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AEDES AEGYPTI VECTORIAL CAPACITY IS DETERMINED BY THE INFECTING GENOTYPE OF DENGUE VIRUS

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Abstract

Dengue viruses causing severe, hemorrhagic disease have displaced less virulent strains in the Americas during the past three decades. The American (AM) genotype of dengue serotype 2 has been endemic in the Western Hemisphere and South Pacific, causing outbreaks of dengue fever (DF), but has not been linked to dengue hemorrhagic fever (DHF). The Southeast Asian (SEA) genotype of dengue was introduced into this hemisphere in 1981, has caused outbreaks with numerous cases of DHF, and has displaced the AM genotype in several countries. We investigated the effect of viral genotype on the potential for transmission by infecting *Aedes aegypti* mosquitoes collected in South Texas with six viruses, representing these two genotypes. Viral replication in the midgut was significantly higher in SEA-infected mosquitoes, and virus-specific proteins could be detected in salivary glands 7 days earlier in SEA- than AM-infected mosquitoes. This much earlier appearance of dengue virus in salivary glands resulted in an estimated 2- to 65-fold increase in the vectorial capacity of these mosquitoes for the viruses that can cause DHF. This may be one of the mechanisms through which more virulent flaviviruses spread and displace others globally.

INTRODUCTION

The 4 serotypes of dengue virus (Flaviviridae; genus *Flavivirus*) are responsible for up to 50 million cases of dengue fever (DF) annually; approximately 500,000 cases progress to the more severe dengue hemorrhagic fever (DHF).[1] Infection by one serotype does not confer immunity to the other 3, and sequential infections may predispose one to developing DHF.[2] Thus, severe dengue disease has been traditionally associated with the human host's immune response ("immunopathogenesis") rather than with viral genetic or phenotype differences. Because humans are the only hosts shown to develop disease, the contribution of these and other factors to pathogenesis have been impossible to measure. Currently, dengue viruses are maintained in a human-mosquito-human cycle, with *Aedes aegypti* serving as the principal vector. Disease control methods have been limited to mosquito abatement, and there are no licensed vaccines or antivirals.

Dengue serotype 2 viruses (DENV-2) are phylogenetically grouped into 4 genotypes.[3] The American (AM) genotype has been endemic in the Western Hemisphere and South Pacific islands for 5 decades.[4] However, to date, there have been no documented cases of DHF attributed to this genotype,[5] even when causing a secondary infection.[6] In contrast, the Southeast Asian (SEA) genotype was first detected in the Americas during an epidemic in Cuba

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in 1981, during which thousands of DHF cases were also documented.[7,8] Since then, the SEA genotype has rapidly displaced the AM genotype, which until recently had only been detected in Peru and northern Mexico.[5] Indeed, during the most recent DF and DHF epidemic in northern Mexico and southern Texas (fall/winter of 2005), SEA genotype viruses were isolated and seem to have displaced the AM genotype virus that had been isolated up until 1995 (Smith BR, unpublished data; Anderson JR and Rico-Hesse R, unpublished data). The displacement of the AM genotype by the SEA genotype may be due, in part, to differential infection, dissemination, and replication rates in the mosquito. Southeast Asian strains have been shown to infect a larger proportion of mosquitoes than AM strains,[9] and a greater proportion of SEA-infected mosquitoes develop disseminated infections.[10] Mosquitoes that were fed on both genotypes simultaneously were much more likely to develop an infection with SEA genotype viruses than with AM viruses (21 versus 3%). Also, human dendritic cells infected with SEA viruses produce larger amounts of negative-strand RNA (a surrogate for measuring replication) and a higher output of viral progeny per infected cell than those infected with AM viruses, which may explain higher viral load in human blood.[11]

In terms of epidemiology, these differences in transmission may be quantified by examining various entomological parameters that contribute to the transmission of a parasite from an infected host to secondary, uninfected individuals. Vectorial capacity is the average rate at which potentially infective mosquito bites arise following the introduction of a single infectious host.[12,13] It incorporates the feeding habits and survival of the vector species and the time required for the pathogen to develop to infectivity, termed the extrinsic incubation period (EIP). Vectorial capacity (*C*) is given by the equation

$$C = ma^2 p^n b \Big/ -\ln p$$

where *m* is vector density with respect to the host, *a* represents the daily probability of being fed upon (host preference index multiplied by frequency of feeding), *p* is the probability of daily survival, *n* is the length of the EIP in days, and *b* corresponds to vector competence (the proportion of vectors that will eventually become infective).[13] Vectorial capacity is largely controlled by intrinsic, vector factors; for example, vector competence[14] and host preference[15] are, to various degrees, controlled by genetics. In addition, extrinsic factors play a significant role: temperature and humidity influence the probability of daily survival and the length of the EIP.[16-18] As a result, quantification of *C* is useful only for site- and/or time-specific comparisons, or for assessing the effectiveness of intervention campaigns.[19] Missing from the interpretation of vectorial capacity is the contribution of the infecting strain of pathogen (though this may be included in the vector competence component also).

We investigated differences in dissemination between DENV-2 strains of SEA and AM origin and determined what effect these differences would have on transmission dynamics. To date, studies on the influence of the arthropod-borne viral strain are lacking.[20] Here we show that SEA and AM genotype DENV-2 viruses have drastically different EIPs and that the shorter EIP for SEA viruses contributes to genotypic displacement by increasing the vectorial capacity of *Ae. aegypti*. This is the first report to demonstrate how replication and dissemination dynamics of virus strains contribute to the overall vectorial capacity of *Ae. aegypti*.

MATERIALS AND METHODS

Mosquito infections

McAllen (Texas) strain *Aedes aegypti* females of the F_4 generation were reared at 28°C, 75% humidity, and a 14:10 hour light:dark cycle. Larvae were fed bovine liver powder *ad libitum*, and pupae were transferred to cages to allow adult emergence. Adults were maintained on a 4% sucrose solution, which was removed from the cage 24 hours prior to infections.

Approximately 7–10 days post-emergence, females were exposed to an infectious bloodmeal via a water-jacketed membrane feeding apparatus. The bloodmeal consisted of defibrinated rabbit blood and a single DENV-2 virus, of either SEA or AM genotype, at a final concentration of 2.5×10^8 genome equivalents/mL. The viruses used in this study are listed in Table 1. Following blood-feeding, mosquitoes were held at 30°C for up to 14 days. All infected mosquitoes came from the same oviposition paper.

Indirect immunofluorescent assay

To determine dissemination rates, we focused on detection of viral protein in tissues relevant to transmission, namely the midguts and salivary glands. Each day through Day 14 post-infection (PI), mosquitoes were cold anesthetized, and midguts and salivary glands were dissected from 6–10 mosquitoes and washed 3 times in *Aedes* saline (154 mM NaCl, 1.36 mM $CaCl_2$, 2.68 mM KCl, 1.19 mM $NaHCO_3$, pH 7.0). Midguts were spread apart in a single well, while salivary glands were fixed whole to the slide; the tissues were fixed in cold acetone and air-dried. Anti-DENV-2 polyclonal mouse ascitic fluid (obtained from the Centers for Disease Control and Prevention), diluted 1:200 in phosphate-buffered saline (PBS), was spotted onto each well and incubated at 37°C for 30 minutes in a humid chamber. Slides were washed twice in PBS, 5 min each, and overlaid with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma, St. Louis, MO) diluted 1:200 in PBS. After 30 minutes at 37°C, the slides were washed twice in PBS, 5 minutes each, and air dried; coverslips were fixed with glycerol:PBS (5:1). Fluorescence was observed at 200–400× with a Nikon (Melville, NY) E400 microscope.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from midguts of dengue-infected mosquitoes on Days 1–14 and triturated in 50 μL Trizol (Invitrogen, Carlsbad, CA). Triturated tissues were brought to a final volume of 500 μL with Trizol, extracted with 100 μL chloroform, and incubated overnight at −20°C with 10 μg glycogen in 250 μL isopropanol. Pelleted RNA was washed with 1 mL 75% ethanol, air dried, and resuspended in a final volume of 30 μL DEPC-H_2O. Dengue viral RNA (positive- and negative-strand) was quantified in triplicate with the RNA UltraSense kit (Invitrogen, Carlsbad, CA) using a previously described qRT-PCR protocol.[21] Each 25-μL reaction contained 5 μL 5× UltraSense Reaction Mix, 1.25 μL Enzyme Mix, 0.5 μL ROX reference dye, 100 nM sense primer (5′-GCTGAAACGCGAGAGAAACC-3′), 200 nM antisense primer (5′-CAGTTTTAITGGTCCTCGTCCCT-3′), and 100 nM probe (FAM-5′-AGCATTCCAAGTGAGAATCTCTTTGTCAGCTGT-3′-TAMRA); 5 μL total RNA served as template. Amplification was performed on an ABI Prism 7700 Sequence Detection Instrument at 50°C for 15 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The threshold C_t value was set at 10 times the standard deviation of cycles 3–15. To quantify negative-strand viral RNA, cDNAs were generated with the SuperScript III First Strand kit (Invitrogen, Carlsbad, CA), using only the sense primer and 2 μL total RNA, following the manufacturer's directions. qPCR was then performed with the Platinum Supermix-UDG kit (Invitrogen, Carlsbad, CA) with 5 μL cDNA and the conditions described above.

Dengue RNA standards were produced by RT-PCR amplification of a 94-bp fragment from a SEA virus (strain K0049), followed by cloning into pCR2.1 using the TOPO cloning kit (Invitrogen, Carlsbad, CA). The plasmid was digested with *HindIII*, and RNA transcripts were generated with the T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's instructions. Concentrations of transcribed RNA were determined using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR) according to the supplied directions. Sample RNA copy number was interpolated from a standard curve of serial 10-fold dilutions of this *in vitro*-transcribed standard.

To directly compare dengue viral copy number in dissected midguts, a qRT-PCR assay was developed for an *Ae. aegypti* housekeeping gene to quantify tissue and normalize samples. The ribosomal protein S17 (RpS17) RNA has been used as a gene expression standard in other experiments;[22,23] we have adapted this for use as a qRT-PCR standard, which requires a shorter amplicon. Primer and probe sequences were identified from the LF272 EST sequence (Genbank accession number BM005484) using PrimerExpress (Applied Biosystems, Foster City, CA). Primers were tested on RNA extracted from various adult mosquito tissues and larvae and pupae using Trizol reagent, following the manufacturer's instructions. Adult tissues were resuspended in 30 μL DEPC-treated water, while larvae and pupae were resuspended in 100 μL. A 101-base pair fragment was amplified with the Superscript III One-step RT-PCR kit (Invitrogen, Carlsbad, CA) in a 50-μL reaction containing 1× reaction mix, 200 nM forward primer (RpS17F; 5′-ACATCTGATGAAGCGCCTGC-3′), 200 nM reverse primer (RpS17R; 5′-ACACTTCCGGCACGTAGTTGT-3′), 2 μL enzyme mix; 5 μL of each RNA extraction was added as template. Amplification was performed at 50°C for 30 minutes, 94°C for 2 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 30 seconds, followed by a final extension at 68°C for 5 minutes.

Quantitative RT-PCR reactions followed the conditions described previously, with 50 nM forward primer, 50 nM reverse primer, 100 nM RpS17 probe (TET-5′-CACTCCCAGGTCCGTGGTATCTCCATC-3′-TAMRA), in a 25 μL reaction. Amplification was performed and analyzed as described previously. The number of RpS17 copies present in the samples was estimated from an *in vitro*-transcribed RNA generated as described previously for the dengue standard; a 101-bp product amplified from total mosquito RNA served as the template for cloning.

Statistical analyses

Infection rates for midguts and salivary glands examined by IFA were tested for variation with logistic regression, defining the logit as ln(total number infected/total number uninfected), to model the proportion positive against the day of infection and tested whether the slopes and intercepts differed between genotypes. Dengue viral RNA equivalents were normalized by dividing them by the number of RpS17 copies and multiplying the result by the mean RpS17 value for all individuals. Comparisons of viral RNA by genotype at each time point were analyzed by 2-way analysis of variance of data for the first 7 days of infection (when data were available for all 6 strains). Early time points (1–12 hr) were compared by *t* tests. Differences were considered significantly different at $P < 0.05$. The *t* tests were performed using GraphPad (San Diego, CA) Prism, logistic regression with R version 2.3.1,[24] and 2-way ANOVA with SigmaStat version 3.1 (Richmond, CA).

RESULTS

Midgut and salivary gland infections

Because they are the most important mosquito organs in dengue replication and transmission, we focused on the midguts and salivary glands. These organs were dissected from 6–10 females each day through Day 14 PI. Mosquitoes infected with SEA viruses developed midgut infections rapidly, and by Day 4, approximately 80% of midguts were positive by IFA (Figure 1). Salivary glands first showed evidence of SEA antigen on Day 2, and 50% were infected by Day 7 PI. The presence of antigen in salivary glands at Days 2 and 3 is unusual; however, the fluorescence was focal, relatively weak, and distinct from uninfected controls. The washing should have removed any contamination by infected fat body, hence it is likely that this reflects a more rapid dissemination by SEA viruses. In contrast, midguts infected with AM viruses lagged significantly behind SEA infections. At Day 4 PI, only 45% of midguts were positive for antigen, and 12 days were required to reach an 80% infection rate (Table 2, Figure 1A).

For salivary glands, dissemination rates were similar, where 50% of salivary glands were infected at Day 14 PI, 7 days later than with the SEA genotype viruses (Table 2, Figure 1B).

We analyzed variation between genotypes using logistic regression, with the logit defined as the natural log of the total number of infected organs divided by the total number of uninfected organs from mosquitoes infected by each genotype, at each time point PI. In the midguts, both the slopes and intercepts were significantly higher ($P < 0.01$ and $P < 0.05$, respectively) for the SEA genotype than the AM genotype (Figure 2A). In the salivary glands, the intercept was significantly higher for SEA viruses ($P < 0.05$), but the slopes were not significantly different (Figure 2B).

Viral RNA in midgets

To determine whether these differences in virus dissemination were due to differences in viral replication, we quantified viral positive- and negative-strand RNA in midguts on Days 1–14 PI. (Figure 3A). These values were corrected for the amount of tissue extracted by using the amount of RpS17 RNA as a surrogate; RpS17 RNA was detected in all tissues tested (data not shown). Southeast Asian viruses replicated to a higher level than did AM viruses (2-way ANOVA, $P < 0.001$, $F = 15.958$, $df = 1$), with a difference of means of 1.2 $\log_{10}$ genome equivalents. Using a Holm-Sidak post-test, there was also a significant effect of day postinfection ($P < 0.005$), where RNA levels at days 2–4 were lower than days 1 and 5–7 for each strain. Although the assumption of equal variance was not met, differences were evident among strains, where CO489 and K0049 (SEA) and IQT2913 (AM) generally produced more genome equivalents than did the remaining strains.

Because our previous work with human dendritic cells had shown differences in viral RNA quantities at early time points PI, we then infected mosquitoes with either IQT2913 (AM strain) or CO489 (SEA strain) and quantified positive- and negative-strand viral RNA in midguts at 1, 4, and 12 hours PI (Figure 3B). Total viral RNA, representing viral genomes and replicative forms, differed significantly only at 4 hours PI ($P < 0.0001$), where there were more genome equivalents in the midgut of IQT2913 (AM)-infected mosquitoes than in CO489 (SEA)-infected mosquitoes (Figure 3A). The IQT2913 strain also produced significantly more negative-strand RNA, representing only viral replicative intermediates, at 4 and 12 hours PI ($P = 0.0303$ and $P = 0.0396$, respectively) than did the CO489 strain (Figure 3B).

Effect of infecting strain on vectorial capacity

Because the probability of daily survival and the length of the EIP interact exponentially, the differences shown above may have a dramatic impact on the likelihood of transmission. To illustrate this, we compared the value p^n between genotypes using the results we obtained above. Values of n were held at the time when 50% of salivary glands became infected: 7 days for SEA and 14 days for AM. Here we assume that those mosquitoes with viral antigen in the dissected salivary glands are capable of transmitting the virus, as validated by others.[18,25] Values for p, which are largely dependent on environmental conditions, were obtained from the literature: Australia, 0.86 and 0.91;[26] China, 0.763;[27] Kenya, 0.824,[28] 0.89;[29] Puerto Rico, 0.356 and 0.735,[30] 0.55 and 0.69;[31] Tanzania, 0.656 and 0.849;[32] Thailand, 0.805, 0.814, 0.834, 0.843.[30] Figure 4 shows the effect that the EIP differences have on p^n, and thus on vectorial capacity: SEA strains are 2- to 65-fold more likely to be transmitted than AM strains, when all other components of C are constant.

DISCUSSION

Dengue viruses causing severe disease continue to spread globally, apparently displacing autochthonous, less virulent viruses. For example, SEA DENV-2 viruses have largely

supplanted AM viruses in the Western Hemisphere and South Pacific Islands;[3,5] the latter genotype may now be endemic only in Peru and Northern Mexico. A recent epidemic due to a SEA genotype DENV-2 virus in northeastern Mexico and southern Texas may indicate that the AM genotype has been displaced from Mexico as well. The results reported here explain one of the factors that could cause viral displacement and increased transmission of those genotypes causing DHF.

A previous study examining intraserotype variation in susceptibility to dengue viruses demonstrated potential differences in infection rates, depending on both the infecting virus and vector strain;[33] however, that study did not examine dissemination, instead focusing on infection of the vector, and used long-established *Ae. aegypti* colonies, which have likely lost genetic variability in susceptibility.[9] In our study, dengue virus strains from both genotypes readily infected mosquito midguts, and dengue antigen was evident in nearly 100% of infected mosquitoes, though there was a significant time lag when AM genotype viruses were fed to mosquitoes. However, an AM strain produced significantly more viral RNA at early time points than did a SEA strain, which suggests that AM viruses may infect a larger proportion of midgut cells. Similar results have been seen in primary human dendritic cells (DCs) infected *ex vivo*: a larger percentage of DCs are infected by AM strains, but SEA strains generate substantially more viral progeny per infected cell, and thus produce a much higher virion output.[11] It is unclear whether the two genotypes differ in their ability to bind to midgut receptors and/or infect midgut epithelial cells. Here we have shown that potential differences in binding lead to significant variation in replication rates.

The much greater dissemination rate of SEA viruses compared with AM viruses confirms our earlier results using IFA of head squashes.[10] The higher viral replication in midguts likely contributes to this increased dissemination, since the slopes of the regression lines for salivary gland infections are not different. That is, once virus disseminates from the midgut, SEA and AM viruses infect the salivary glands at equal rates. Others have suggested that translational differences may contribute to growth or virulence phenotypes; thus, viral RNA may not increase, but the increased expression of viral proteins from this viral RNA could produce more virions. Translational differences between viral strains have been shown in human cell culture,[34] and these differences may be due to interactions between the 5′- and 3′-non-translated regions (NTRs).[35-37] Consistent nucleotide differences between the SEA and AM genotype viruses have been shown to alter the secondary structure of the 5′- and 3′-NTRs,[38] which may affect translation levels and, as a result, influence packaging and export (i.e., dissemination) of virions from infected midgut cells. Replacement of SEA NTRs with AM NTRs leads to a decrease in viral output in human DCs,[39] and we intend to test the contribution of these NTRs to the ability of these viruses to disseminate through *Ae. aegypti*.

Vectorial capacity for a virus/vector system describes the number of host cases that will arise from the introduction of a single case in a particular area. Estimations of *C* have implicitly assumed that the infecting strain of a particular virus has little or no impact on either *b* (the ability to be transmitted by the vector) or *n* (the length of the EIP), although Kramer and Ebel[20] recently suggested that this be investigated. Both of these components are influenced by extrinsic factors (e.g., temperature, humidity, infectious dose, etc.). However, we have shown that, holding all other factors constant, the infecting strain may have a substantial effect on *C*. This is largely due to the exponential relationship between *p* and *n*. When the value of *p* (survival) is relatively low, mosquitoes infected with SEA viruses are up to 65 times more likely to survive the EIP and transmit virus. These strain differences may also manifest in *b* (vector competence); assuming that those mosquitoes with antigen present in the salivary glands are capable of transmitting virus, approximately 80% of SEA-infected mosquitoes will transmit at the end of a standard 14-day EIP, versus 60% of AM-infected mosquitoes. We assumed that mosquitoes with antigen in the salivary glands will transmit the virus orally, but

did not validate this in our study. However, a previous study with DENV-2 in *Aedes albopictus* showed that oral transmission was correlated with the amount of salivary gland tissue positive for dengue antigen,[25] and oral transmission did not occur until virus was recovered from *Ae. aegypti* salivary glands.[18] Similarly, only 7 of 100 *Culex pipiens quinquefasciatus* mosquitoes with West Nile virus antigen in their salivary glands did not have detectable virus in the saliva.[40]

In summary, we have shown that SEA strains of DENV-2 virus disseminate through the *Ae. aegypti* vector faster than AM strains and are presumably transmitted much sooner to the human host. This difference in dissemination may be due to differences in virus replication but may also be determined by rates of translation of viral "messenger" RNA. This is the first report documenting the effect of viral infecting strain on the vectorial capacity of a mosquito. These differences in transmission may explain how many more human cases can occur, including a rise in the number of hemorrhagic fever cases, and how one dengue virus can be displaced by another, with a significant effect on the epidemiology of disease across the globe.

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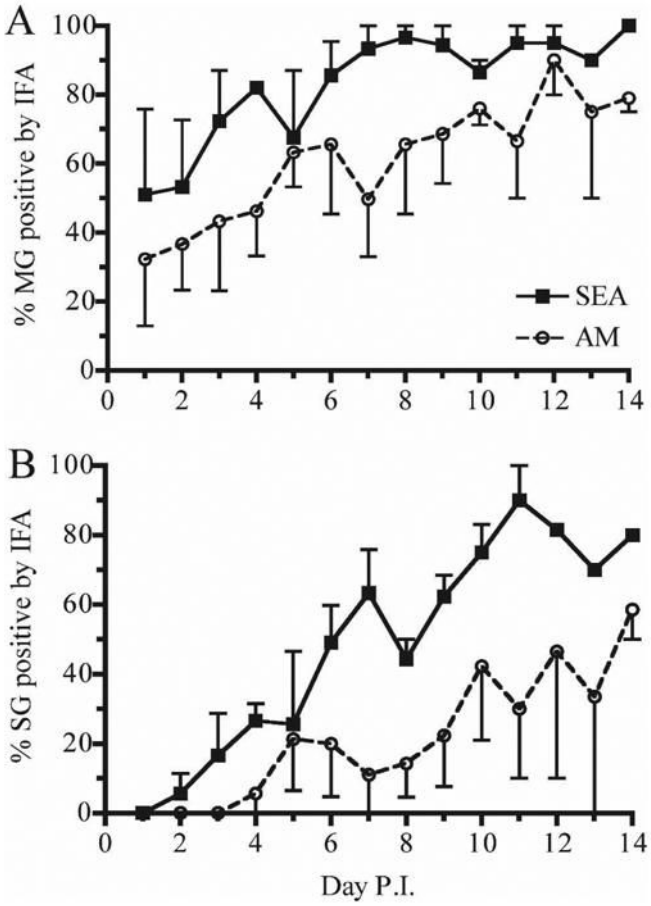

FIGURE 1.

Infection rates for (**A**) midguts and (**B**) salivary glands of *Aedes aegypti* females singly infected with each of three Southeast Asian or American genotype dengue 2 viruses. Six to ten individuals per virus strain were examined at each time point. Error bars represent the standard error of the mean from three virus strains for each genotype. SEA, Southeast Asian genotype; AM, American genotype; MG, midgut; SG, salivary glands.

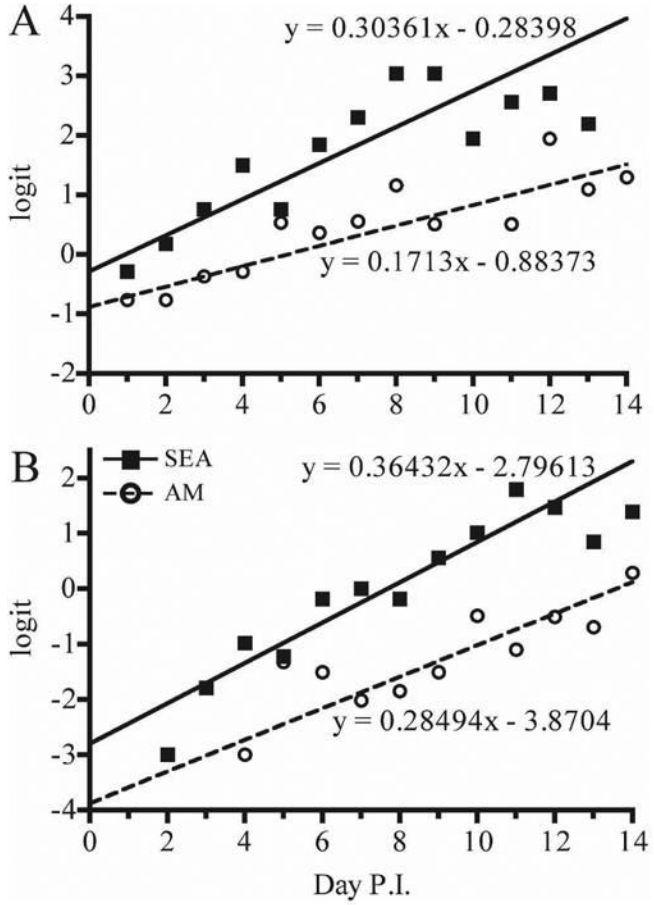

FIGURE 2.

Comparison of regression lines for DENV-2 infections of *Aedes aegypti* midguts (**A**) and salivary glands (**B**) by three strains each of Southeast Asian (SEA; solid squares, solid line) or American (AM; open circles, dashed line) origin. On the y-axis, the logit represents ln (total number infected/total number uninfected) for each genotype. In midguts (A), both the slope and intercept differ significantly between genotypes ($P < 0.05$). In salivary glands (B), only the intercepts differ significantly ($P < 0.05$).

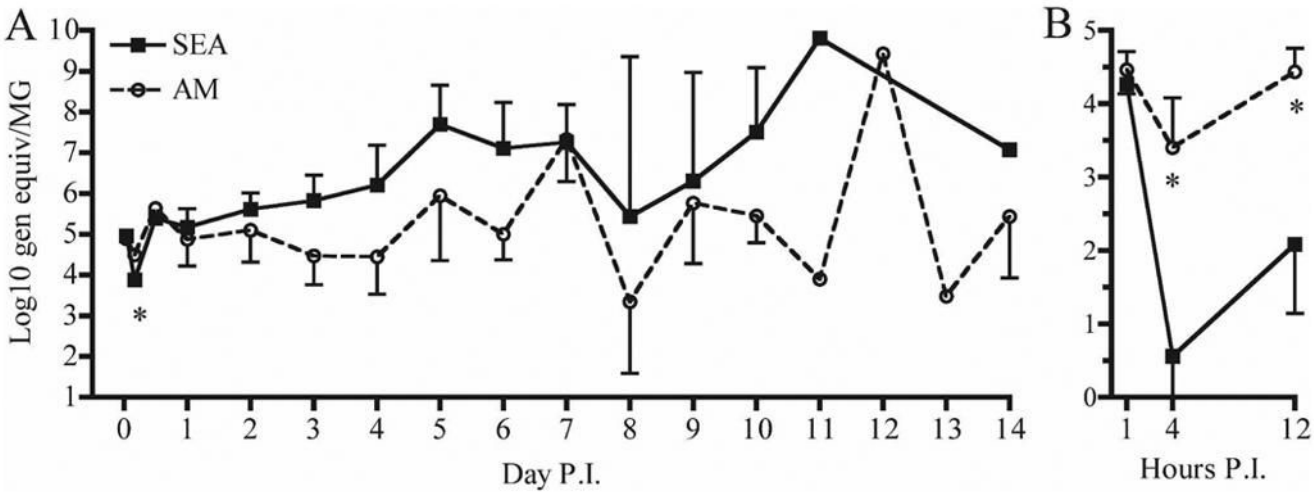

FIGURE 3.

(**A**) Viral RNA quantities in midguts of *Aedes aegypti* females singly infected with each of three Southeast Asian or three American genotype dengue 2 viruses. Six to ten individuals for each of six virus strains were examined at each time point, for day 1–14 P.I. (**B**) For time points 1, 4, and 12 hr, six individuals were examined for each of two virus strains. Asterisks indicate times at which differences are significant by unpaired *t* test. Error bars represent the standard error of the mean. SEA, Southeast Asian genotype; AM, American genotype.

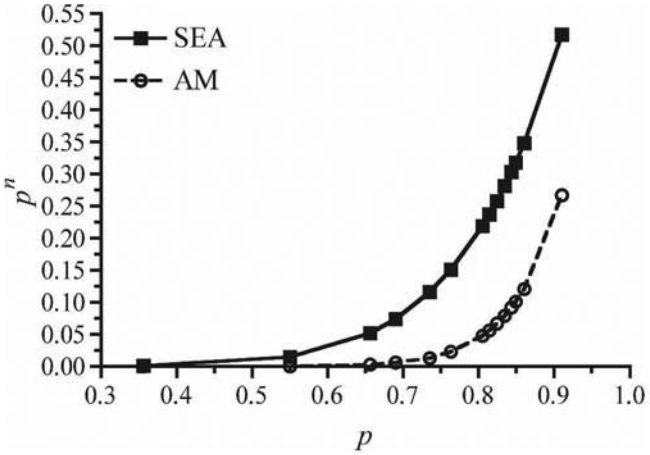

FIGURE 4.

Likelihood of an infected mosquito surviving the extrinsic incubation period (EIP) for Southeast Asian and American genotypes of dengue 2 virus. The probability of surviving the EIP, p^n, is plotted against the probability of daily survival, p. Values for p were obtained from the literature.[26-32]

TABLE 1

Dengue 2 viruses used to infect McAllen strain *Aedes aegypti*

Strain	Genotype*	Passage†	Location	Year	Diagnosis‡
IQT2913	AM	C4	Iquitos, Peru	1996	DF
Ven2	AM	A2, C3	Maracay, Venezuela	1987	DF
131	AM	C3	Sonora, Mexico	1992	DF
CO489	SEA	C4	Bangkok, Thailand	1996	DF
Mara3	SEA	C3	Maracay, Venezuela	1990	DF
K0049	SEA	C4	Kamphaeng Phet, Thailand	1995	DHF

*American (AM) or Southeast Asia (SEA) genotype, determined as described in Ref. 11.

†Number of passages (numerals) in AP61 (A) or C6/36 (C) mosquito cells.

‡Dengue fever (DF) or dengue hemorrhagic fever (DHF).

TABLE 2

Proportion of midguts and salivary glands singly infected with three Southeast Asian (SEA) or three American (AM) genotype dengue 2 viruses, detected by IFA

	Midguts								Salivary glands							
	SEA			AM					SEA			AM				
Day PI	CO489	K0049	Mara3	IQT2913	131	Ven2	SEA total	AM total	CO489	K0049	Mara3	IQT2913	131	Ven2	SEA total	AM total
1	2/10*	5/5	2/6	3/10	4/6	0/6	9/21	7/22	0/10	0/6	0/6	0/10	0/6	0/6	0/22	0/22
2	6/10	1/6	5/6	1/10	3/6	3/6	12/22	7/22	0/10	0/5	1/6	0/9	0/6	0/6	1/21	0/21
3	5/10	4/6	6/6	3/10	1/6	5/6	15/22	9/22	1/10	0/6	2/5	0/10	0/6	0/6	3/21	0/22
4	8/10	5/6	5/6	2/9	3/6	4/6	18/22	9/21	3/10	1/6	2/6	0/9	0/6	1/6	6/22	1/21
5	7/10	2/6	6/6	4/7	3/6	5/6	15/22	12/19	1/10	0/6	4/6	1/7	0/6	3/6	5/22	4/19
6	9/10	4/6	6/6	3/10	4/6	6/6	19/22	13/22	3/10	3/6	4/6	1/10	0/6	3/6	10/22	4/22
7	8/10	6/6	6/6	2/6	2/6	5/6	20/22	9/18	4/10	4/6	5/6	0/6	0/5	2/6	11/22	2/17
8	9/10	6/6	6/6	3/10	4/6	6/6	21/22	13/22	5/10	3/6	2/6	1/10	0/6	2/6	10/22	3/22
9	10/10	5/6	6/6	4/10	5/6	5/6	21/22	14/22	7/10	3/6	4/6	0/10	1/6	3/6	14/22	4/22
10	9/10	5/6	ND	7/9	4/6	5/6	14/16	16/21	6/9	5/6	ND	1/9	2/6	5/6	11/15	8/21
11	9/10	4/4	ND	5/10	5/6	ND	13/14	10/16	8/10	4/4	ND	1/10	3/6	ND	12/14	4/16
12	9/10	6/6	ND	8/10	6/6	ND	15/16	14/16	8/10	5/6	ND	1/10	5/6	ND	13/16	6/16
13	9/10	ND†	ND	3/6	6/6	ND	9/10	9/12	7/10	ND	ND	0/6	4/6	ND	7/10	4/12
14	10/10	ND	ND	6/8	5/6	ND	10/10	11/14	8/10	ND	ND	4/8	4/6	ND	8/10	8/14

*Number positive/number tested.

†Not determined due either to mortality or poor feeding.