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Original Article

# American and American/Asian genotypes of dengue virus differ in mosquito infection efficiency: candidate molecular determinants of productive vector infection

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## ABSTRACT

**Introduction.** Genotype replacement has been associated with dramatic increases in epidemic dengue and severe dengue cases in Latin America, but little is known concerning the determinants of these genotypic sweeps. More efficient productive infection of *Aedes aegypti* mosquitoes could condition such sweeps. Viruses that more efficiently infect and are transmitted by mosquitoes will have a better chance of being transmitted to the next susceptible host and would thus have greater epidemic potential.

**Objectives.** The objectives were to: determine differences in vector infection potential for DENV-2 isolates representing two DENV-2 genotypes from the Yucatan Peninsula of Mexico and to identify candidate viral molecular determinants of phenotypic differences.

**Materials and Methods.** DENV-2 American and American/Asian isolates from the Yucatan Peninsula were characterized *in vivo* in mosquitoes. Midgut infection and virus dissemination to infect salivary glands for productive mosquito infection was determined by indirect inmunofluorescence assay and mosquitoes were titrated to determine viral load. The E gene and 3´ UTR were sequenced to investigate potential molecular correlates of efficient vector infection.

**Results.** All viruses from both genotypes were equivalent in their ability to infect mosquito midguts. However, the viruses from the America/Asian genotype efficiently disseminated to infect the salivary glands in *Aedes aegypti* mosquitoes but the American genotypes did not. Sequence analysis of the E protein gene and the 3' UTR of the viruses suggested candidate molecular determinants of the vector infection phenotypes.

**Conclusions.** DENV-2 virus genotypes differ in vector competence; American/Asian genotypes efficiently disseminate from the midgut to infect salivary glands to be transmitted. Some of the amino acid changes in E protein and nucleotides in the 3´UTR may be related to this phenotype.

**Key words:** DENV, *Aedes aegypti* infection, salivary glands, E protein gene, 3´UTR

## RESUMEN

**Los genotipos americano y americano/asiático del virus dengue difieren en la eficiencia de infección del mosquito: determinantes moleculares candidatos para infección productiva del vector**

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**Introducción.** La sustitución de genotipos parece jugar un papel crítico en el incremento dramático de las epidemias y la severidad del dengue en Latinoamérica; sin embargo, la información acerca de los determinantes de competencia entre estos genotipos es escasa. La infección productiva en *Aedes aegypti* puede ser uno de los elementos que condicionen dichas sustituciones. Los virus que infectan de forma más eficiente y se diseminan en los mosquitos tienen mejor probabilidad de ser transmitidos al siguiente hospedero susceptible y pueden, así, tener un potencial epidémico mayor.
**Objetivos.** Los objetivos de este trabajo fueron establecer las diferencias en el potencial para infectar al vector de varios aislados virales de la Península de Yucatán, México, así como identificar candidatos moleculares que determinan dichas diferencias fenotípicas.
**Materiales y Métodos.** Diferentes aislados virales de la Península de Yucatán, México, se caracterizaron *in vivo* en mosquitos. La infección de los intestinos medios y la diseminación a las glándulas salivales de los mosquitos se determinó por inmunofluorescencia indirecta y los títulos virales por ensayos en placa. El gen de la proteína E (envoltura) y el 3´UTR (región no traducida 3´) se secuenciaron para identificar sustituciones en las secuencias de nucleótidos o aminoácidos que correlacionan con la infección del vector.
**Resultados.** Ambos genotipos examinados fueron equivalentes en la capacidad para infectar intestinos medios. Sin embargo, ellos difirieron significativamente en su capacidad para diseminarse a las glándulas salivales. Los virus correspondientes al genotipo Americano/Asiático se diseminaron de forma eficientemente en mosquitos *Aedes aegypti* colectados en México. El análisis de las secuencias del gen de la proteína E y del 3´UTR reveló determinantes moleculares que pueden estar relacionados con los fenotipos observados en la infección del vector.
**Conclusiones.** El genotipo del VDEN-2 condiciona la capacidad vectorial e impacta las tasas de diseminación hacia tejidos clave como las glándulas salivales en el mosquito. Algunos de los cambios en aminoácidos de la proteína E y nucleótidos en el 3´UTR pudieran estar relacionados con este fenotipo.

**Palabras clave:** dengue, infección del vector, glándulas salivales, gen de la proteína E, 3´UTR

## INTRODUCTION

DENV infections are a serious cause of morbidity and mortality in tropical and subtropical areas in the world, representing a public health problem in 112 countries (1). All four dengue serotypes generate clinically indistinguishable disease, and all can cause dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In the Americas, the resurgence of the mosquito vector has been followed by the circulation of multiple DENV serotypes and genotypes resulting in dengue hyperendemicity (2). The number of dengue cases in the Americas has increased dramatically in the past 30 years (3). Concomitant with the dramatic increase in dengue cases has been the remarkable increase in the proportion of severe dengue infections (4). Phylogenetic analyses have revealed that American/Asian genotypes have been efficiently displaced the local American genotypes in many countries in the Americans including Mexico (5-9). Five different genotypes of DENV2 have been established: American, Asian (1 and 2), American/Asian, Cosmopolitan and Sylvatic (10). In Mexico, recent phylogenetic analysis of isolates suggests that DENV evolution in Mexico is characterized by frequent lineage and genotype replacement (7,11,12).

The determinants of DENV sweeps in nature are poorly understood. The virus must successfully be transmitted between mosquitoes and humans to maintain and amplify the virus in nature. In humans, DENV viremia titer correlates with disease severity in humans (13,14) and higher viremia titer could clearly result in more productive vector infections, thereby more efficiently amplifying the virus in nature. Some DENV

genotypes (*e.g*. Southeast Asian) exhibit greater replication and dissemination efficiency in mosquitoes (15-17), which could also amplify virus in nature. DENV-2 infection and dissemination rates in *Aedes aegypti* infected with some Southeast Asian viruses were about 3-fold higher than those infected with American genotypes (15,17).

In these studies five DENV-2 strains representing the American/Asian and American genotypes were characterized in their ability to infect productively recently colonized strains of *Ae. aegypti* from Mexico. The viruses, which were isolated from cases of different clinical severity ranging from DF to DSS and were low passage to avoid genetic effects from virus passage, were phenotyped for their ability to infect and replicate in mosquito midguts and to disseminate from midguts and infect the salivary glands. All viruses efficiently infected mosquito midguts, but the American/Asian genotype viruses more efficiently disseminated from the midgut to infect the salivary glands.

## MATERIALS AND METHODS

**DENV-2 strains.** Six different DENV-2 strains were studied. Five of these strains clustered into the American/Asian genotype and one of them into the American genotype (**Table 1**). All the Yucatan (Yuc) strains and the Quintana Roo (QRoo) strains had low passage histories, but the prototype strain DENV-2 Jam 1409, included as a control virus, was a high passage (Jam1409hp). The DENV-2 Yuc viruses belonging to the American/Asian genotype were isolated from patients in Mexico between 2001 and 2002 from anonymous patients with different disease severity. The DENV-2 QRoo3315 American genotype strain was isolated from a patient from Quintana Roo in 1994. All the viruses from the Yucatan Peninsula were kindly provided by investigators at the Laboratorio de Arbovirologia in the Centro de Investigaciones Regionales "Dr. Hideyo Noguchi" in the Universidad Autónoma de Yucatán (UADY). Additionally some other DENV-2 sequences from the GeneBank were used in some of the analysis (**Table 1**). The initial classification of virus genotypes was based on genetic relatedness determined by the nucleotides located at the E/NS1 junction (19) or the prM/E junction (20).

**Mosquito infection and assay.** Recently colonized Chetumal mosquitoes (F-3 to F-6) were used to phenotype the viruses. The Chetumal colony was established from collections made in Chetumal, Yucatan Peninsula, in Southeast Mexico, where the mosquitoes are extremely vector competent[21]. Mosquitoes were raised, orally challenged, and assayed as previously reported (21). Briefly, to prepare blood meals, the respective virus stock (Multiplicity of infection-0.001) was amplified in C6/36 mosquito cells for 7 days at 28°C. At day 7, the medium was replaced, and at 12 days post-infection virus was harvested for titration and to prepare artificial bloodmeals for mosquito oral infections. Similar titers (~1 x $10^7$ PFU/ml) of the DENV-2 strains were provided to 50-100 *Ae. aegypti* females in artificial blood meals (22). Aliquots of bloodmeals were collected before and after the feeding for titration by plaque assay. Engorged mosquitoes were sorted, placed in cartons, and maintained on sugar and water until processed (22). At 14 days, post blood meal, mosquitoes were dissected to analyze midguts, salivary glands, and head tissues for the presence of DENV antigen using indirect immunofluorescence (22). Blood meals were titrated in LLC-MK2 cells by plaque assay (22).

**Viral RNA extraction and cDNA synthesis.** Viral RNA was isolated from stocks using the QIAamp viral RNA minikit from Qiagen following manufacturer's instructions. Viral RNA was and then stored at -70ºC until cDNA synthesis. For the first strand of cDNA synthesis we used SuperScript™ III retrotranscriptase (RT) from Invitrogen (Carlsbad, CA) and primers D2-2479R (5'A$^{A}_{G}$ATCCCGCTGCCACATTTT3') to obtain the cDNA fragment to sequence E gene and D2-10723R

**Table 1**

| Virus name | City/ Province | Year of isolation | Clinical case | Genotype | Reference | GeneBank accesion number |
|---|---|---|---|---|---|---|
| 044 | México | 1983 | DF | American | 5 | AF100151[&] |
| SML6420 | Texas, USA | 1995 | DF | | 18 | AF309965[*] |
| IQT1950 | Perú | 1995 | DF | | 5 | AF100467[*] |
| Mex94QRoo (QRoo3315) | Cancún, México | 1994 | DF | | This study | &,* |
| Yuc12914 | Tekax, México | 2001 | DF | American/ Asian | 7 This study | &,* |
| Yuc11936 | Sta. Elena, México | 2001 | DFHM | | 7 This study | &,* |
| Yuc14757 | Mérida, México | 2002 | DHFII | | This study | &,* |
| Yuc14497 | Mérida, México | 2002 | DHFIII | | This study | &,* |
| 383 | México | 1995 | | | 5 | AF100147[&] |

(5'AGAACCTGTTGATTCAACAGCACCA3') to obtain the cDNA to sequence 3´UTR following manufacturer's instructions.

**Sequence analysis. E gene**. The entire region for the E protein genes of the Yucatan DENV-2 viruses and the reference DENV-2 Jam1409 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). Sequence of used primers were:
**1)** D2-1146F:
5'ATGAAGAGCAGGACAAAAGGTT3';
**2)** D2-1233F:
5'AGGATGGGGAAATGGATGTGGAT3';
**3)** D2-2018F:
5'AAGATAGCCCAGTCAACATAGAAG3';
**4)** D2-1245R:
5'ATTTCCCCATCCTCTGTCTACCAT3';
**5)** 5'TTCTTCGCGTGGGGATTTTTG3';
**6)** D2-2076R:
5'TTCA$^{A}/_{G}$TTGTCCCGGCTCTACTACTCT3';
**7)** D2-2461R:
5'A$^{A}/_{G}$ATCCCGCTGCCACATTT3'

The sequences were aligned using the ClustalW program and amino acid changes found were place on the E protein model (23) using the DENV-2 QRoo3315 sequence as backbone.

**Sequence analysis. 3' UTR**. Sequences were assembled using SeqMan program from Lasergene (DNASTAR Inc.). Assembly of the entire 3'UTR sequence (454 nt) required to use 4 primers:
**1)** D2-10146F:
5'GGCAAAGAACATCCAAACAGC3';
**2)** D2-10304F:
5'GTCAGGTCGGATTAAGCCATAGTA3';
**3)** D2-10522R:
5'CCCGTTGTTGCTGCGATTTGTA3';
**4)** D2-10723R:
5'AGAACCTGTTGATTCAACAGCACCATTCCA3'

The sequences were obtained in the Genomics Core Facility at Colorado State University. The sequences were aligned using the ClustalW program.

## RESULTS

**Infection of *Ae. aegypti* (Chetumal strain) mosquitoes with American and American/Asian genotype viruses.** To investigate the influence of virus genotype on vector infection, the midgut infection and dissemination rates caused by the American and American/Asian genotype viruses. Mosquitoes were then allowed to ingest artificial blood meals of equivalent virus titers (**Figure 1**) and the ability of the respective viruses to productively infect mosquitoes from Chetumal, Yucatan Peninsula, was determined. Infections of midguts and dissemination to salivary glands and head tissues were used as the measure of productive infection ability of the viruses. Midgut infections were determined at 7 days post ingestion of the infectious bloodmeal. Salivary glands and head tissue infection rates were assessed 14 days after the bloodmeal.

Despite equivalent midgut infection rates, significant differences ($P<0.01$ by ANOVA one way comparison test) in dissemination rates to the salivary glands and head tissues were revealed. DENV-2 QRoo3315 virus (American genotype) exhibited equivalent midgut infection rates (**Figure 1a**). However, dissemination rates to either salivary glands or head tissues were dramatically low. The DENV-2 Yucatan viruses (American/Asian genotype) exhibited dissemination rates to head tissues of 50-80% and to salivary glands of 60-80%. Meanwhile the American genotype (QRoo3315) showed only ~3% of dissemination to head tissues and ~19% of dissemination to salivary glands (**Figure 1b and 1c**).

**Differences found in E protein gene among the different Yucatan isolates.** The analysis of the sequencing data showed that a total of 148 nucleotide substitutions occurred in the different E genes, however most of these changes originated sysnonymous substitutions (data not shown). Both conservative and non conservative amino acid substitutions in the sequence were located by multiple alignment analysis using Clustal W program (**Figure 2**). Fourteen amino acid changes were identified in total in the E protein (**Table 2**). Of the fourteen changes observed, 5 occurred in domain I (E-6, E-46, E-139, E-162, and E-340); 6 in domain II (E-55, E-71, E-81, E-91, E-129, and E-131); and 3 in domain III (E-390, E-484, and E-491) (**Table 2**). The substitution in E-6 was only observed in the DENV-2 Jam 1406 strain. Three of the observed changes have been previously reported: E-6, E-71, and E-390. Only four non conservative changes among the studied strains (E-46, 131, 340 and 390) were found (**Figure 3**).

**Genotype changes in the 3' UTR among the Yucatan isolates and other sequences reported.** Comparison of the 3' UTR nucleotide sequence of DENV-2 strains revealed multiple and potentially important differences between the examined American and American/Asian genotypes (**Figure 4**). The 3' UTR (454 nt) of the DENV-2 (from nt 10270 to 10723 in viral genome) encompasses three main regions[29]: i) a variable region (VR) adjacent to the stop codon of the viral polyprotein, ii) a core region containing two predicted secondary structures, the DB1 (containing CS2) and DB2 (containing RCS2), and iii) a 3'- terminal region enclosing the cyclization sequence (CS1) and the terminal long stable stem-loop (the 3'SL formed by SLA and SLB). The CS1 sequence at the 3'UTR is complimentary to the cCS1 motif located in the 5'UTR. Viral genome circularization allows RNA synthesis and alterations of either CS1 or cCS1 severely diminished viral genome replication[30,31]. The 3'SL binds both virus-encoded and cellular proteins and is required for virus replication and translation.

Numerous changes including point mutations and deletions differentiated viruses from the two genotypes (**Figure 4a**). Four point mutations were located in the DB1 (CS2) and two in the SLB region of the 3'SL. Other affected secondary structures included HP-2 (**Figure 4b**). We observed several deletions in the regions linking DB2/DB1 and DB1/CS1, in addition to those found at the beginning

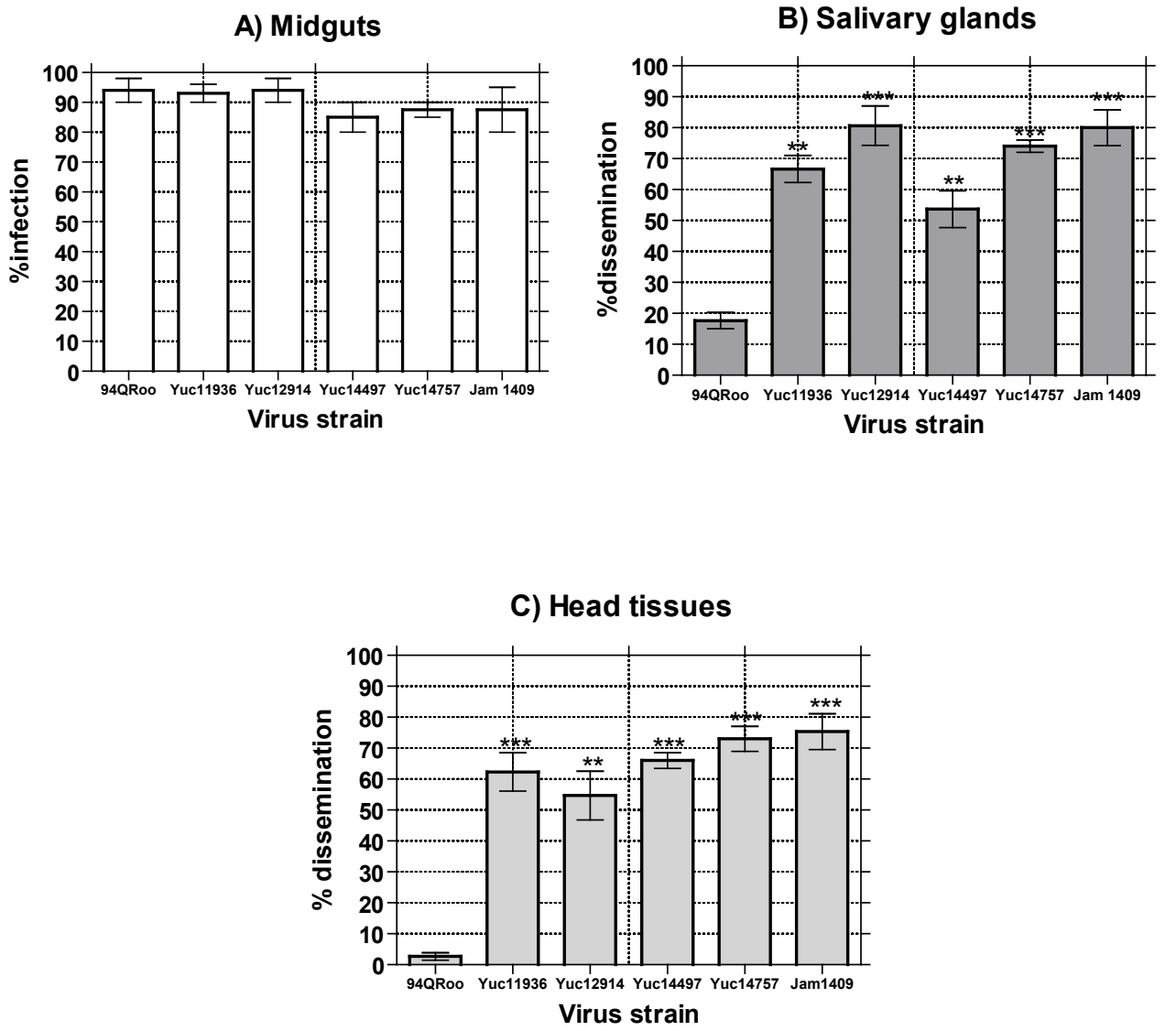



**Figure 1. DENV-2 American and American/Asian genotypes differed in their dissemination but not in their midgut infection rates in Aedes aegypti Chetumal mosquitoes. DENV-2 antigen was detected in Chetumal mosquito tissues by indirect immunofluorescence assays using the serotype-specific 3H5-1 (ATCC number HB-46) as primary antibody. (A) Midgut infection and dissemination rates to salivary glands (B) and head tissues (C) were determined in three independent experiments. Bars represent mean ± SEM from two independent experiments (n=30-40 mosquitoes for each experiment). ** P<0.01 or *** P<0.001 by ANOVA one-way test. Virus titers in bloodmeals were: 2.0±1.0x107 PFU/ml for QRoo3315; 1.0±0.2x107 PFU/ml for Yuc11936; 2.4±1.4x107 PFU/ml for Yuc12914; and 1.6±0.8x107 PFU/ml for Yuc14497, 1.7±0.9x107 PFU/ml for Yuc14757**

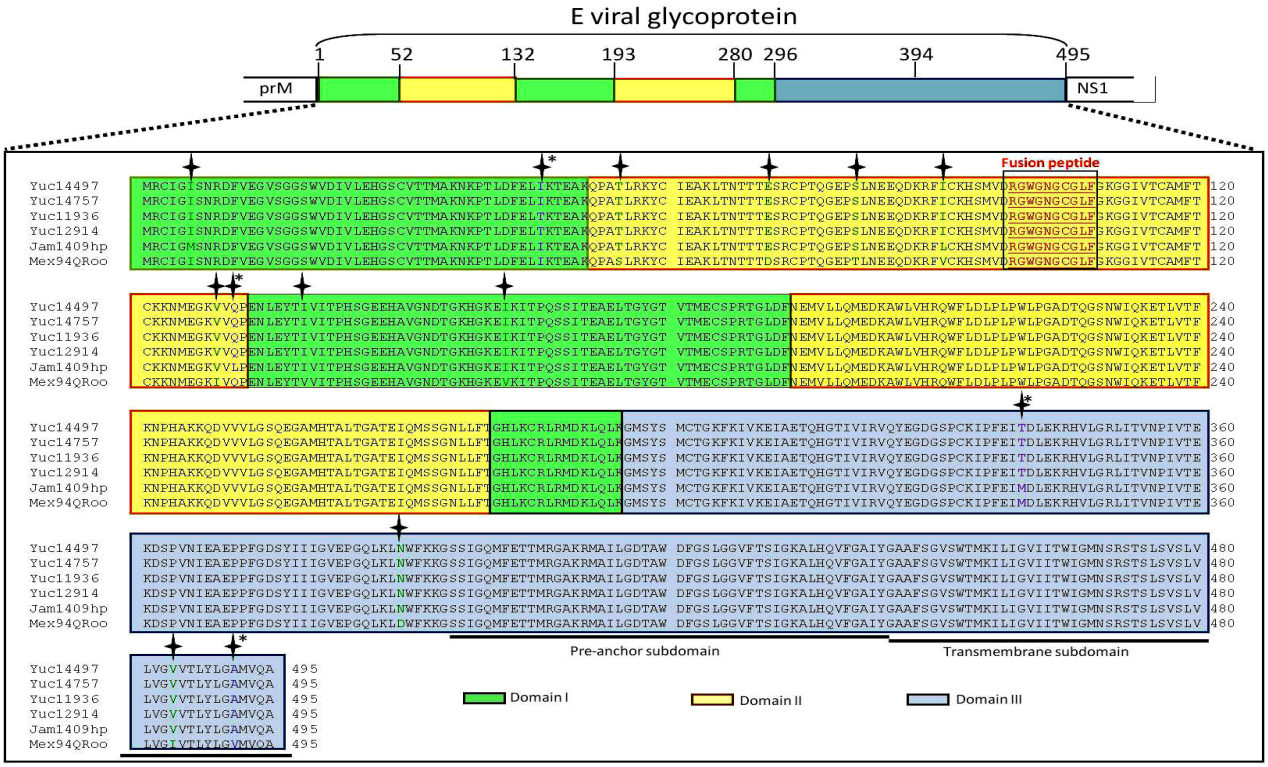



**Figure 2. Alignment of the different sequences obtained for the E proteins. Sequence alignment of the E proteins from all the DENV-2 Yucatan strains analyzed as well as the DENV-2 Jam 1409. The domains of this protein are indicated as well. *Non-conservative amino acid substitutions**

**Table 2**
**Amino acid changes detected in American and American/Asian genotypes examined. *Non-conservative changes. In different color changes between American and American/Asian isolates from the Yucatan Peninsula are shown**

| | American Genotype | American/Asian genotype | | | |
|---|---|---|---|---|---|
| **AA changes** | QRoo3315 | Yuc11936, Yuc12914 Yuc14497, Yuc14757 | Jam1409 | Domain | Previous reference |
| **E-6** | I | I | M | I | 24,25 |
| **E-46*** | I | I/T | I | I | |
| **E-55** | S | T | T | II | |
| **E-71** | D | E | E | II | 26,27 |
| **E-81** | T | S | S | II | |
| **E-91** | V | I | L | II | |
| **E-129** | I | V | V | II | |
| **E-131*** | Q | Q | L | II | |
| **E-139** | V | I | I | I | |
| **E-162** | V | I | I | I | |
| **E-340*** | M | T | M | I | |
| **E-390*** | D | N | N | III | 28 |
| **E-484** | I | V | V | III | |
| **E-491** | V | A | A | III | |

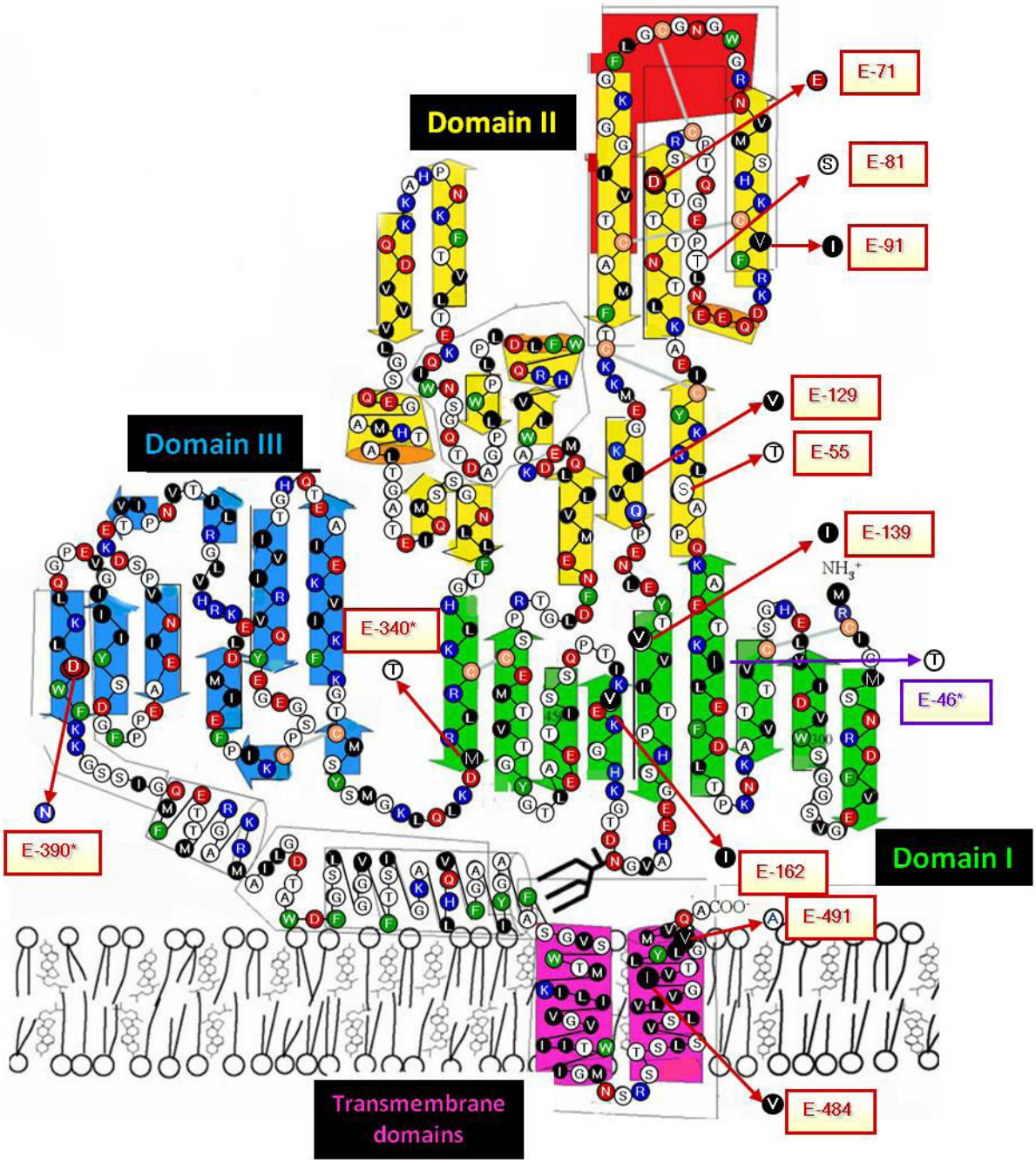
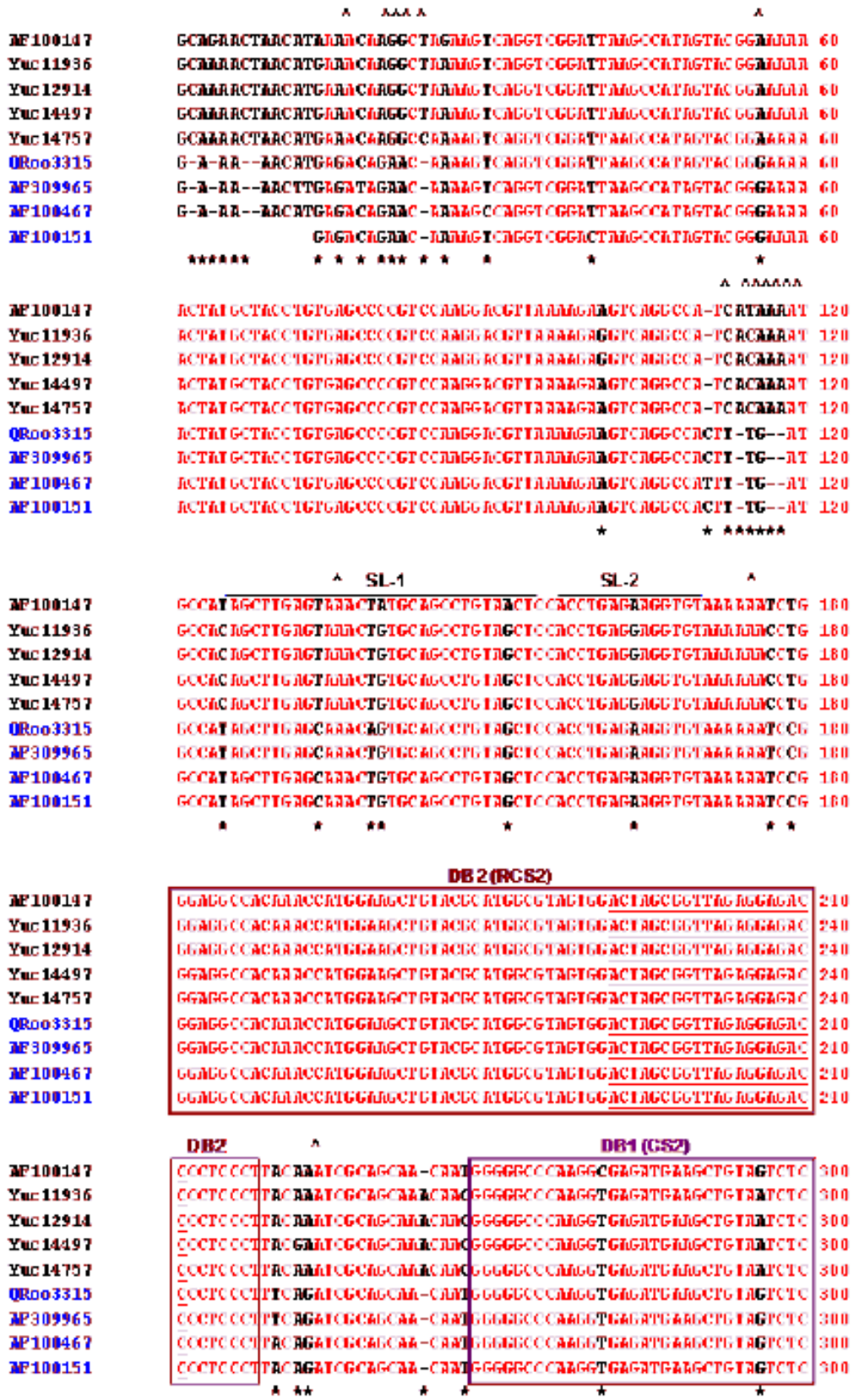



**Figure 3. Affected domains and each of the amino acid changes observed in the analyzed isolates. The backbone corresponds to the DENV-2 QRoo3315 American genotype. All the amino acid substitutions found between the DENV-2 QRoo3315 (American genotype) and Yucatan strains (American/Asian genotype) are shown. *Non-conservative changes (backbone structure adapted from Hrobowski *et al*., 2005)**

A) Figure 4



**A) Figure 4 continued**

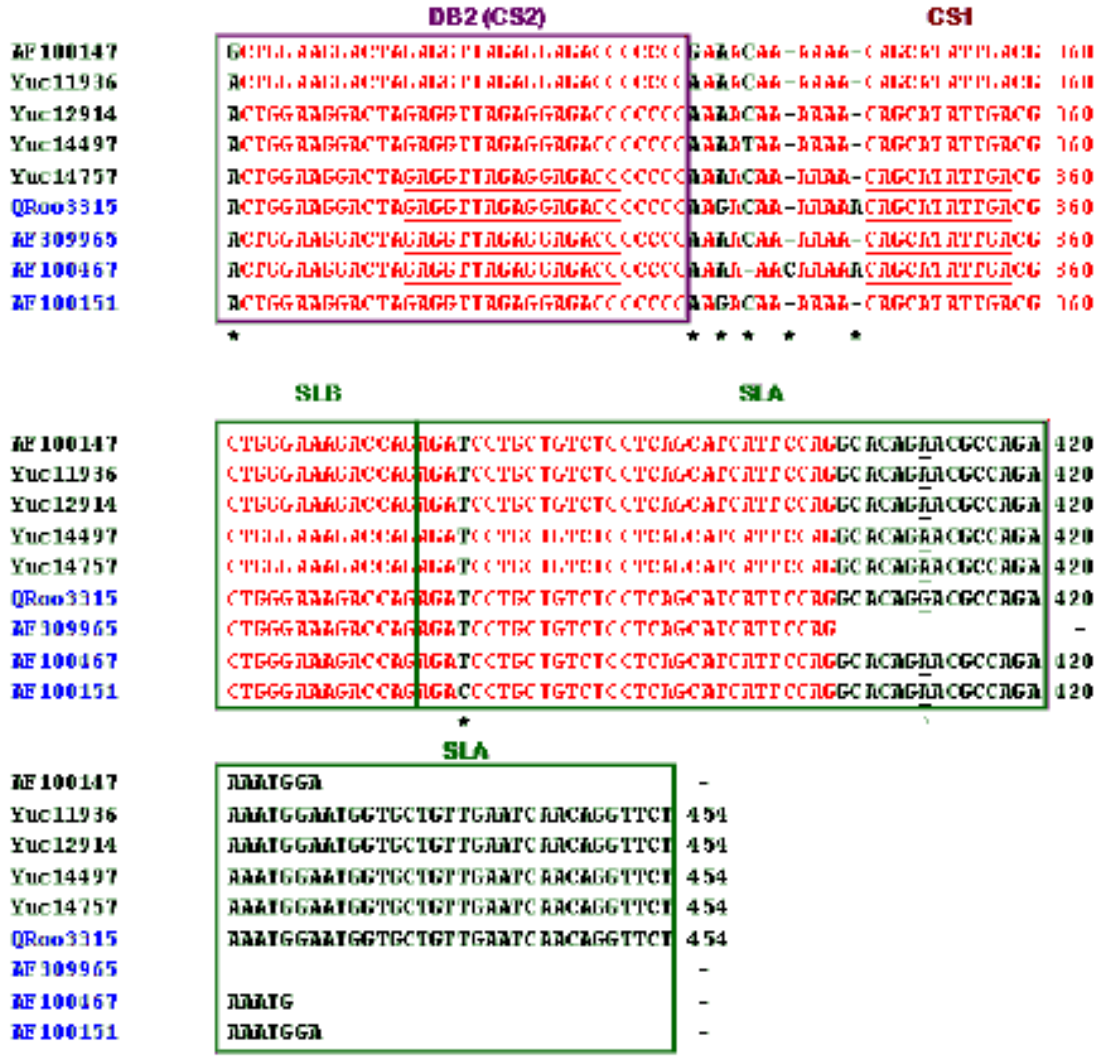
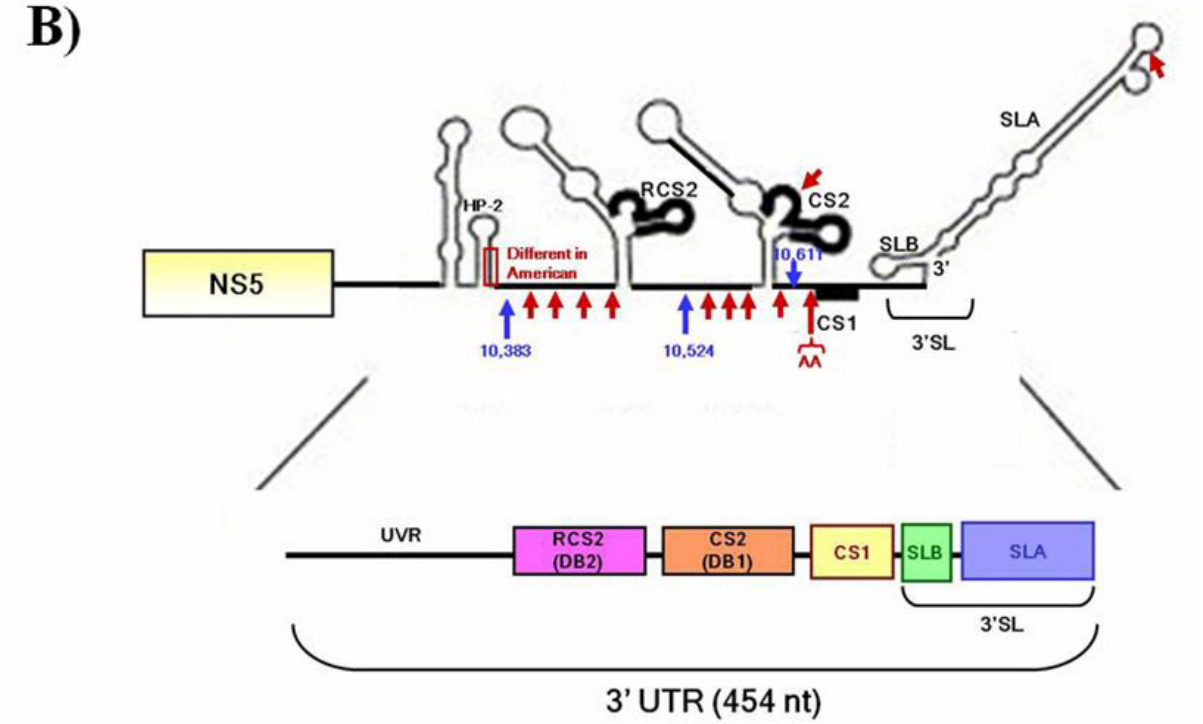



**Figure 4. The 3'UTR of examined isolates of DENV-2 from the Yucatan Peninsula differ notably. A) Alignment of the different examined viruses belonging to either the American or American/Asian genotypes. Functional domains are shown in boxes or underlined. (*) Indicates the position of each difference found in the comparison. (^) Denotes the sites where nucleotide changes seem to be genotype specific. The sequence comparison was performed using the Clustal W alignment algorithm. Unique mutations can be observed in the QRoo3315 virus that belongs to the American genotype. B) The secondary structure of the 3'UTR and the different functional domains are shown 30,47. Arrows point out the sites where the principal secondary structures were affected by mutations. The 3´UTR consists of: 1) a variable region (VR) adjacent to the stop codon of the viral polyprotein that encloses 2 hairpin structures (HP), 2) a core region containing two predicted secondary structures, the DB1 (containing CS2) and DB2 (containing RCS2), and 3) a 3'- terminal region enclosing the CS1and the 3'SL (formed by SLA and SLB)**

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of the 3'UTR. We identified three regions that were more variable, these enclosed nucleotides 1 to 26 (region 1), 111 to 118 (region 2), and 337 to 346 (region 3). Region 1 was located right after the NS5 stop codon and region 3´ upstream of the cyclization domain. Some nucleotides were related to a specific genotype (^ in **Figure 4a**).

## DISCUSSION

Multiple studies have tried to determine the relationships among DENV genotype, disease severity and epidemic potential. DENV genetic variation alone does not completely explain the incidence of severe disease or the magnitude of outbreaks, but the evidence is compelling for differences in virulence and epidemic potential among DENV lineages (32). Virus epidemic potential could be conditioned by many factors, including replication efficiency and vector infection and transmission potential. Dengue viremia titers are positively correlated with disease severity, and virus titer with vector infection (33-35).

Our results showed that American/Asian genotype viruses much more efficiently infected mosquitoes than the American genotype virus. Interestingly, all the viruses efficiently infected (**Figura 1a**) and replicated to similar titers in the midguts of *Ae. aegypti* Chetumal mosquitoes. However, the QRoo3315 DENV-2 did not efficiently disseminate from the midgut to infect secondary target organs including the salivary glands even in this recently colonized mosquito strain

(Figure 1b). Unfortunately, in these analyses we only characterized one American genotype virus. Nonetheless our results add to and confirm he findings for other American genotype dengue viruses using laboratory reared mosquitoes (15).

Productive infection of the vector is the major determinant of transmission potential. Virus genotypes able to infect more efficiently mosquitoes will have a better chance of being transmitted and to have a greater epidemic potential. We previously demonstrated that *Ae. aegypti* from the Yucatan Peninsula were genetically homogeneous and extremely vector competent for DENV-2 Jam 1409 (21,36). In this regard, we also determined the ability of the Yuc11936, 12914, 14497, and 14757 (American/Asian) viruses to infect mosquitoes from a colony established from collections in Baja California. This *Ae. aegypti* Loreto strain showed to be much less vector competent than the Chetumal strain (37). All four of the Yuc viruses corresponding to the American/Asian genotype very efficiently infected, replicated to similar titers and disseminated as or more efficiently in the *Ae. aegypti* Loreto as in the Chetumal mosquitoes (data not shown).

Partial sequence analyses of the viruses were conducted to identify candidate molecular determinants of the productive infection of vectors. The rational to study the E protein was supported by the evidence that specific virulence phenotypes have been associated to amino acid changes in this protein (10,24-28). We found twelve amino acid changes between the QRoo3315 (American) and the other viruses corresponding to the American/Asian genotype.

Two of the observed changes had been previously reported, these were: E-71 that relates to escape neutralization mutants; and E-390, which is located in the motif that may govern binding to host cell and associates also with genotype identity (**Table 2, Figure 2**). In the E protein, domain I contains the molecular hinge region involved in the conformational changes that take place at low pH in the endosome. Domain II contains the fusion peptide and is involved in the dimerization process. Finally, domain III is involved in virus attachment to its receptor and contains the carboxi-terminal Ig-like section. There are two glycosylated asparagines (Asn) on each E subunit: Asn-153 on domain I and Asn-67 on domain II. 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 dimmers (38).

Genotype identity seems to reside in E-390 (Asn-Asp), thus this is critical in the transition from the Asian to American genotype. We consistently found that this change differentiated American from the American/Asian genotypes in our studied viruses. A very important amino acid change observed between the American (QRoo3315) and the American/Asian viruses was E-71 (Glu-Asp), which not only relates to neutralization escape mutants but also to neurovirulence. Thus, the Yuc American/Asian genotype viruses have at least this signature for a more virulent virus strain.

Five out of the twelve changes amino acid changes found occurred in domain II, this domain has been recently associated with antibody binding by crystal structure analysis of the molecular complexes (F. Rey, pers. commun.). Thus domain II is strongly related to the emergence of neutralization escape mutations. Some of the changes in domain I and II of the E protein could alter fusion efficiency and affect virulence as well. However, the effect on functionality of the several of the other identified changes (mostly the non-conservative) remains to be established.

Virus replication efficiency could condition the vectorial capacity and transmission potential by *Ae. aegypti*. Flavivirus genome translation and replication requires genome circularization with the intervention of the 5' and 3'UTR sequence and structural elements, the non structural viral proteins (mostly NS5), and of some host cell protein (39-42). The 3'UTR contains motifs that are critical for translation and RNA synthesis

(30,43,44). The secondary structure in the 3'UTR is a central determinant of virus replication efficiency and may also influence cell tropism, vector/host specificity, pathogenesis, and virulence (29,45-47).

Some of the differences between American and American/Asian genotype viruses in the 3'UTR might be responsible of the different ability that dengue virus genotypes exhibit to replicate and disseminate in mosquito vector. So far, a more extended analysis using 3´UTR sequences reported to the GeneBank, showed coincidences in several of the found mutations supporting that this region may be an important determinant of the phenotypic differences between these two genotypes (**Figure 4**). However, further studies will be necessary to establish the actual role of each affected region in the 3´UTR.

The differences between the American and American/Asian genotype viruses in the E gene and the 3'UTR are provocative, but further research will be necessary to determine if any of the differences itself condition efficient vector infection.

In addition, with the current availability of large scale sequence analysis, entire genomes can be analyzed, which will undoubtedly reveal additional candidates for mosquito infectivity (48,49). Infectious clone technology for flaviviruses (6) now permits unprecedented power to identify the specific mutations in the virus genomes that determine efficient productive vector infection by American/Asian genotype viruses and that condition DENV genotypic sweeps through tropical regions of the world.

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