivity and dissemination in the mosquitoes. The recovery of dissemination rates observed at 14dpiBM (Figure 4.3.8B) may be explainable by the generation and/or selection of viral quasispecies occurring at midgut level that may need to become enriched and disseminate at a slower rate as consequence. The greatest RNA genotypic diversity of La Crosse virus is generated in virus that infects and replicates in the midgut (6).

The pH changes can affect the overall charge/conformation of surface proteins and probably the receptor-ligand interactions. The charge on the protein represents a sum of the individual amino acid charges in the side chains. The charge on amino acid side chains depends on the pH of the solution and the pK_A (acid-ionization constant) of the side chains. Therefore, pH can significantly influence the molecular conformation of proteins. Although the pK_A of COO^- and $NH3^+$ termini in a protein occur at extreme pH (<3.1 and >8.0 respectively), some other amino acid groups have pK_A values in the biological range, such as cysteine 8.3-8.5 and histidine 6.0-6.5. Changes in temperature and pH may alter some amino acid residues charge and configuration. How they

influence the overall protein conformation could depend on amount, position and exposure of these residues in the protein.

Virus-cell interactions in the mosquito midgut may differ from those that occur in the human host, since these interactions must occur not only at a different pH (7.4 versus ~9.0) but also at different temperature (37°C versus ≤30°C). Additionally, cholesterol that makes cellular membranes more fluid may be significantly depleted in the cholesterol auxotrophs insect cells (51). Thus, it is reasonable to speculate that virus binding, and probably entry mechanisms, differ in human and mosquito hosts. There is evidence that a region in domain III (E-380 to 389) of the E protein is involved in DENV-2 and WNV binding to mosquito but not mammalian cells (45, 132). Treatment of cell surfaces with certain glycosidases significantly reduces DENV-2 binding to mammalian cell lines, but not to the insect cell lines. Furthermore, heparin and polysulfates compete with and inhibit viral infection of mammalian cells, but have no effect on virus binding or infection of insect cells (284, 287).

Presumably following receptor-ligand binding on the plasma membrane, viruses are taken up by endocytosis. However, exceptions to the endocytic entry have been observed for some flaviviruses, which can fuse directly to the plasma membrane of insect cells (280). Direct fusion of DENV-2 with plasma membrane may also occur for human monocytes and BHK-21 at non acidic pH (153). The presence of neutralizing antibodies on the virus alter this mode of entry (265). All fusion proteins existing on the virion surface are in an metastable state in which fusion peptides (a hydrophobic sequence) remain hidden or shielded in the glycoprotein (73, 158, 233). Acidic pH induces the E protein dimers on the DENV virion surface to recluster irreversibly into trimers (196, 327), an event that may depend on E protein protonation (addition of H^+) (277). Trimer formation is the crucial stage causing virus-host cell membrane fusion and allowing virus nucleocapsid release from the acidic endosomal compartment to the cell cytoplasm

(196). However the effect of alkaline pH on the E protein conformation has not been determined.

In the midgut, virus-cell recognition must occur at alkaline pH that could affect viral E glycoprotein conformation, thereby influencing entry and fusion as consequence. Virus fusion with the mosquito host cells may occur at the plasma membrane or the endosomal membrane. Optimal fusion from with out (FFWO) induced in C6/36 cells requires exposure of adsorbed virus to pH 6.0 and an incubation at 39°C (280), which are not the natural conditions in the mosquito midgut. WNV early interaction with a macrophage-like cell line induced fusion and endocytosis at pH 6.4, while only endocytosis occurred at pH 8.0 (149). It is clear that trimers are not formed in TBE at alkaline pH (8.0) (277). WNV fusion and uncoating depend on acidic pH, although uncoating occurring outside the cell renders the virus noninfectious (92, 93). The nature of DENV fusion in mosquito cells *in vivo* is still unknown.

In the DENV-2 E protein, two amino acid residue substitutions have been repeatedly noted after multiple virus passage in mosquito cells: E-6 (Ile to Met) (109) and loss of the E-153 (Asn) glycosylation site (110, 139, 168). Interestingly, these changes in E protein alter virus membrane fusogenic activity (139). Since, changes in the glysosylation site (E-Asn-153) are not observed in alternating passages, this may be an indication of its role in recognizing, binding, and infecting mammalian but not mosquito cells.

When the midgut pH was altered using CA (carbonic anhydrase) inhibitors, a negative effect on virus dissemination was observed at 7 dpiBM (Figure 3.3.8A). However, the differences between the treated and control group were not significant at 14 dpiBM (Figure 4.3.9. and Table 4.3.3). To which extent the pH is altered by CA inhibitors and the buffer activity of blood mixed in the meal remain unindentified. Thus whether the lower DENV-2 dissemination rates at early times resulted directly from pH

change in the midgut or as a collateral effect of other activities affected by the treatment requires further investigation.

The dissemination of virus in mosquitoes that ingested either cell-associated virus occurring before viral antigen is detected in midgut is provocative and complex to explain (Figures 4.3.9 -4.3.10 and Table 4.3.4). Dissemination occurred at equivalent levels in cell-associated and cell-free virus portion despite viral titers. There may be some concern about the effect of residual cell-free virus in the cell- associated virus portion, since the latter was not washed. However, the cell-associated virus infectious bloodmeal contained almost 2 Logs less virus than the cell-free virus portion. Further examination of the conditions underlining virus dissemination from cell-associated virus fraction will be necessary.

The C6/36 cells are presumably hemocytic in origin and could transport the virus into the hemocel. In humans, several cells including macrophages, phagocytes, and DCs are able to transverse capillaries and migrate in tissues and organs. However, migration of hemocytes in insect organs has not been fully described (163). Thus, mobility of self-(hemocytes) and non-self phagocytic cells in hematophagous insects needs to be investigated.

Arboviruses may also encounter proteolytic activity of digestive enzymes. Early trypsin is released shortly after digestion and other proteases continue to be liberated into the endoperithrophic space until blood digestion is completed. Thus, escape and proteolytic processing of virus free in the plasma or contained in infected PMBC cells in the midgut might be different. A significant portion of cell-associated virus might be represented by immature viral particles. Trypsin cleavage of E-prM-containing virus particles *in vitro* releases a soluble 45 kDa fragment of E protein that retains cell-binding capacity (304). DENV purified viral particles from cell culture contain a mixture of prM and M proteins, in which prM often predominates (4). Possibly trypsin intervenes in

proteolytic cleavage of immature viral particles released from infected cells during blood digestion.

Whether these dissemination rates caused by DENV-associated with C6/36 cells mimic the natural infection with DENV-infected PBMC (monocytes, macrophages, CD14, and B-cells) remain to be established. Studies to explore vector infection with DENV-2-infected PBMCs will reveal whether cell-associated virus infection of vector is an artifact of virus growing in insect cells or has in fact biological significance in dengue transmission.

Virus infection of vectors is a complex process in which mosquito physiology must be exploited by viruses to infect and to be transmitted. Blood digestion, nutrient transport, innate immune response could all play a role on virus mobilization through the vector. Further and more detailed investigation is necessary to clearly understand virus infection/transmission mechanisms in the vector and to develop new strategies to interrupt these events to control dengue transmission.

5. SUMMARY AND FUTURE DIRECTIONS

5.1. Virogenesis of DENV-2 Jam1409 in orally infected mosquitoes. The kinetics of DENV-2 Jam1409 after oral infection in its primary vector *Ae. aegypti* was investigated. Multiple organs and tissues were analyzed, which included midguts, salivary glands, heads, abdominal and thoracic tissues. DENV-2 antigen was initially detected in midgut, trachea and abdominal fat body between 2 and 3 days post-infectious blood meal (dpiBM) (Figure 2.3.1, 2.3.9 and Table 2.3.2). The presence of DENV-2 antigen in tracheal system (Figure 2.3.8) was significantly correlated with early virus dissemination (χ^2=16.27, df=1, P<0.001), thus the tracheal system may allow the virus to "bypass" the midgut and disseminate. The tracheal system has been related to the systemic spread of insect viruses and other arboviruses as discussed. However, the exact mechanism that viruses use remains unknown. The tracheal epidermal cells and the tracheoblasts are the only cellular components of this system. Likely some physiological functions involving trachea are being exploited by the viruses to disseminate out of the midgut, but further research is necessary to establish the mechanism.

Midgut infections were characterized by lateral spread of initial infection foci throughout the entire midgut with a peak of infection between 7 and 10 dpiBM (Figure 2.3.1). The amount of viral antigen and the viral titers in this organ declined at day 10, whereas surprisingly viral RNA levels remained quite stable (Figure 2.3.6). In contrast to other studies, a second blood meal did not increased viral titers in the midgut (Figure 2.3.7). However, the level at which virus repression/persistence may occur in the midgut requires further investigation. DENV-2 antigen was found in approximately 36-40% of examined mosquito salivary glands as early as 4 dpiBM, while head tissue did not contain detectable viral antigen before 7 dpiBM (Table 2.3.2). DENV-2 Jam1409 exhibited a high dose-dependent tropism for salivary glands and head tissues (Figures

2.3.10 and 2.3.11). Virus transmission studies will be necessary to establish the time after which DENV to start to being transmitted in mosquito saliva.

5.2. Correlation between disease severity in humans and virogenesis in mosquitoes. The correlation between DENV-2 clinical severity in humans and virogenesis in *Aedes aegypti* mosquitoes was investigated. Four viruses of the American/Asian genotype (Yucatan strains), which were isolated from cases of different clinical severity, were characterized in mosquitoes. Dissemination rates to salivary glands/head tissues and viral titers were determined at 14 dpiBM in two newly-established *Aedes aegypti* mosquito populations: Chetumal (coindigenous) and Loreto (non coindigenous). The case severity of the Yucatan strains (American/Asian genotype) was significantly correlated ($P<0.001$ by χ^2 3 df) with the dissemination rates to head tissues of Chetumal mosquito population (Figure 3.3.6A). However, the dissemination rates to the salivary glands correlated with disease severity only for two of the viruses (Yuc11936 and Yuc14497). This correlation was not as clear for mosquitoes of the Loreto population (Figure 3.3.6B). Most of these DENV-2 Yucatan viruses induced significantly ($P<0.05$ or $P<0.001$) higher viral titers in individual Chetumal or Loreto mosquitoes than the high passage laboratory strain DENV-2 Jam1409 (Figure 3.3.8).

The American genotype virus (QR0094) was restricted in its ability to disseminate out of the midgut as compared to viruses from the American/Asian genotype isolated from the same geographic area (the Yucatan Peninsula) (Figure 3.3.10). Viruses from the two genotypes differed in plaque morphology (Figure 3.3.4) and ability to form syncytia (Figure 3.3.5) in cell cultures. Both virus genotypes showed differences in the examined E protein as well as the 3'UTR sequence (Figures 3.3.11-3.3.15). These differences may account for the apparent fitness advantage for replication in mosquitoes that American/Asian genotype viruses have over the American genotype tested in this study. Potentially, other differences may be in regions that were not

examined could also condition the dramatic phenotypic differences between the American and American/Asian genotypes. Full-length sequence of these viruses could identify other potential genetic determinants. The effect of candidate genetic determinants could be characterized by site-directed-mutagenesis on an infectious clone. Once the changes are generated, produced viruses could be tested in both cell culture and mosquitoes to confirm the gene sequence and function relationships.

5.3. Physiological determinants of DENV-2 infection in *Aedes aegypti* mosquitoes. The effects of the pH in the midgut and the presence of DENV-2 infected cells on the kinetics of virus infection were investigated. The pH in the midgut of adult mosquitoes had not been investigated. The pH in the midgut lumen was established for different adult female mosquito species. In the midgut lumen of *Ae. aegypti* the pH value ranged between 8.5 and 9.5 (Figure 4.3.1), and was between 8.0 and 9.5 for *Aedes triseriatus*, *Anopheles gambiae* and *Culex tarsalis* mosquito species (Figure 4.3.2). The pH in the *Ae. aegypti* midgut is greatly determined by the activity of carbonic anhydrase (CA), which is broadly distributed along the mosquito midgut (Figure 4.3.5C). A short term inhibition of CA activity using CA inhibitors profoundly affected the overall pH (Figures 4.3.3. and 4.3.4). The midgut pH influenced DENV-2 infectivity *in vitro* and *in vivo* (Figures 4.3.7- 4.3.9). Fusogenic activity *in vitro* depended on the pH level during the initial infection. Some DENV-2 strains produced polykaryotes when infection occurred at either acidic or neutral conditions, whereas others caused them only at acidic conditions (Figure 4.3.7). The reduction of the pH in the mosquito midgut using CA inhibitors consistently reduced DENV-2 dissemination rates at 7 dpiBM (4.3.9A). Cell-associated virus seems to play a significant role in early DENV dissemination but not in midgut infection (4.3.11). The possible mechanism and the biological significance of these observations remain to be investigated. Apparently, mosquito physiology is effectively exploited by viruses to sustain their transmission cycle in nature.

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APPENDICES

Appendix 7.1. DENV-2 genomic components

DENV-2						
Genomic sequence			**Protein**			
	Begin at	Length (nt)		Begin at	Length (aa)	Approx. molecular weight (176)
5'UTR	1	96				
C	97	342	C	1	114	11 Kd
prM	439	273	prM	115	91	26 Kd
M	712	225	M	206	75	8 kd
E	937	1485	E	281	495	50 kd
NS1	2422	1056	NS1	776	352	46 Kd
NS2A	3478	654	NS2A	1128	218	22 Kd
NS2B	4132	390	NS2B	1346	130	14 kd
NS3	4522	1854	NS3	1476	618	70 Kd
NS4A	6376	450	NS4A	2094	150	16 Kd
NS4B	6826	744	NS4B	2244	248	27 kd
NS5	7570	2700	NS5	2492	900	103 kd
3'UTR	10270	454				
Total	10723					

Appendix 7.2. Substitutions in DENV E protein previously reported.

VIRUS	CHANGES IN E PROTEIN	CHANGE TYPE	*	OVERALL EFFECT	SELECTION METHOD	OTHER CHANGES	CITED
DENV-1	E_196 Met▶Val	conserv.	I-II	increase in mouse neurovirul. (apoptosis)	Passage in suckling mice	NS3_435 Leu▶Ser	Duarte dos Santos *et. al.*, 2000
	E_365 Val▶□Ile	conserv.	III				
	E_405 Thr▶Ile	non-conserv.	III				
	E_69 Ile▶other		I	growth	Phenotypic	C_114 Ala▶other	Ishak, *et al* 2001
DENV-2	E_6 Ile▶Met	conserv.	I	Altered membrane fusion	NH_4Cl and FFWI assays	None reported	Guirakhoo *et al.*, 1993
	E_134 Asn▶Ser	conserv.					
	E_153 Asn▶Tyr	non-conserv.					
	E_153 ** Asn▶Asp	non-conserv.	I	Resistant to low pH	low pH (4.0) treatment		
	E_390 Asp▶His	non-conserv.	III	increased neurovirul. in mice	Passaging in mice	No reported	Sanchez & Ruiz, 1996
	E_71 Glu▶Asp	non-conserv.	I	increased neurovirul. in mice	Passage in suckling mice	preM_55 Leu▶Phe preM_57 Arg▶Lys NS1_105 Arg▶Glu	Bray, *et al.*, 1998
	E_126 Glu▶Lys						
	E_126 Glu▶Lys	non-conserv.	I	Neurovirul. in mice	Passaging	prM non related	Gualano, *et al.*, 1998
	E_390 Asn▶Asp (Asian to American)	non-conserv.	III	Less replication in MDM	Phenotypic	No reported	Pryor, *et al.*, 2001
DENV-4	E_155 ** Thr▶Ile	non-conserv.	I	Increase neurovirul.	Site direct mutagen.	C_81 Thr▶Ile prM78 Val▶Ala	Kawano, *et al.*, 1993
	E_401 Phe▶Leu	conserv.	III				

Appendix 7.3. Plaque assay test

Quantities of each reagent required to prepare the primary and secondary overlays for plaque assays are provided. Amounts according to the amount of plates needed.

1st Nutrient overlay. 4 ml to each well in a 6-well plate

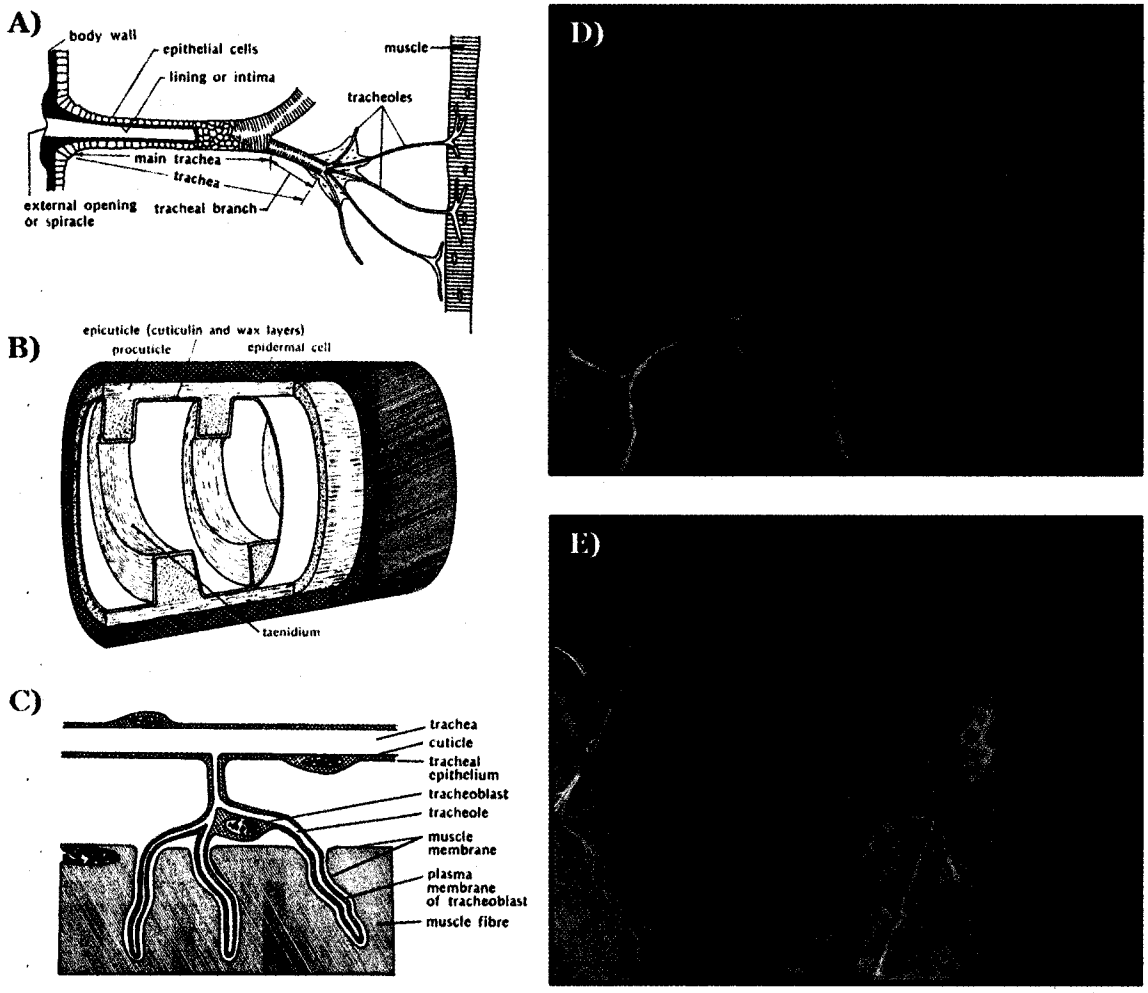
Agar suspension to sterilize in the autoclave								
Agar	3 g	4 g	5 g	6 g	7.5 g	10 g	12 g	15 g
dH_2O	245 ml	326 ml	407 ml	489 ml	611 ml	815 ml	977 ml	1221 ml
Cool down to 45-50°C and add the next sterilized solutions								
Earl's BSS (10X)	30 ml	40 ml	50 ml	60 ml	75 ml	100 ml	120 ml	150 ml
YE-LAH	9.9 ml	13.2 ml	16.5 ml	19.8 ml	24.8 ml	33.0 ml	39.6 ml	49.5 ml
FBS	6.0 ml	8.0 ml	10.0 ml	12.0 ml	15.0 ml	20.0 ml	24.0 ml	30.0 ml
7.5% $NaHCO_3$	9.0 ml	12.0 ml	15.0 ml	18.0 ml	22.5 ml	30.0 ml	36.0 ml	45.0 ml
Gentamycin (50mg/ml)	0.3 ml	0.4 ml	0.5 ml	0.6 ml	0.75 ml	1.0 ml	1.2 ml	1.5 ml
Fungizone (1 mg/ml)	0.6 ml	0.8 ml	1.0 ml	1.2 ml	1.5 ml	2.0 ml	2.4 ml	3.0 ml
Total volume	300 ml	400 ml	500 ml	600 ml	750 ml	1000 ml	1200 ml	1500 ml
No. plates	12	16	20	25	30	40	50	60

2nd Nutrient overlay. 2 ml of the next solution for each well in a 6-well plate

Agar suspension to sterilize in the autoclave								
Agar	1.6 g	2.0 g	2.5 g	3.2 g	4.0 g	5.0 g	6.4 g	8.0 g
H_2O	143 ml	152 ml	215 ml	276 ml	345 ml	430 ml	550 ml	690 ml
Cool down to 45-50°C and add the next sterilized solutions								
10X Earl's solution	16 ml	20 ml	25 ml	32 ml	40 ml	50 ml	64 ml	80 ml
Neutral red	6.0 ml	7.5 ml	9.5 ml	12 ml	15 ml	19 ml	24 ml	30 ml
Gentamycin (50mg/ml)	0.16 ml	0.2 ml	0.25 ml	0.32 ml	0.4 ml	0.5 ml	0.64 ml	0.8 ml
Total Volume	160 ml	200 ml	250 ml	320 ml	400 ml	500 ml	640 ml	800 ml
No. plates	12	16	20	25	30	40	50	60

To prepare one liter of the 10X Earl's BSS solution, 68g NaCl, 4 g of KCl, 1.25g $NaH_2PO_4.H_2O$ and 10 g Glucose in 800 ml of water were mixed and sterilized. Onc the solution cooled, 100ml of $CaCl_2.2H_2O$ 0.155M and 100ml of $MgSO_4.7H_2O$ 0.083M were added. The YE-LAH solution contained 2% w/v of yeast extract and 10% w/v lactoalbumin hydrolysate (ratio 1:1) autoclaved for 55 min).

Appendix 7.4. The mosquito tracheal system.



Tracheal system in mosquitoes. A) Parts of insect trachea (248). B) Semidiagrammatic reconstruction of a tracheal wall showing the epidermal cells, epicuticle, and taenidia (239). C) Tracheoles indenting muscle membrane (41). D and E) Tracheal system connected to the midgut in *Ae. aegypti*, DENV-2 uninfected and infected mosquitoes, respectively.

Appendix 7.5. Alignment of E protein from DENV-2 Yucatan strains using Clustal W

```
Yuc14497        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
Yuc14757        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
Yuc11936        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELTKTEAKQPATLRKYC 60
Yuc12914        MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELTKTEAKQPATLRKYC 60
Jam1409hp       MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC 60
                *****:********************************************* **********

Yuc14497        IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
Yuc14757        IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
Yuc11936        IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
Yuc12914        IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
Jam1409hp       IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
                ******************************:*****************************

Yuc14497        CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT 180
Yuc14757        CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT 180
Yuc11936        CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT 180
Yuc12914        CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT 180
Jam1409hp       CKKNMEGKVVLPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT 180
                ********** ************************************************

Yuc14497        VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF 240
Yuc14757        VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF 240
Yuc11936        VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF 240
Yuc12914        VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF 240
Jam1409hp       VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF 240
                ************************************************************

Yuc14497        KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS 300
Yuc14757        KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS 300
Yuc11936        KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS 300
Yuc12914        KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS 300
Jam1409hp       KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS 300
                ************************************************************

Yuc14497        MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE 360
Yuc14757        MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE 360
Yuc11936        MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE 360
Yuc12914        MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE 360
Jam1409hp       MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTE 360
                ************************************* ********************

Yuc14497        KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW 420
Yuc14757        KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW 420
Yuc11936        KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW 420
Yuc12914        KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW 420
Jam1409hp       KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW 420
                ************************************************************

Yuc14497        DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV 480
Yuc14757        DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV 480
Yuc11936        DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV 480
Yuc12914        DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV 480
Jam1409hp       DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV 480
                ************************************************************

Yuc14497        LVGVVTLYLGAMVQA 495
Yuc14757        LVGVVTLYLGAMVQA 495
Yuc11936        LVGVVTLYLGAMVQA 495
Yuc12914        LVGVVTLYLGAMVQA 495
Jam1409hp       LVGVVTLYLGAMVQA 495
                ***************
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Appendix 7.6. Alignment of E proteins in Clustal W

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
Yuc14497     MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC
Yuc14757     MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC
Yuc11936     MRCIGISNRDFVEGVSGGSWVDIVLEHuGSCVTTMAKNKPTLDFELTKTEAKQPATLRKYC
Yuc12914     MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELTKTEAKQPATLRKYC
Jam1409hp    MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC
Mex94QRoo    MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPASLRKYC
             *****:************************************ ********:*****

                    70        80        90       100       110       120
                     |         |         |         |         |         |
Yuc14497     IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT
Yuc14757     IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT
Yuc11936     IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT
Yuc12914     IEAKLTNTTTESRCPTQGEPSLNEEQDKRFICKHSMVDRGWGNGCGLFGKGGIVTCAMFT
Jam1409hp    IEAKLTNTTTESRCPTQGEPSLNEEQDKRFLCKHSMVDRGWGNGCGLFGKGGIVTCAMFT
Mex94QRoo    IEAKLTNTTTDSRCPTQGEPTLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT
             **********:*********:*********:******************************

                   130       140       150       160       170       180
                     |         |         |         |         |         |
Yuc14497     CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT
Yuc14757     CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT
Yuc11936     CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT
Yuc12914     CKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT
Jam1409hp    CKKNMEGKVVLPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGT
Mex94QRoo    CKKNMEGKIVQPENLEYTVVITPHSGEEHAVGNDTGKHGKEVKITPQSSITEAELTGYGT
             ********:* *******:**********************:******************

                   190       200       210       220       230       240
                     |         |         |         |         |         |
Yuc14497     VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
Yuc14757     VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
Yuc11936     VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
Yuc12914     VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
Jam1409hp    VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
Mex94QRoo    VTMECSPRTGLDFNEMVLLQMEDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF
             ************************************************************

                   250       260       270       280       290       300
                     |         |         |         |         |         |
Yuc14497     KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
Yuc14757     KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
Yuc11936     KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
Yuc12914     KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
Jam1409hp    KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
Mex94QRoo    KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS
             ************************************************************
```

```
                    310        320        330        340        350        360
                     |          |          |          |          |          |
Yuc14497      MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE
Yuc14757      MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE
Yuc11936      MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE
Yuc12914      MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEITDLEKRHVLGRLITVNPIVTE
Jam1409hp     MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTE
Mex94QRoo     MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTE
              ************************************ ********************

                    370        380        390        400        410        420
                     |          |          |          |          |          |
Yuc14497      KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW
Yuc14757      KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW
Yuc11936      KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW
Yuc12914      KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW
Jam1409hp     KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAW
Mex94QRoo     KDSPVNIEAEPPFGDSYIIIGVEPGQLKLDWFKKGSSIGQMFETTMRGAKRMAILGDTAW
              ****************************:*******************************

                    430        440        450        460        470        480
                     |          |          |          |          |          |
Yuc14497      DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
Yuc14757      DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
Yuc11936      DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
Yuc12914      DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
Jam1409hp     DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
Mex94QRoo     DFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLV
              ************************************************************

                    490
                     |
Yuc14497      LVGVVTLYLGAMVQA
Yuc14757      LVGVVTLYLGAMVQA
Yuc11936      LVGVVTLYLGAMVQA
Yuc12914      LVGVVTLYLGAMVQA
Jam1409hp     LVGVVTLYLGAMVQA
Mex94QRoo     LVGIVTLYLGVMVQA
              ***:******.****
```

Appendix 7.7. Alignment of E protein genes in Clustal W

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
Yuc14497    ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGG
Yuc14757    ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGG
Yuc11936    ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGG
Yuc12914    ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGG
Jam1409hp   ATGCGTTGCATAGGAATGTCAAATAGAGACTTTGTAGAAGGGGTTTCAGGAGGAAGCTGG
Mex94QRoo   ATGCGCTGCATAGGAATATCAAATAGGGACTTTGTGGAAGGAGTGTCAGGAGGGAGTTGG
            ***** *********** ******** ***** ** ***** ** ******** ** ***

                    70        80        90       100       110       120
                     |         |         |         |         |         |
Yuc14497    GTTGACATAGTCTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAACAAACCAACA
Yuc14757    GTTGACATAGTCTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAACAAACCAACA
Yuc11936    GTTGACATAGTCTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAACAAACCAACA
Yuc12914    GTTGACATAGTCTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAACAAACCAACA
Jam1409hp   GTTGACATAGTCTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAATAAACCAACA
Mex94QRoo   GTTGACATAGTTTTAGAACATGGAAGTTGTGTGACGACGATGGCAAAAAATAAACCAACA
            *********** ****************************************** *********

                   130       140       150       160       170       180
                     |         |         |         |         |         |
Yuc14497    TTGGATTTTGAACTGATAAAAACAGAAGCCAAACAACCTGCCACTCTAAGGAAGTACTGT
Yuc14757    TTGGATTTTGAACTGATAAAAACAGAAGCCAAACAACCTGCCACTCTAAGGAAGTACTGT
Yuc11936    CTGGATTTTGAACTGACAAAAACAGAAGCCAAACAACCTGCCACTCTAAGGAAGTACTGT
Yuc12914    CTGGATTTTGAACTGACAAAAACAGAAGCCAAACAACCTGCCACTCTAAGGAAGTACTGT
Jam1409hp   TTGGATTTTGAACTGATAAAAACAGAAGCCAAACAACCTGCCACCCTAAGGAAGTACTGT
Mex94QRoo   CTGGACTTTGAACTGATAAAAACAGAAGCCAAACAACCCGCCTCCTTAAGGAAGTACTGT
             **** ********** ********************** *** *  **************

                   190       200       210       220       230       240
                     |         |         |         |         |         |
Yuc14497    ATAGAAGCAAAGCTGACCAACACAACAACAGAATCGCGTTGCCCAACACAAGGGGAACCC
Yuc14757    ATAGAAGCAAAGCTGACCAACACAACAACAGAATCGCGTTGCCCAACACAAGGGGAACCC
Yuc11936    ATAGAAGCAAAGCTGACCAACACAACAACAGAATCGCGTTGCCCAACACAAGGGGAACCC
Yuc12914    ATAGAAGCAAAGCTGACCAATACAACAACAGAATCGCGTTGCCCAACACAAGGGGAACCC
Jam1409hp   ATAGAAGCAAAGCTGACCAATACAACAACAGAATCTCGTTGCCCAACACAAGGGGAACCC
Mex94QRoo   ATAGAGGCTAAACTGACCAACACGACAACAGACTCGCGCTGCCCAACACAAGGGGAACCC
            ***** ** ** ******** ** ******** ** ** **********************

                   250       260       270       280       290       300
                     |         |         |         |         |         |
Yuc14497    AGTCTAAATGAAGAGCAGGACAAAAGGTTCATCTGCAAACACTCCATGGTAGACAGAGGA
Yuc14757    AGTCTAAATGAAGAGCAGGACAAAAGGTTCATCTGCAAACACTCCATGGTAGACAGAGGA
Yuc11936    AGTCTAAATGAAGAGCAGGACAAAAGGTTCATCTGCAAACACTCCATGGTAGACAGAGGA
Yuc12914    AGTCTAAATGAAGAGCAGGACAAAAGGTTCATCTGCAAACACTCCATGGTAGACAGAGGA
Jam1409hp   AGTCTAAATGAAGAGCAGGACAAAAGGTTCCTCTGCAAACACTCCATGGTAGACAGAGGA
Mex94QRoo   ACCCTGAATGAAGAGCAGGACAAAAGGTTTGTCTGCAAACATTCCATGGTAGACAGAGGA
            *  ** ************************  ********** ******************

                   310       320       330       340       350       360
                     |         |         |         |         |         |
Yuc14497    TGGGGAAATGGATGTGGATTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTTACA
Yuc14757    TGGGGAAATGGATGTGGATTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTTACA
Yuc11936    TGGGGAAATGGATGTGGATTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTTACA
Yuc12914    TGGGGAAATGGATGTGGATTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTTACA
Jam1409hp   TGGGGAAATGGATGTGGATTATTTGGAAAGGGAGGCATTGTGACCTGTGCTATGTTTACA
Mex94QRoo   TGGGGAAATGGATGTGGATTGTTTGGAAAAGGAGGCATCGTGACCTGTGCTATGTTCACA
            ********************* ******** ******** ***************** ***

                   370       380       390       400       410       420
                     |         |         |         |         |         |
Yuc14497    TGCAAAAAGAACATGGAAGGAAAAGTCGTGCAGCCAGAAAACTTGGAATACACCATCGTG
Yuc14757    TGCAAAAAGAACATGGAAGGAAAAGTCGTGCAGCCAGAAAATTTGGAATACACCATCGTG
Yuc11936    TGCAAAAAGAACATGGAAGGAAAAGTCGTGCAGCCAGAAAATTTGGAATACACCATCGTG
Yuc12914    TGCAAAAAGAACATGGAAGGAAAAGTCGTGCAGCCAGAAAATTTGGAATACACCATCGTG
```

```
Jam1409hp     TGCAAAAAGAACATGGAAGGAAAAGTCGTGCTGCCAGAAAATTTGGAATACACCATCGTG
Mex94QRoo     TGCAAAAAGAACATGGAAGGAAAAATTGTGCAGCCAGAAAACCTGGAATACACTGTCGTG
              ************************* * **** *********  **********  *****

                       430       440       450       460       470       480
                       |         |         |         |         |         |
Yuc14497      ATAACACCTCACTCAGGAGAAGAGCACGCTGTGGGTAATGACACAGGAAAGCATGGCAAG
Yuc14757      ATAACACCTCACTCAGGAGAAGAACACGCTGTGGGTAATGACACAGGAAAGCATGGCAAG
Yuc11936      ATAACACCTCACTCAGGAGAAGAGCACGCTGTAGGTAATGACACAGGAAAGCATGGCAAG
Yuc12914      ATAACACCTCACTCAGGAGAAGAGCACGCTGTAGGTAATGACACAGGAAAGCATGGCAAG
Jam1409hp     ATAACACCTCACTCAGGAGAAGAGCACGCTGTAGGTAATGACACAGGAAAACATGGCAAG
Mex94QRoo     ATAACACCTCATTCAGGGGAAGAACATGCAGTCGGAAATGACACAGGAAAACATGGTAAA
              *********** ***** ***** ** ** ** ** ************** ***** **

                       490       500       510       520       530       540
                       |         |         |         |         |         |
Yuc14497      GAAATCAAAATAACACCACAGAGTTCCATCACAGAAGCAGAACTGACAGGCTATGGCACT
Yuc14757      GAAATCAAAATAACACCACAGAGTTCCATCACAGAAGCAGAACTGACAGGCTATGGCACT
Yuc11936      GAAATCAAAATAACACCACAGAGTTCCATCACAGAAGCAGAACTGACAGGCTATGGCACT
Yuc12914      GAAATCAAAATAACACCACAGAGTTCCATCACAGAAGCAGAACTGACAGGCTATGGCACT
Jam1409hp     GAAATCAAAATAACACCACAGAGTTCCATCACAGAAGCAGAACTGACAGGCTATGGCACT
Mex94QRoo     GAAGTCAAGATAACACCACAGAGCTCCATCACAGAGGCGGAACTGACAGGCTATGGCACT
              *** **** ************** *********** ** **********************

                       550       560       570       580       590       600
                       |         |         |         |         |         |
Yuc14497      GTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGCTGCTGCAG
Yuc14757      GTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGCTGCTGCAG
Yuc11936      GTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGATTTCAATGAGATGGTGCTGCTGCAG
Yuc12914      GTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGCTGCTGCAG
Jam1409hp     GTCACGATGGAGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGCTGCTGCAG
Mex94QRoo     GTTACGATGGAGTGCTCTCCAAGAACGGGCCTCGACTTCAATGAGATGGTGTTGCTGCAA
              ** ***************** ************** *************** *******

                       610       620       630       640       650       660
                       |         |         |         |         |         |
Yuc14497      ATGGAAGACAAAGCTTGGCTGGTGCACAGGCAATGGTTCCTAGACCTGCCGTTACCATGG
Yuc14757      ATGGAAGACAAAGCTTGGCTGGTGCACAGGCAATGGTTCCTAGACCTGCCGTTACCATGG
Yuc11936      ATGGAAGACAAAGCTTGGCTGGTGCACAGACAATGGTTCCTAGACCTGCCGTTACCATGG
Yuc12914      ATGGAAGACAAAGCTTGGCTGGTGCACAGACAATGGTTCCTAGACCTGCCGTTACCATGG
Jam1409hp     ATGGAAGACAAAGCTTGGCTGGTGCACAGGCAATGGTTCCTAGACCTGCCGTTACCATGG
Mex94QRoo     ATGGAAGACAAAGCTTGGCTGGTGCACAGACAATGGTTCCTAGACCTACCGTTGCCATGG
              ****************************** ***************** ***** ******
Prim.cons.    ATGGAAGACAAAGCTTGGCTGGTGCACAG2CAATGGTTCCTAGACCTGCCGTTACCATGG

                       670       680       690       700       710       720
                       |         |         |         |         |         |
Yuc14497      CTACCCGGAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACTTTC
Yuc14757      CTACCCGGAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACTTTC
Yuc11936      CTACCCGGAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACTTTC
Yuc12914      CTACCCGGAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACTTTC
Jam1409hp     CTACCCGGAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACTTTC
Mex94QRoo     CTGCCCGGAGCAGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTCACCTTC
              ** ******** ********************************************** ***

                       730       740       750       760       770       780
                       |         |         |         |         |         |
Yuc14497      AAAAATCCCCACGCGAAGAAACAGGATGTCGTTGTTTTAGGGTCTCAAGAAGGGGCCATG
Yuc14757      AAAAATCCCCACGCGAAGAAACAGGATGTCGTTGTTTTAGGGTCTCAAGAAGGGGCCATG
Yuc11936      AAAAATCCCCACGCGAAGAAACAGGATGTTGTCGTTTTAGGGTCTCAAGAAGGGGCCATG
Yuc12914      AAAAATCCCCACGCGAAGAAACAGGATGTTGTCGTTTTAGGGTCTCAAGAAGGGGCCATG
Jam1409hp     AAAAATCCCCACGCGAAGAAACAAGATGTCGTTGTTTTAGGATCTCAAGAAGGGGCCATG
Mex94QRoo     AAAAATCCCCATGCGAAAAAACAGGATGTTGTTGTCTTAGGATCCCAAGAAGGGGCTATG
              *********** ***** ***** ***** ** ** ***** ** ********** ***

                       790       800       810       820       830       840
                       |         |         |         |         |         |
Yuc14497      CACACGGCACTCACAGGGGCCACAGAAATCCAGATGTCATCAGGAAACTTACTGTTCACA
Yuc14757      CACACGGCACTCACAGGGGCCACAGAAATCCAGATGTCATCAGGAAACTTACTGTTCACA
Yuc11936      CACACGGCACTCACAGGGGCCACAGAAATCCAGATGTCATCAGGAAACTTACTGTTCACA
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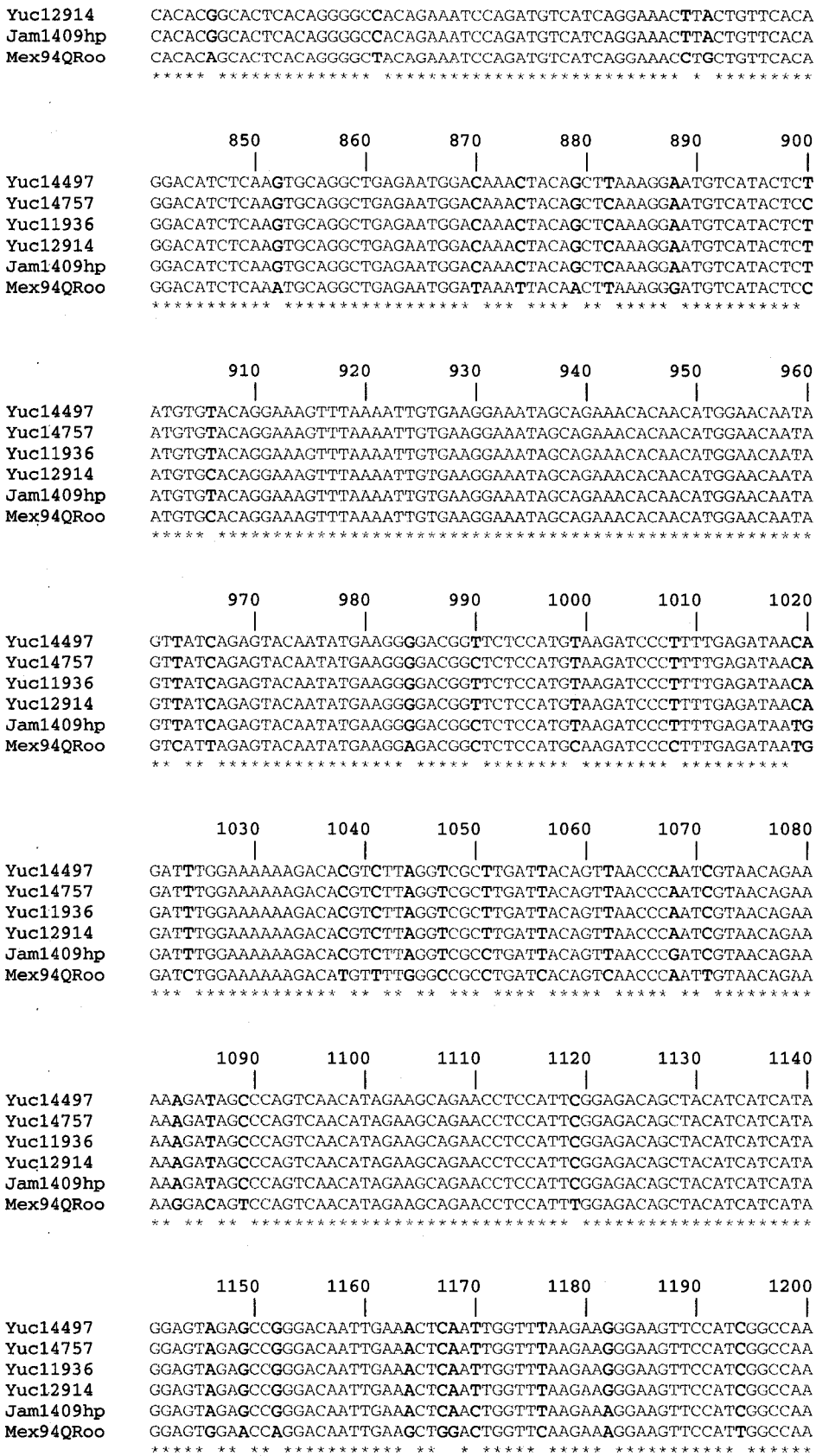

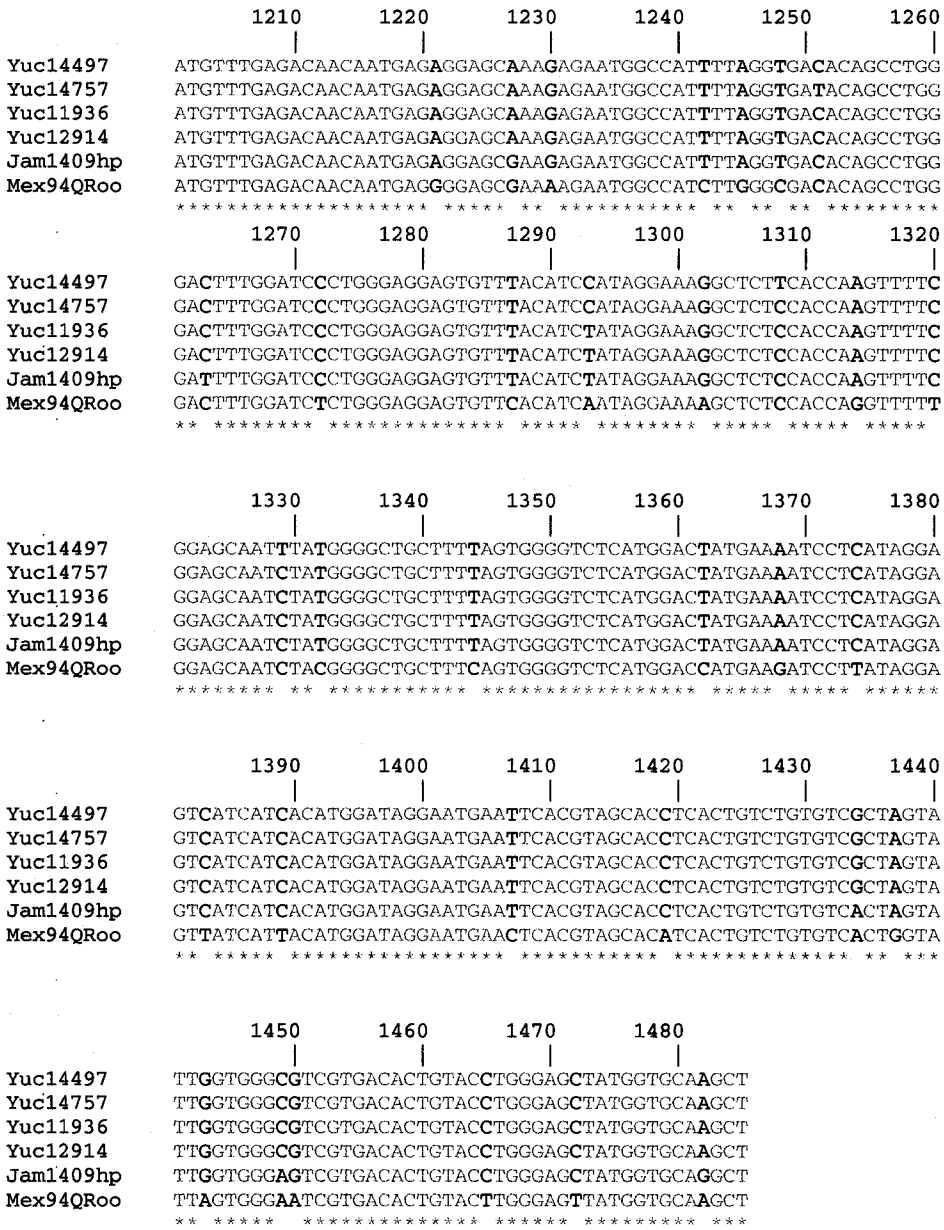

Appendix 7.8. Clustal W for 3'UTR sequence of DENV-2 analyzed

```
Jam1409     GAAGGCA-AAACTAACATGAAACAAGGCCAAAAGTCAGGTCGGATTAAGCCATAGTACGG
Yuc11936    GAAGGCA-AAACTAACATGAAACAAGGCTAAAAGTCAGGTCGGATTAAGCCATAGTACGG
Yuc12914    GAAGGCA-AAACTAACATGAAACAAGGCTAAAAGTCAGGTCGGATTAAGCCATAGTACGG
Yuc14497    GAAGGCA-AAACTAACATGAAACAAGGCTAAAAGTCAGGTCGGATTAAGCCATAGTACGG
Yuc14757    GAAGGCA-AAACTAACATGAAACAAGGCCAAAAGTCAGGTCGGATTAAGCCATAGTACGG
94QRoo      GT-GGTAGAAA--AACATGAGACAGAAC-AAAAGTCAGGTCGGATTAAGCCATAGTACGG

Jam1409     AAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCA-TCAC
Yuc11936    AAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAGGTCAGGCCA-TCAC
Yuc12914    AAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAGGTCAGGCCA-TCAC
Yuc14497    AAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCA-TCAC
Yuc14757    AAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCA-TCAC
94QRoo      GAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCACTT-T
Jam1409     AAAATGCCACAGCTTGAGTAAACTGTGCAGCCTGTAGCTCCACCTGAGGAGGTGTAAAAA
Yuc11936    AAAATGCCACAGCTTGAGTAAACTGTGCAGCCTGTAGCTCCACCTGAGGAGGTGTAAAAA
Yuc12914    AAAATGCCACAGCTTGAGTAAACTGTGCAGCCTGTAGCTCCACCTGAGGAGGTGTAAAAA
Yuc14497    AAAATGCCACAGCTTGAGTAAACTGTGCAGCCTGTAGCTCCACCTGAGGAGGTGTAAAAA
Yuc14757    AAAATGCCACAGCTTGAGTAAACTGTGCAGCCTGTAGCTCCACCTGAGGAGGTGTAAAAA
94QRoo      GA--TGCCATAGCTTGAGCAAACAGTGCAGCCTGTAGCTCCACCTGAGAAGGTGTAAAAA

Jam1409     ACCTGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG
Yuc11936    ACCTGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG
Yuc12914    ACCTGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG
Yuc14497    ACCTGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG
Yuc14757    ACCTGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG
94QRoo      ATCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAG

Jam1409     GAGACCCCTCCCTTACAAATCGCAGCAAACAACGGGGGCCCAAGGTGAGATGAAGCTGTA
Yuc11936    GAGACCCCTCCCTTACAAATCGCAGCAAACAACGGGGGCCCAAGGTGAGATGAAGCTGTA
Yuc12914    GAGACCCCTCCCTTACAAATCGCAGCAAACAACGGGGGCCCAAGGTGAGATGAAGCTGTA
Yuc14497    GAGACCCCTCCCTTACGAATCGCAGCAAACAACGGGGGCCCAAGGTGAGATGAAGCTGTA
Yuc14757    GAGACCCCTCCCTTACAAATCGCAGCAAACAACGGGGGCCCAAGGTGAGATGAAGCTGTA
94QRoo      GAGACCCCTCCCTTTCAGATCGCAGCAA-CAATGGGGGCCCAAGGTGAGATGAAGCTGTA

Jam1409     ATCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAA--CAGCATAT
Yuc11936    ATCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAA--CAGCATAT
Yuc12914    ATCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAA--CAGCATAT
Yuc14497    ATCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAATAAAAAA--CAGCATAT
Yuc14757    ATCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAA--CAGCATAT
94QRoo      GTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAAGACAAAAAAAACAGCATAT

Jam1409     TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACG
Yuc11936    TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACG
Yuc12914    TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACG
Yuc14497    TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACG
Yuc14757    TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACG
94QRoo      TGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGGACG

Jam1409     CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
Yuc11936    CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
Yuc12914    CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
Yuc14497    CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
Yuc14757    CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
94QRoo      CCAGAAAATGGAATGGTGCTGTTGAATCAACAGGTTCT
```

Appendix 7.9. Author's material reproduction permission

De: "Stephen C Harrison" <harrison@crystal.harvard.edu>

Asunto: Re: Permission

Fecha: Fri, 16 Dec 2005 19:13:32 -0500

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

Dear Ms Salazar-Sanchez
I am happy to give permission to use the illustration from Modis et al. (2004) for your dissertation.
Good luck.
Stephen Harrison

De: "John Gamble" <john.gamble@virgin.net>

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

Asunto: Re: Permission

Fecha: Sat, 17 Dec 2005 14:27:01 -0000

Dear Isabel,

Thank you for your email seeking permission to use your Fig 1.1 from my paper in Clinical Science [(2000) 98;211-216], I'm glad that you found the information useful.
You do have my permission to use that figure, of course! However, I had to sign over the copyright for the original material in that paper, to The Biochemical Society. I have little doubt that they will also give their permission.
With regards,
John

Fecha: Sat, 17 Dec 2005 13:16:18 -0500

Asunto: Re: Author's permission

De: "Richard J. Kuhn" <kuhnr@purdue.edu>

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

Dear Isabel,

You have my permission to use the images that you mentioned for your thesis. Best wishes on your thesis defense!

Sincerely,

Richard J. Kuhn

Fecha: Sun, 18 Dec 2005 20:27:31 -0500

Asunto: Re: Author's permission

De: "Scott Michael" <smichael@fgcu.edu>

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>, yhrobows@tulane.edu, rfgarry@tulane.edu

Estimada Dra. Salazar Sanchez,

The figures you are interested in adapting for use in your thesis were originally drawn by Dr. Robert Garry. I'm confident that he will have no problem allowing you to use them, but it would be best for him to make that decision himself. Congratulations on defending you thesis.

Best regards,

Scott

Fecha: Mon, 19 Dec 2005 08:24:30 +0200

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

De: "Philippe Despres" <pdespres@pasteur.fr>

Asunto: Re: Permission

you have my permission for using the Mexican mosquito from Leticia's lab.
Good luck for your dissertation.
Best regards
Philippe

Fecha: Mon, 19 Dec 2005 09:08:29 -0600

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>, yhrobows@tulane.edu, rfgarry@tulane.edu, "Scott Michael" <smichael@fgcu.edu>

De: "Robert Garry" <rfgarry@tulane.edu>

Asunto: Re: Author's permission

Dear Dr. Salazar-Sanchez,

You have my permission to use figures from Hrobowski YM, Garry RF and Michael SF. (2005) in your dissertation.

Sincerely,

Bob G.

Fecha: Mon, 19 Dec 2005 13:24:23 -0600 (CST)

Asunto: Re: Permission

De: lcedillo@cinvestav.mx

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

Dear Isabel:
Not problem. Good luck.
Leticia.>

De: "Dennis Knudson" <dennis.knudson@colostate.edu>

Asunto: Re: Permission

Fecha: Mon, 19 Dec 2005 12:54:43 -0700

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

Thank you for kindly asking. While we have no problems with the image's use, please be advised that the copyright holder is now the Journal in which it was published.

Dennis L. Knudson
Professor of Entomology/Microbiology
Department of Bioagricultural Sciences and Pest Management
College of Agricultural Sciences
Colorado State University
Fort Collins, CO 80523

Fecha: Mon, 19 Dec 2005 14:58:57 -0800

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

De: "Sarjeet Gill" <sarjeet.gill@ucr.edu>

Asunto: Re: Author's permission

Isabel

I am authorizing the use of the Figure (1.13) in your thesis from the Sanders paper.

Wish you the best.

Sarjeet

Fecha: Tue, 20 Dec 2005 10:16:34 -0500

Asunto: Re: Author's permission

De: "Scott Michael" <smichael@fgcu.edu>

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

I believe that Bob already gave his permission in the intervening time. I'll forward you his email in case there was a problem and you did not receive it directly.

Best,

Scott

Fecha: Wed, 21 Dec 2005 10:42:49 -0500

Asunto: Re: Permission

De: "mlorena" <mlorena@jhsph.edu>

Para: "Isabel Salazar-Sanchez" <isalazarsan@yahoo.com>

As far as I am concerned you have my permission for the quote. However, I do not know if there are any copyright issues with the publisher.

Best luck for your future career!

Marcelo