

EFFICIENCY OF DENGUE SEROTYPE 2 VIRUS STRAINS TO INFECT AND DISSEMINATE IN *AEDES AEGYPTI*

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Abstract. Dengue serotype 2 (DEN-2) viruses with the potential to cause dengue hemorrhagic fever have been shown to belong to the Southeast (SE) Asian genotype. These viruses appear to be rapidly displacing the American genotype of DEN-2 in the Western Hemisphere. To determine whether distinct genotypes of DEN-2 virus are better adapted to mosquito transmission, we classified 15 viral strains of DEN-2 phylogenetically and compared their ability to infect and disseminate in different populations of *Aedes aegypti* mosquitoes. Envelope gene nucleotide sequence analysis confirmed that six strains belonged to the American genotype and nine strains were of the SE Asian genotype. The overall rate of disseminated infection in mosquitoes from Texas was 27% for the SE Asian genotype versus 9% for the American genotype. This pattern of infection was similar in another population of mosquitoes sampled from southern Mexico (30% versus 13%). Together, these findings suggest that *Ae. aegypti* tends to be more susceptible to infection by DEN-2 viruses of the SE Asian genotype than to those of the American genotype, and this may have epidemiologic implications.

INTRODUCTION

Dengue virus belongs to the family Flaviviridae and comprises four antigenically distinct groups, designated as serotypes 1–4. Human infections are acquired by the bite of the mosquito, usually by *Aedes aegypti*, and often present as a self-limited febrile illness, dengue fever. Some of these infections may progress to a more severe condition, dengue hemorrhagic fever (DHF), characterized by thrombocytopenia and plasma leakage. All four dengue virus serotypes have the potential to cause DHF. However, studies in Southeast (SE) Asia suggest that secondary infection with dengue serotype 2 (DEN-2) virus is more likely to cause severe disease than do other serotypes.^{1–3}

Strains of DEN-2 virus may be classified into distinct phylogenetic clusters or genotypes, each with different epidemiologic associations. The sylvatic genotype comprises strains from West Africa and is maintained in a cycle involving non-human primates and forest-dwelling mosquitoes.^{4,5} This genotype appears to have evolved independently of viral strains involved in outbreaks of human disease.^{6,7} Epidemic strains of DEN-2 virus may be grouped into three distinct genotypes, previously defined as the American, SE Asian, and Indian Subcontinent genotypes.^{6,8,9} The American genotype of DEN-2 virus has been detected in the Western Hemisphere since the 1950s and in the South Pacific during the 1970s, and has been isolated from patients presenting solely with dengue fever.^{6,10–12} Dengue viruses of SE Asian genotype, in contrast, have the potential to cause DHF and were first detected in the Americas in the 1980s.^{6,10} The SE Asian genotype appears to have displaced the American genotype throughout much of the Western Hemisphere, suggesting that dengue virus genotypes may differ in transmission potential.¹⁰

Dengue virus strains may differ in transmission potential by several possible mechanisms. Some strains may infect and replicate more efficiently in target cells, thereby sustaining higher viremias in the human host and infecting more mosquitoes.^{3,13,14} Another possibility is that some viral strains may infect and be transmitted by vector mosquitoes more efficiently than other strains. It has been demonstrated that dengue virus serotypes and strains within a serotype may vary in their ability to infect and disseminate in mosquitoes.^{15,16} An analysis of six viruses indicated that DEN-2 virus strains

of the SE Asian genotype tended to infect *Ae. aegypti* more efficiently than members of the American genotype.¹⁷ However, the efficiency of vector infection also varied among viral strains within a genotype, indicating the need to test more viruses to ascertain trends in this transmission phenotype.

Despite an increasingly detailed knowledge of the phylogenetic relationships of dengue viruses, little is known about the biology or phenotype of such strains. In this report, we explore the relationship between viral genotype and phenotype by comparing the transmission potential of DEN-2 viruses representing distinct genotypes. Accordingly, we analyzed the phylogenetic relationships of 15 strains of DEN-2 virus and then compared their ability to infect and disseminate in different populations of *Ae. aegypti* mosquitoes.

MATERIALS AND METHODS

Mosquito colonies. Mosquito eggs were collected from McAllen, Texas during the fall of 2001 and from Tehuantepec, Oaxaca, Mexico during the summer of 2002 to generate colonies of *Ae. aegypti*. The F₁ (Tehuantepec) and F₂ (McAllen) generation females were used in this study. Mosquitoes were maintained in an insectary at 28°C, a relative humidity of 70–80%, and a 12:12 hour light-dark cycle. Larvae were reared in pans of water at a density of 200–400 larvae per liter and fed a mixture of ground rabbit chow: liver powder: yeast (4:2:1). Pupae were transferred to screened cages and emergent adults were maintained on a diet of 4% sugar water. Colonies were cycled every 3–5 months by providing female mosquitoes meals of human blood. Eggs were collected and kept moist for at least 24 hours and then air-dried for storage.

Virus strains. Fifteen low-passage isolates of DEN-2 virus were used in this study (Table 1). Virus stocks were prepared as follows: monolayers of C6/36 cells were grown to 90% confluency in 75-cm² flasks, then inoculated with dengue virus at a multiplicity of infection of approximately 10 genome equivalents/cell, and incubated for 1 hour at 28°C in an atmosphere of 5% CO₂. Flasks were supplemented with 15 mL of maintenance medium (minimal essential medium, 2% fetal bovine serum [FBS], 1× non-essential amino acids, 100 U/mL of penicillin, and 100 µg/mL of streptomycin) and maintained at 28°C in an atmosphere of 5% CO₂. Infection was moni-

TABLE 1
Geographic origin, year of isolation, and passage history of dengue virus strains used in this study

Strain	Passage history	Location	Year	Genbank accession no.
780447	C6/36 3	Trinidad	1978	AY158327
Ven2	AP61 2, C6/36 3	Maracay, Venezuela	1987	AY158328
Mara3	C6/36 3	Maracay, Venezuela	1990	AY158329
102954	C6/36 3	Aragua, Venezuela	1991	AY158330
360236	C6/36 2	Bucaramanga, Colombia	1992	AY158331
131	C6/36 3	Sonora, Mexico	1992	AY158332
132	C6/36 2	Sonora, Mexico	1992	AY158333
CO385	C6/36 2	Bangkok, Thailand	1994	AY158334
CO576	C6/36 2	Bangkok, Thailand	1994	AY158335
K0005	C6/36 2	Kamphaeng Phet, Thailand	1994	AY158336
K0049	C6/36 2	Kamphaeng Phet, Thailand	1995	AY158337
328298	C6/36 3	Reynosa, Mexico	1995	AY158338
Iqt2913	C6/36 3	Iquitos, Peru	1996	AY158339
124B	C6/36 2	Santa Cruz, Bolivia	1998	AY158340
Oax468	C6/36 2	Juchitan, Mexico	2000	AY158341

tored daily by an indirect fluorescent antibody test (IFAT) and cell supernatants were harvested when more than 90% of the cells expressed dengue viral antigen, usually seven or eight days post-infection. Virus stocks were stored as individual 1-mL aliquots in 20% FBS at -70°C .

Indirect fluorescent antibody test. Immunofluorescence was used to test for dengue viral infection in both C6/36 cells and in mosquito head tissues.^{18,19} The C6/36 cells were prepared for IFAT by spotting 10 μL of cell suspension onto a multi-well slide and incubating slides in a humid chamber for 20 minutes at 37°C . Mosquitoes were prepared by removing heads onto individual wells of a multi-well slide and then squashing the heads with a cover slip. Slides with C6/36 cells or head tissue were acetone-fixed, air-dried, and incubated for 30 minutes at 37°C with anti-DEN-2 mouse ascites fluid diluted 1:200 in phosphate-buffered saline (PBS) with 5% FBS. Slides were washed twice in PBS and then overlaid with fluorescein isothiocyanate-labeled, goat anti-mouse IgG (Sigma, St. Louis, MO) diluted in 1:200 in PBS. Slides were incubated for 30 minutes at 37°C , washed twice in PBS, and mounted with glycerol containing 20% PBS. Slides were examined at $200\times$ using a Nikon (Melville, NY) E400 microscope with an epi-fluorescence attachment.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The concentration of viral RNA was estimated by a real time RT-PCR as previously described; this method was chosen to standardize virus quantities in mosquito blood meals.¹⁷ The ratio of plaque-forming units (assayed in LLC-MK₂ cells) to genome equivalents is approximately 1:1,500 for low-passage strains of dengue virus; however, this ratio varies widely among isolates.¹⁷ This is not surprising since plaque formation is a laboratory phenotype that does not necessarily correlate with the number of infectious particles.²⁰ In contrast, the number of genome equivalents (estimated by a real time RT-PCR) was shown to correlate strongly with titers of infectious virus by mosquito inoculation.²¹ Based on these considerations, we standardized infectious blood meals on the basis of genome equivalents, which correlated with number of mosquito infectious doses, a ratio of approximately 10:1 (genome equivalents: 50% mosquito infectious doses).

RNA was extracted from viral stocks in duplicate using the Viral RNA kit (Qiagen, Valencia, CA). The RNA was added

to a 50- μL RT-PCR using the TaqMan Gold RT-PCR kit (PE Applied Biosystems, Foster City, CA). Each reaction contained the following: $1\times$ TaqMan buffer, 5.5 mM MgCl_2 , 300 μM ATP, 300 μM CTP, 300 μM GTP, 300 μM UTP, 100 nM PA-1 (5'-CAGATCTCTGATGAATAACCAACG-3'), 100 nM PA-4 (5'-CATTCCAAGTGAGAATCTCTTTGTCA-3'), 100 nM PRB (TET-5'-ATGCTGAAACGCGAG-AGAAACCGC-3'-TAMARA), 1.25 units of Amplitaq Gold DNA polymerase, 12.5 units of Multiscribe reverse transcriptase, and 20 units of RNase inhibitor. Amplification was performed using a ABI Prism 770 Sequence Detection Instrument (PE Applied Biosystems) as follows: one cycle at 50°C for two minutes, one cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 64°C for one minute. The RNA copy number was estimated from a standard curve generated by *in vitro*-transcribed RNA standards.

Vector competence. Mosquitoes were exposed to dengue virus by oral feeding as described elsewhere with minor modifications.¹⁷ *Aedes aegypti* females, 7–10 days old, were transferred to small holding cages and deprived access to sugar water for 20–30 hours. Each dengue virus strain was diluted to a final concentration of 2.5×10^8 viral RNA copies/mL in defibrinated rabbit blood and offered to mosquitoes by means of a water-jacketed membrane feeder. Mosquitoes were exposed to membrane feeders for 30 minutes and fully engorged mosquitoes were then selected and maintained at 30°C for 14 days on a diet of 4% sugar water. For each feeding trial, the entire panel of 15 virus strains was tested within a 12-hour time frame. Mosquitoes exposed to rabbit blood without virus served as a negative control.

Nucleotide sequencing and phylogenetic analysis. The envelope gene nucleotide sequences were determined for the 15 dengue virus strains listed in Table 1. RNA was extracted from the same virus stocks used in the vector competence studies. The RNA was amplified in a 50- μL RT-PCR using the Titan System (Roche Diagnostics, Mannheim, Germany). Each reaction contained a final concentration of the following reagents: $1\times$ RT-PCR buffer, 5 mM DTT, 200 μM ATP, 200 μM CTP, 200 μM GTP, 200 μM TTP, 500 nM D2/618V (5'-ACCAGAAGACATAGATTGTTGGTGC-3'), 500 nM D2/2578 (5'-TTACTGAGCGGATTCCACAGATGCC-3'), 10 units of RNase inhibitor, and $1\times$ enzyme mixture. Amplification was performed as follows to generate a 1,984-basepair

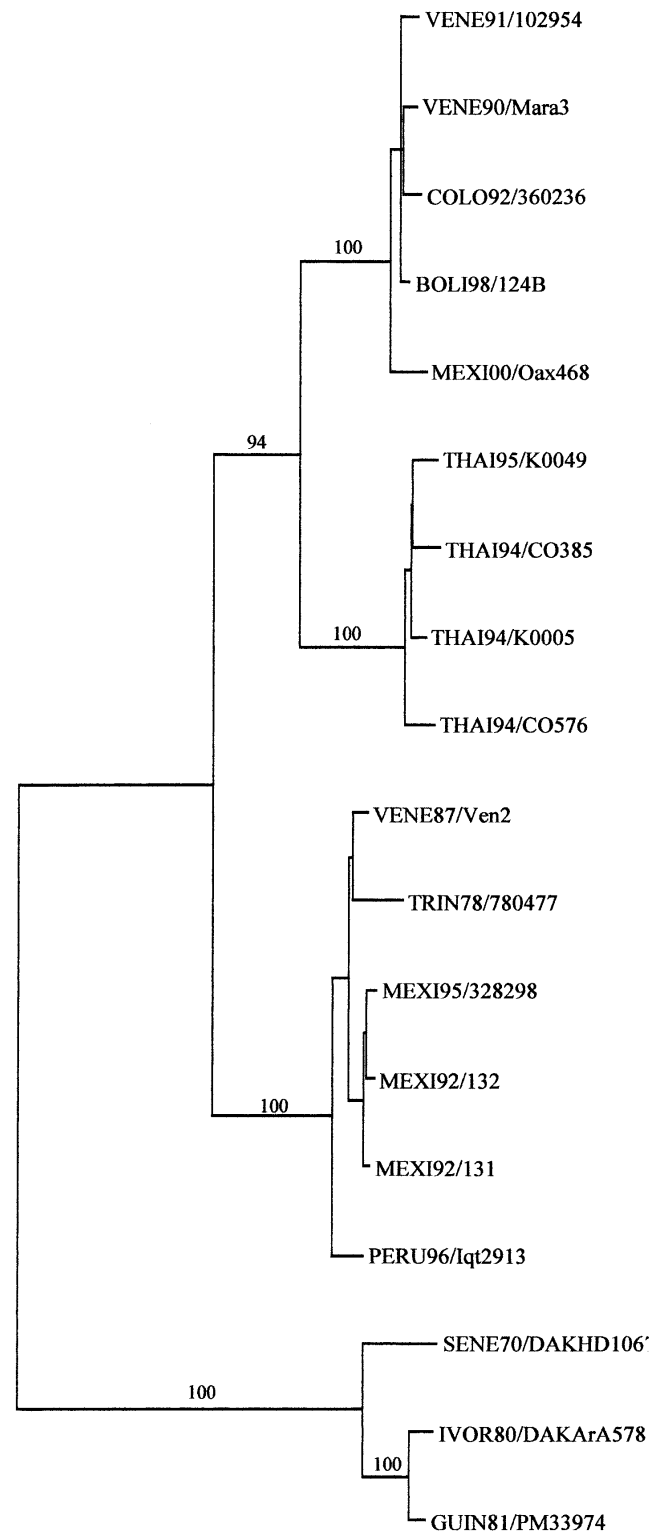
fragment: one cycle at 45°C for 30 minutes, 10 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for 2 minutes 30 seconds, followed by 25 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for 2 minutes 30 seconds, plus one five-second extension time for each subsequent cycle. Amplified cDNA was purified and sequenced using procedures previously described.²² Overlapping nucleotide sequences were edited and aligned with the Lasergene software package (DNASTAR, Madison, WI). Phylogenetic analysis of these sequences was by maximum likelihood (PAUP*)²³ using three sylvatic strains of DEN-2 virus as an outgroup. Trees were estimated by the heuristic search method, with the transition/transversion ratio estimated using the HKY85 model. Confidence values of groupings in the tree were evaluated by performing 1,000 bootstrap replicates.

RESULTS

Maximum likelihood analysis of the envelope gene was performed on the 15 DEN-2 virus strains used in this study to evaluate their evolutionary relationships. In addition, three sylvatic strains of DEN-2 virus from West Africa served as an outgroup to root the phylogenetic tree. This analysis confirmed that the 15 DEN-2 virus strains segregate into either of two distinct genotypes as previously defined (Figure 1).^{6,8,10} Earlier isolates from Trinidad, Venezuela, and southern Mexico and more recent isolates from Peru and northern Mexico clustered together in the American genotype. Other DEN-2 virus strains from Venezuela and southern Mexico were distinct from earlier isolates in these countries and clustered with isolates from Bolivia and Colombia. These latter five isolates were more closely related to strains from Thailand and together they formed the SE Asian genotype. Viruses representing the SE Asian genotype exhibited more genetic variation in the envelope gene than American genotype viruses.

To determine whether distinct genotypes of DEN-2 virus are better adapted to infect and disseminate in *Ae. aegypti*, we allowed female mosquitoes of the McAllen colony to feed on virus blood meals and tested them for the presence of dengue viral antigen in head tissue (Figure 2). Disseminated infection frequencies for individual strains within the American genotype were more uniform, ranging from 6% to 12%. The SE Asian genotype, in contrast, exhibited more phenotypic variation and disseminated infection frequencies ranged from 8% to 58%. The overall rate of disseminated infection was 27% of 296 mosquitoes tested for the SE Asian genotype versus 9% of 209 mosquitoes tested for viral strains of the American genotype. To account for all sources of variation, including variation among individual strains within a genotype, we analyzed vector infection data by analysis of variance. Significant differences in disseminated infection rates were detected between the American and SE Asian genotype ($F_{1,13} = 7.29$, $P = 0.02$, by analysis of variance [ANOVA]). The most infectious dengue viral isolates included strains of both Thai (CO576) and Venezuelan (102954, Mara3) origin and did not form a discrete phylogenetic cluster within the SE Asian genotype.

To determine whether a similar pattern of dengue virus infection could be observed in another, geographically distinct, population of mosquitoes, *Ae. aegypti* of the Tehuante-



— 1% divergence

FIGURE 1. Maximum likelihood tree depicting the phylogenetic relationships of 18 dengue serotype 2 viruses, based on nucleotides of the envelope gene (1,485 bases). Numbers on branches represent bootstrap support for each branch. Horizontal branch lengths are proportional to the percentage of divergence in the nucleotide sequence. Viruses are denoted by the first four letters of the country of origin, the last two digits of year of isolation, and the strain number. Sylvatic strains from Senegal, Guinea, and Côte d'Ivoire served as an outgroup.

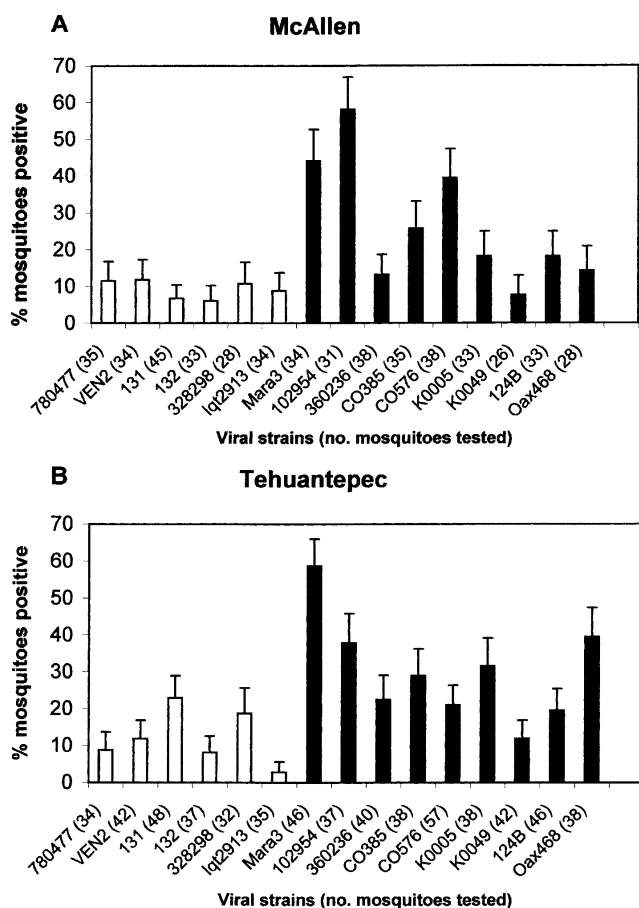


FIGURE 2. Infection and dissemination of dengue serotype 2 viruses in the **A**, McAllen and **B**, Tehuantepec colonies of *Aedes aegypti*. Mosquitoes were scored as positive if dengue virus antigen was detected in the head tissues by an indirect fluorescent antibody test. American genotype viruses are depicted by white bars and Southeast (SE) Asian genotypes are depicted by black bars. Error bars show the standard deviation.

pec colony were exposed to the same panel of DEN-2 viruses. Overall, mosquitoes of the Tehuantepec colony also tended to be more susceptible to infection by members of the SE Asian genotype (30% of 382 mosquitoes) than the American genotype viruses (13% of 228 mosquitoes), and differences among genotypes were statistically significant ($F_{1,13} = 8.93$, $P = 0.01$, by ANOVA). The pattern of infection among individual viral strains was similar to that of the McAllen colony with two significant exceptions (Figure 2). Disseminated infection rates of a co-indigenous dengue virus strain (Oax468) from nearby Juchitan, Mexico increased almost three-fold in the Tehuantepec colony of *Ae. aegypti* compared with the McAllen colony ($P = 0.03$, by Fisher's exact test). Likewise, the rate of disseminated infection of another isolate from Mexico (131) was more than three-fold greater in the Tehuantepec colony than in the McAllen colony of *Ae. aegypti* ($P = 0.04$, by Fisher's exact test).

DISCUSSION

Dengue viruses exhibit a considerable amount of variation in their efficiency to infect and disseminate in vector mosqui-

toes. In this study, we found that this variation is not randomly distributed across the two genotypes; specifically, the most infectious dengue virus strains were of the SE Asian genotype, including strains of both Thai and Venezuelan origin. By analysis of variance, we found that mean variation between genotypes exceeded mean variation within genotypes and from this we conclude that strains of the SE Asian genotype tend to infect mosquitoes more efficiently despite considerable variation within this genotype. This trend was maintained in two geographically separated *Ae. aegypti* populations derived from Texas and Mexico.

This study also raised the possibility that dengue virus may become locally adapted to its mosquito vector. Indeed, we found that dengue virus strain Oax468 isolated from a patient in Juchitan, Mexico was more infectious for mosquitoes acquired from nearby Tehuantepec than for mosquitoes obtained from McAllen, Texas. However, other evidence argues against local adaptation; specifically, dengue virus strain 328298, which was isolated along the Texas-Mexico border region, was about equally infectious for mosquitoes derived from nearby McAllen as for those from Tehuantepec. This phenomenon merits further study by evaluating patterns of differential infection in a larger collection of sympatric viral-vector pairs.

The susceptibility of *Ae. aegypti* to infection by dengue virus is determined by an array of vector, viral, and environmental factors. To minimize the effect of environmental variation, we infected each colony of mosquitoes with all 15 strains of dengue virus within a 12-hour time period, and mosquitoes were held together in incubators maintained at 30°C. Mosquitoes were randomly assigned to each experimental group and sufficiently large sample sizes were used to apply statistical analysis across viral genotypes. Nonetheless, some of the variation we observed may have been influenced by environmental and/or vector variation among experimental groups. In reference to a previous study, we found that mosquitoes derived from McAllen, Texas were more susceptible to infection by some of the same dengue virus strains used in this study.¹⁷ Moreover, infection rates for each of the dengue virus strains were more uniform in the prior study. This discrepancy may be due to differences in the genetic background of the two McAllen colonies used. In the former study, mosquitoes were collected from the field during a different year (1999 versus 2001 in this study) and were maintained in the laboratory for a greater number of generations (F_4 versus F_2 here) prior to oral infection experiments.

Entomologic parameters that influence the force of dengue virus transmission include vector density, proportion of vector mosquitoes feeding on the reservoir host, daily survival rate, vector competence, and extrinsic incubation period.²⁴ Viral genetics may directly influence two of these variables: vector competence and extrinsic incubation period. Extrinsic incubation period is the time interval from vector infection to vector infectiousness which varies from 7 to 12 days for dengue virus, depending largely on temperature.²⁵ Higher temperatures generate faster viral replication rates in the mosquito and thus accelerate viral dissemination to the mosquito salivary glands. Viral genetics could also influence this parameter; however, we previously found that viral replication rates in *Ae. aegypti* appear to be similar among different dengue viral genotypes.¹⁷ In contrast, vector competence clearly varies as function of the genetic characteristics of the infecting

strain and we therefore focused exclusively on this parameter, which estimates the proportion of mosquitoes that can physiologically acquire, maintain, and transmit a pathogen. We evaluated vector competence by estimating the proportion of mosquitoes expressing dengue viral antigen in the head tissue and did not directly measure the proportion of mosquitoes that secrete virus in saliva. Prior experiments indicated that after dissemination of dengue virus to head tissue, mosquitoes strains are equally capable of transmitting the virus.¹⁵ In this context, disseminated infection rates served as a surrogate for vector transmission potential.

Phylogenetic analysis of past and contemporary dengue virus isolates in Colombia, Venezuela, Brazil, and southern Mexico suggest that the SE Asian genotype of DEN-2 virus has replaced the American genotype in these regions.¹⁰ All of the most recent DEN-2 virus isolates thus analyzed have proven to be of the SE Asian genotype except for isolates derived from Iquitos, Peru and the Texas-Mexico border region. These observations suggest that transmission of the SE Asian genotype is more robust than that of the American genotype. Differences in transmission potential may be reflective of *Ae. aegypti* being more efficient at acquiring, maintaining, and transmitting viruses of the SE Asian genotype. Such a difference in vector competence could sustain transmission of the SE Asian genotype when conditions are sub-optimal. For example, as herd immunity increases against DEN-2 virus in the host population, dengue virus strains of the American genotype could become locally extinct before the SE Asian genotype. This assumes, however, that viruses of both genotypes are equivalent in sustaining viremias in the human host; this factor is being addressed to fully understand the mechanisms behind the displacement phenomenon.

Relatively few genetic differences among dengue virus strains may have profound consequences for their subsequent infection of and transmission by vector mosquitoes. For example, the parental strain of a live-attenuated DEN-2 vaccine proved to be about five times more infectious to mosquitoes than its vaccine derivative.²⁶ Whole genome sequencing revealed that the vaccine strain actually represents a mixture of two genetic variants that vary at one amino acid position and these variants differ from the parental strain by seven or eight amino acids.²⁷ The SE Asian and American genotypes of DEN-2 virus may be distinguished by 11 amino acid changes in the coding region and by 7 nucleotide substitutions and 10 deletions in the non-coding regions of the genome.²² Many of these substitutions are predicted to confer changes in antigenicity and RNA secondary structure of untranslated regions. Such differences could have dominant effects on viral infection, replication, and dissemination in the mosquito vector; however, genetic differences within a genotype also appear to influence vector competence. We are currently developing dengue virus infectious clones to map viral genetic traits that affect dengue virus transmission; this work provides a foundation for such studies on the molecular basis of vector competence.

Received October 9, 2002. Accepted for publication February 12, 2003.

Acknowledgments: We thank Dr. Alejandro Cisneros for facilitating field work in Mexico, Uriel Martinez for helping with mosquito collections in Tehuantepec, and Dr. Jack Hayes for providing mosquito eggs from McAllen, Texas.

Financial support: This work was supported by grants from the National Institutes of Health (AI-10427 and AI-50123) and the Quillin Foundation.

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