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Dengue-1 Virus Clade Replacement in Thailand Associated with Enhanced Mosquito Transmission

RUNNING TITLE: Role of vectors in dengue virus evolution

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Abstract

Dengue viruses (DENV) are characterized by extensive genetic diversity, which can be organized in multiple, genetically distinct lineages that arise and die out on a regular basis in endemic regions. A fundamental question for understanding DENV evolution is the relative extent to which stochastic processes (genetic drift) and natural selection acting on fitness differences among lineages contribute to lineage diversity and turnover. Here, we used a set of recently collected and archived low-passage DENV-1 isolates from Thailand to examine the role of mosquito vector-virus interactions in DENV evolution. By comparing the ability of 23 viruses isolated on different dates between 1985 and 2009 to be transmitted by a present-day *Aedes aegypti* population from Thailand, we found that a major clade replacement event in the mid-1990s was associated with virus isolates exhibiting increased titers in the vector's hemocoel, which is predicted to result in a higher probability of transmission. This finding is consistent with the hypothesis that selection for enhanced transmission by mosquitoes is a possible mechanism underlying major DENV clade replacement events. There was significant variation in transmission potential among isolates within each clade, indicating that in addition to vector-driven selection other evolutionary forces act to maintain viral genetic diversity. We conclude that occasional adaptive processes involving the mosquito vector can drive major DENV lineage replacement events.

INTRODUCTION

Worldwide, dengue viruses (DENV) are the most important mosquito-borne viral pathogens of humans. The four antigenically distinct DENV serotypes (DENV-1 to -4) cause a broad spectrum of clinical manifestations. An estimated 50 million people experience dengue illness each year, approximately 500,000 of which are associated with severe, life-threatening disease (18). In addition, a significant portion of infections can be inapparent and, thus, go undetected by surveillance programs (15). Despite the large disease burden imposed by dengue on the human population, there is currently no commercially available DENV vaccine or antiviral therapy (46). In endemic regions where multiple serotypes co-circulate, DENV epidemiological dynamics are characterized by complex oscillations in incidence and serotype prevalence (6, 32, 43). A variety of ecological (10, 24) and immunological factors (1, 36) are thought to govern these complex spatio-temporal dynamics. There is also compelling evidence for the influence of virological factors in disease incidence and severity (reviewed in ref. 37). DENV are single-stranded, positive-sense RNA viruses of the genus *Flavivirus* (family *Flaviviridae*) with extensive genetic diversity (21). Each serotype can be divided into large, genetically diverse phylogenetic clusters, which, in turn, consist of multiple, distinct lineages (22). Hereafter, we use the terms clade and lineage interchangeably. In the last two decades, in-depth phylogenetic analyses have significantly improved understanding of DENV epidemiological and evolutionary dynamics (reviewed in refs. 35, 45).

73 One of the most striking features of DENV evolutionary dynamics is that viral lineages
74 within serotypes arise and die out on a regular basis (22). This lineage turnover is
75 detected in phylogenetic analyses in two different, non-mutually exclusive forms: (i)
76 continuous, "ladder-like" temporal structure within clades and (ii) more dramatic, major
77 clade replacement events. Within a clade, the majority of sub-lineages present at a
78 specific time-point are not detected at later time-points, resulting in a ladder-like tree
79 topology (8, 13, 26). Occasionally, an entire clade that persisted for a number of years at
80 a given location goes extinct as an entirely new clade takes over. Such major clade
81 replacement events have been recurrently documented at regional scales, for example
82 DENV-3 in Sri Lanka in the late 1980s (30), DENV-4 in Puerto Rico during the 1980s-
83 1990s (8), DENV-1 in Thailand in the mid-1990s (48), DENV-3 in Thailand in the early
84 1990s (47), DENV-1 in Myanmar in the late 1990s (31), DENV-2 in Vietnam in the early
85 2000s (43), and DENV-1 in Cambodia in the early 2000s (14). Major clade replacement
86 events have also been reported at larger scales. For example, DENV-2 lineages from
87 Southeast Asia displaced a native DENV-2 lineage in the Americas during the early
88 1990s (38). Although successive clades may be temporally non-overlapping (9), in most
89 cases there is a transition period of co-circulation (14, 30, 31, 43, 47, 48).
90
91 Understanding the causes of DENV lineage birth-and-death has important implications
92 for dengue epidemiology and control because it is often associated with changes in
93 disease incidence and severity (16, 17, 30, 31, 38, 40). Elucidating the mechanisms
94 underlying lineage turnover helps to retrospectively understand spatio-temporal patterns
95 of dengue incidence and to make predictions about the risk of future epidemics (43). It

96 also has implications for vaccine design because DENV lineages may differ in their
97 antigenic properties (39, 44).
98
99 Despite their epidemiological significance, the evolutionary forces underlying major
100 clade replacement events are especially unclear. In particular, the extent to which clade
101 replacement events result from the random sampling of viral variants (genetic drift) due
102 to the stochastic nature of DENV transmission and/or from differences in fitness among
103 variants (i.e., adaptive evolution) is an unresolved question. Analyses of DENV gene
104 sequence data have shown that clade replacement events are sometimes associated with
105 signals of positive natural selection, such as an increase in the rate of nonsynonymous
106 substitutions (7), but not always (26, 47). Some phylogenetic studies have concluded that
107 major clade replacements were primarily due to stochastic events (31, 47), whereas others
108 found potentially causal differences in viral fitness between lineages, such as higher
109 viremia level in the human host (43) or enhanced infectivity to mosquito vectors (3, 5,
110 19).
111
112 Here, we addressed the question whether DENV evolution could be driven by adaptation
113 to mosquito vectors using a set of 23 low-passage DENV-1 isolates from Thailand
114 collected during the period 1985-2009. The 23 isolates were recovered from human
115 serum as part of the routine dengue diagnostic and surveillance services performed at the
116 Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok. We
117 compared the ability of the 23 isolates to be transmitted by *Aedes aegypti*, the principal
118 DENV vector species, in experimental *in vivo* vector competence assays. We

119 hypothesized that if viruses are positively selected for enhanced mosquito transmission, a
120 relative increase in transmission potential should be observed for viruses collected over
121 advancing years. All experiments were carried out during 2009-2010 using the first
122 laboratory-reared generation of mosquitoes derived from a wild *Ae. aegypti* population
123 collected in Kamphaeng Phet, Thailand. Although we cannot be sure that these
124 mosquitoes exactly reproduce the vector-virus interactions that occurred at different dates
125 in the past, they are the most relevant vector population of reference available to test our
126 hypothesis. We estimated virus transmissibility with two distinct, complementary vector
127 competence indices: the proportion of *Ae. aegypti* females that developed a DENV
128 infection that disseminated from their midgut into their hemocoel, a requirement for virus
129 transmission by a mosquito, and the infectious titer of disseminated virus (27).
130

131 # MATERIALS AND METHODS

132

133 **Mosquitoes.** Wild *Ae. aegypti* immatures (larvae and pupae) were collected from a
134 variety of artificial containers in several households in Nhong Pling, Kon Tee, Nakorn
135 Choom, Na Bo Kham, and Thep Na Korn sub-districts, Muang district, Kamphaeng Phet
136 Province, Thailand during July 2009 (experiment 1) and March 2010 (experiment 2). In a
137 preliminary experiment, immatures were collected from containers in villages of
138 Ladkrabang district, Bangkok, and Muang district, Kamphaeng Phet Province, in January
139 2009. F_0 adults were allowed to emerge in the laboratory, mate randomly, and feed on
140 defibrinated sheep blood (National Laboratory Animal Center, Mahidol University,
141 Bangkok, Thailand) through a membrane feeding system. F_1 eggs, which were collected
142 and stored on dry pieces of paper towel and maintained under high humidity, were
143 hatched synchronously by placing them under low pressure for 30 min. Larvae were
144 reared in 24 x 34 x 9 cm plastic trays filled with 2.0 l of dechlorinated tap water at a
145 density of approximately 200 first instars per tray and fed a standard diet of
146 approximately 1.0 g of fish food pellets (C.P. Hi Pro®, Perfect Companion Group Co.
147 Ltd, Bangkok, Thailand) per tray. After emergence, F_1 adults were housed in plastic 30 x
148 30 x 30 cm cages (Megaview Science Education Service Co. Ltd, Taichung, Taiwan)
149 with permanent access to 10% sucrose. They were maintained under standard insectary
150 conditions at 28±1°C, 80% humidity, and with a 12:12 hour light:dark cycle.

151

152 **Virus isolates.** Viruses were originally isolated and archived as seed stocks from serum
153 samples collected during routine surveillance for diagnostic public health testing at

154 AFRIMS from clinically ill dengue patients attending Kamphaeng Phet Provincial
155 Hospital in Kamphaeng Phet (experiment 1) and Queen Sirikit National Institute of Child
156 Health in Bangkok (experiment 2). Virus isolation and identification was performed as
157 previously described (25). Each isolate underwent 3-4 passages in cell culture (Table 1),
158 which, according to standard procedures in our AFRIMS laboratory, is the minimum
159 required to obtain a viral titer sufficiently high to infect mosquitoes orally using an
160 artificial blood meal.
161
162 **Oral challenge.** Two sets of two-day-old confluent cultures of *Aedes albopictus* cells
163 (C6/36, ATCC #CRL-1660^{TM}) in 25 cm^2 flasks (approximately 10^7 cells/flask) were
164 inoculated with 1.0 ml of stock virus per flask and incubated at 35°C under 5% CO_2. A
165 similar titer can be reached by growing virus in C6/36 cells at 35°C for 4-6 days instead
166 of the usual 7-10 days at 28°C. In experiment 1, supernatant was harvested at 5 and 6
167 days post-inoculation to prepare the infectious blood meal of experimental blocks 1 and
168 2, respectively. In experiment 2, supernatant was harvested at 4 days post-inoculation to
169 prepare the infectious blood meal of both experimental blocks. The protocol difference
170 between experiments 1 and 2 was due to logistical constraints. The artificial blood meal
171 consisted of 1:1 mix of defibrinated sheep blood (National Laboratory Animal Center,
172 Mahidol University, Bangkok, Thailand) and virus suspension. Three- to seven-day-old
173 *Ae. aegypti* F_1 females deprived of sucrose and water for 24 hours were offered an
174 infectious blood meal for 30 min through pieces of desalted porcine intestine stretched
175 over water-jacketed glass feeders maintained at 37°C. Samples of the blood meal were
176 saved for subsequent titration by plaque assay. After blood feeding, mosquitoes were

177 briefly sedated with CO_2 from dry ice and fully engorged females were transferred to
178 clean paper cups. Unfed or partially fed females were discarded. Engorged females were
179 maintained under standard insectary conditions and provided cotton soaked with 10%
180 sucrose *ad libitum*.
181
182 **Vector competence.** The ability of DENV isolates to be transmitted by *Ae. aegypti* was
183 assessed at 7 and 14 days post blood meal (pbm) with two vector competence
184 phenotypes: (i) the proportion of mosquitoes that developed a disseminated infection and
185 (ii) the infectious titer of disseminated virus. These two complementary indices of vector
186 competence represent two successive, non-overlapping aspects of the infection process in
187 mosquitoes that lead to their ability to transmit virus, namely that virus disseminates
188 beyond the midgut and the magnitude of infectious titer in the hemocoel. As such, we
189 analyzed them separately. The two periods of extrinsic incubation (7 and 14 days pbm)
190 were chosen to represent early and late phases of dissemination kinetics (28).
191 Relationship between the infectious titer of disseminated virus and the potential for
192 transmission *in vitro* was established in a preliminary experiment (see below). Upon
193 harvest, the head of each female was cut off and placed individually in 1.0 ml of
194 mosquito diluent (MD), consisting of RPMI 1640 medium with 10% heat-inactivated
195 FBS with 100 units/ml penicillin and 100 μg/ml streptomycin. Bodies were kept
196 separately in 1.0 ml of MD. Samples were stored at –70°C before processing. Body and
197 head samples were quickly thawed in a water bath at 35±2°C and homogenized in a
198 mixer mill (Qiagen) at 24 cycles/sec for 2 min. Infected bodies were screened by
199 serotype-specific RT-PCR (experiment 1) or plaque assay (experiment 2). In experiment

200 1, total RNA was extracted from 140 µl of body homogenates using QIAamp viral RNA
201 mini kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed
202 with 5 µl of extracted RNA following a standard protocol (29) with the following
203 modifications: (i) 1X PCR buffer II supplied with Amplitaq DNA Polymerase (Applied
204 Biosystems) was used instead of the original buffer (50 mM KCl, 10 mM Tris pH 8.5 and
205 0.01 mM gelatin) in both the first round RT-PCR and the second round PCR (nested
206 PCR); (ii) the first round RT-PCR reaction contained Avian myeloblastosis virus reverse
207 transcriptase (Promega) instead of rav-2 recombinant reverse transcriptase; (iii) the 1:50
208 dilution of the first round RT-PCR product was used as the template in the nested PCR;
209 (iv) the nested PCR reaction contained 12.5 pmol of each primer instead of 50 pmol; and
210 (v) the number of the nested PCR cycles was increased from 20 cycles to 25 cycles (29).
211 Plaque assay was performed in rhesus monkey kidney cells (LLC-MK_2, ATCC #CCL-
212 7^{TM}) as described previously (42). Briefly, the homogenized samples were passed
213 individually through a 0.22 µm syringe filter unit and 1:2, 1:10 and 1:100 dilutions were
214 prepared in MD. The samples were placed in an ice bath and 100 µl/well were inoculated
215 into a monolayer of LLC-MK_2 cells in 24-well plates. The virus was adsorbed for 1 hour
216 at room temperature (20-28ºC) on a rocker platform. The inoculum was removed and 0.5
217 ml/well of a first overlay of medium was added. The cells were incubated for 5 days at
218 35±1ºC in a 5±0.5% CO_2 incubator. The cells were stained with a second overlay of
219 medium containing 4% neutral red (Sigma). Plaques were counted and plaque forming
220 units (PFU)/ml were calculated. Head samples of infected bodies were titrated by plaque
221 assay in LLC-MK_2 cells. Mosquitoes whose bodies were negative by RT-PCR

222 (experiment 1) or plaque assay (experiment 2) were considered uninfected and their
223 heads were not processed further.
224
225 ***In vitro* transmission.** A preliminary experiment was carried out to define the
226 relationship between the titer of disseminated virus and the potential for *in vitro* virus
227 transmission. This experiment involved two F_1 populations of *Ae. aegypti* sampled during
228 January-February 2009 in Bangkok and Kamphaeng Phet, respectively. Both populations
229 were experimentally exposed to two 2009 DENV-1 isolates from Bangkok (ID#
230 00076/09 and 00088/09) and two 2009 DENV-1 isolates from Kamphaeng Phet (ID#
231 30015/09 and 30025/09). The relationship between the titer of disseminated virus and *in*
232 *vitro* transmission success was evaluated both at 8 and 14 days pbm. Mosquitoes were
233 anesthetized with triethylamine (Sigma-Aldrich), and their legs were removed
234 individually and placed into 1.0 ml of MD. Saliva samples were collected using a forced
235 salivation technique (2). Briefly, mosquito mouthparts were inserted for 15 min into a
236 glass microcapillary tube filled with approximately 10 µl of FBS. After 15 min, the
237 contents of the microcapillary tube were placed into 0.3 ml of MD and snap-frozen in dry
238 ice. The remainder of the mosquito bodies were placed individually into 1.0 ml of MD,
239 and all samples were stored at –70°C before processing. Body and leg samples were
240 quickly thawed in a water bath at 35±2°C and homogenized in a mixer mill. Infected
241 bodies were screened by serotype-specific RT-PCR as described above. The titer of
242 disseminated virus in the legs of mosquitoes whose body was positive by RT-PCR was
243 determined by plaque assay in LLC-MK_2 cells as described above. Mosquitoes whose
244 bodies were negative by RT-PCR were considered uninfected and their legs were not

245 processed further. The presence of virus in the saliva samples from mosquitoes with a
246 disseminated infection was tested by plaque assay in LLC-MK_2 cells.
247
248 **Sequencing and phylogenetic analysis.** Viral RNA extracted by QIAamp viral RNA
249 mini kit was converted to cDNA using Transcriptor High Fidelity kit (Roche) and random
250 hexamer oligonucleotides according to the manufacturer's instructions. Sequencing of *E*
251 genes and complete genomes was performed following previously described methods
252 (48). The DNA fragments of the *E* gene and the overlapping DNA fragments covering the
253 entire DENV-1 genome were amplified using Amplitaq DNA Polymerase (Applied
254 Biosystems) and purified using QIAquick PCR purification kit and QIAquick gel
255 extraction kit (Qiagen). Purified DNA fragments were used as templates in cycle
256 sequencing reactions using the DYEnamic ET dye terminator sequencing kit (GE
257 Healthcare) according to the manufacturer's instructions. The sequencing primers were
258 described previously (48). The sequencing products were purified by standard ethanol
259 precipitation before sequencing in a MegaBACE 500 automated DNA sequencer (GE
260 Healthcare). The overlapping sequences obtained from forward and reverse primers were
261 combined for analysis and edited by using the Sequencher software (Gene Code
262 Corporation). Phylogenetic trees were constructed with PAUP* version 4.0 (41) using a
263 maximum likelihood (ML) method. ML trees included the new sequences generated in
264 this study and 17 "background" DENV-1 sequences from GenBank. The best model of
265 nucleotide substitution was chosen with jModelTest (33) based on the lowest akaike
266 information criterion (AIC) value (34). The TIM2+Γ and GTR+Γ models of nucleotide
267 substitution were applied to construct the trees for *E* gene and complete genome

268 sequences, respectively. Reliability of particular phylogenetic groupings was calculated
269 using bootstrap resampling analysis with 1,000 replicate neighbor-joining (NJ) trees
270 under the ML substitution models. All sequences have been submitted to GenBank and
271 assigned accession numbers JN638322-JN638344.
272
273 **Data analysis.** All statistical analyses of vector competence data included the
274 confounding effects of the experiment and the experimental block. The study was run in
275 two separate experiments that involved two different sets of DENV isolates and used a
276 population of *Ae. aegypti* that was sampled at two different times. In both experiments,
277 the same batch of mosquitoes was exposed twice to the same set of DENV isolates on
278 two successive days (i.e., two experimental blocks). In the two blocks, viruses came from
279 the same passage in cell culture but were harvested separately (see above). The effect of
280 the block was thus nested within each experiment and accounted for the difference in titer
281 between the two harvests, the effect of the one-day difference in mosquito age, and the
282 effect of any uncontrolled differences between the two days. The isolate effect was nested
283 within each experiment and clade because each isolate belonged to only one phylogenetic
284 clade and each experiment involved a different set of isolates. Blood meal titers were log-
285 transformed (to ensure normal distribution of the residuals) and compared using a
286 multifactorial analysis of variance (MANOVA) accounting for the effects of clade,
287 experiment, block and their interactions. The proportion of mosquitoes with a
288 disseminated infection was analyzed with a nominal logistic regression that included the
289 effects of blood meal titer, experiment, experimental block, clade, isolate and their
290 interactions. The titer of disseminated virus was log-transformed (to ensure normal

291 distribution of the residuals) and analyzed with a MANOVA that included the effects of

292 blood meal titer, experiment, experimental block, clade, isolate and their interactions.

293 Differences were considered statistically significant at $P<0.05$. Analyses were performed

294 with the software JMP version 5.1.2.

295

296 ## RESULTS

297

298 **Estimation of transmission potential.** Transmission potential was defined as the

299 presence of infectious virus in the mosquito's saliva. In a preliminary experiment, a total

300 of 322 individual *Ae. aegypti* females that developed a disseminated infection following

301 oral challenge were tested for virus titer in their legs and virus presence in their saliva

302 collected *in vitro*. The data included two F_1 *Ae. aegypti* populations (sampled in Bangkok

303 and Kamphaeng Phet, respectively), four DENV-1 isolates (two from Bangkok and two

304 from Kamphaeng Phet), and two extrinsic incubation periods (8 and 14 days pbm). Leg

305 titers ranged from 1×10^1 to 2.3×10^4 plaque forming units per ml (PFU/ml) and

306 infectious virus was detected in 7.5% of saliva samples. The probability of detecting

307 infectious virus in saliva was positively correlated with the infectious titer of

308 disseminated virus (Fig. 1). Logistic regression showed that the leg titer was a highly

309 significant predictor of virus detection in saliva (df=1; L-R χ^2=15.48; P<0.0001). In

310 particular, no virus was detected in any of the saliva samples from mosquitoes with a leg

311 titer < 1×10^3 PFU/ml. For the rest of the study, the infectious titer of disseminated virus

312 was used as an estimate of transmission potential. Because the number of mosquito legs

313 may vary among individuals (they can lose them over time), the head titer was used

314 instead of the leg titer. Previous data showed that the leg titer and the head titer are

315 strongly correlated in individual *Ae. aegypti* (27).

316

317 **Phylogenetic relationships between DENV isolates.** All but two of the 23 DENV-1

318 isolates were passaged four times in cell culture (Table 1) prior to their use in

319 experimental mosquito infections; two were passaged three times. The study was run in
320 two separate experiments involving two different sets of virus isolates (11 in the first
321 experiment and 12 in the second) and *Ae. aegypti* mosquitoes sampled at two different
322 times. Phylogenetic analysis based on the complete DENV *E* gene sequence indicated
323 that the virus isolates could be divided into two major phylogenetic clades within the
324 genotype I of DENV-1 (Fig. 2A). The first clade included 8 isolates sampled between
325 1985 and 1995; the second clade included 15 isolates sampled between 1992 and 2009.
326 Hereafter, the first and second clades are termed "early clade" and "new clade",
327 respectively, in reference to the chronological order of their detection. Complete genome
328 sequences obtained for a selection of 9 isolates sampled between 1986 and 1997 (6 from
329 the early clade and 3 from the new clade) confirmed the phylogenetic relationships
330 inferred using the *E* gene sequence (Fig. 2B).
331
332 **Variation in transmission potential among DENV isolates.** Vector competence assays
333 included a total of 738 individual *Ae. aegypti* females. Transmission potential was
334 characterized based on 7-44 (mean 32) individuals per isolate. Infectious titer of artificial
335 blood meals used in vector competence assays varied among isolates (Fig. 3), ranging
336 from 3.5×10^4 PFU/ml to 3.0×10^6 PFU/ml. Blood meal titers were significantly higher
337 in the second experiment (SS=3.251; df=1; F=19.63; P<0.0001), but they were not
338 significantly different between blocks within experiments (SS=0.574; df=2; F=1.734;
339 P=0.191). The higher blood meal titers in experiment 2 may have resulted from the
340 different harvest date post-inoculation in cell culture (day 4) compared to experiment 1
341 (day 5-6). Importantly, blood meal titers did not differ between clades (SS=0.439; df=1;

342 F=2.649; P=0.112) (Fig. 2). In the early clade, blood meal titers ranged from 4.5 x 10^4

343 PFU/ml to 3.0 x 10^6 PFU/ml (mean 7.0 x 10^5 PFU/ml). In the new clade, blood meal

344 titers ranged from 3.5 x 10^4 PFU/ml and 2.4 x 10^6 PFU/ml (mean 8.2 x 10^5 PFU/ml).

345

346 Overall, about two thirds of mosquitoes were infected (65.6% at 7 days pbm and 69.6%

347 at 14 days pbm). Nearly all infected females developed disseminated infections within the

348 first two weeks pbm (52.9% at 7 days pbm and 94.6% at 14 days pbm). The percentage

349 of females with a disseminated infection (including uninfected individuals) varied

350 significantly among isolates at both 7 and 14 days pbm (Table 2). It ranged from 0 to

351 81% at 7 days pbm (mean 32%) and from 20 to 100% (mean 64%) at 14 days pbm. The

352 percentage of females with a disseminated infection was not significantly influenced by

353 the blood meal titer, but it was significantly higher in the first than in the second

354 experiment at 14 days pbm (Table 2). The higher level of dissemination in the second

355 experiment may have been due to either the different geographical origin of viruses

356 (Bangkok *vs*. Kamphaeng Phet) or any uncontrolled, environmental difference between

357 the two experiments. There was no significant difference between the early and new

358 clade in the mean percentage of females with a disseminated infection at either 7 or 14

359 days pbm (Table 2). Dissemination tended to increase as a function of the year of virus

360 isolation in the early clade whereas it tended to decrease as a function of the year of virus

361 isolation in the new clade, both at 7 days pbm (Fig. 4A) and 14 days pbm (Fig. 4B).

362 These trends may have been a consequence of variation in blood meal titers, which

363 showed similar patterns (Fig. 3).

364

365 Among females with a disseminated infection, head titers ranged from 1.0 x 10^1 to 2.3 x
366 10^4 PFU/ml at 7 days pbm (N=128, mean 5.4 x 10^2 PFU/ml, median 3.0 x 10^1 PFU/ml),
367 and from 1.0 x 10^1 to 2.9 x 10^4 PFU/ml at 14 days pbm (N=243, mean 3.4 x 10^3 PFU/ml,
368 median 1.5 x 10^3 PFU/ml). The titer of disseminated virus was not significantly
369 influenced by the blood meal titer; it differed significantly among isolates at 14 days pbm
370 (Table 3). The mean head titer of the new clade was significantly higher than that of the
371 early clade at 14 days pbm (Table 3; Fig. 5). At 7 days pbm, the new clade had a higher
372 mean head titer than the early clade, but the difference was marginally insignificant
373 (Table 3; Fig. 5). The statistical power at 7 days pbm, however, was reduced compared to
374 14 days pbm because the number of mosquitoes with a disseminated infection was half as
375 large. At 7 days pbm the head titer tended to increase slightly as a function of the year of
376 virus isolation in the new clade, whereas it did not vary temporally in the early clade (Fig.
377 4C). At 14 days pbm, the head titer tended to increase as a function of the year of virus
378 isolation in the early clade, whereas it did not vary temporally in the new clade (Fig. 4D).
379
380
381

382 **DISCUSSION**

383

384 By comparing the ability of 23 DENV-1 isolates from Thailand spanning a 24-year
385 period to infect and be transmitted by *Ae. aegypti*, we found that a major clade
386 replacement event in the mid-1990s was associated with a higher transmission potential
387 of the isolates belonging to the new clade. Higher transmissibility was mainly due to a
388 higher infectious titer of virus in the vector's hemocoel, which is predicted to result in a
389 higher probability of transmission. This finding supports the hypothesis that major clade
390 replacement events can be driven by natural selection and emphasizes the potentially
391 important role of vector-virus interactions in DENV evolution.

392

393 The major clade replacement event revealed by the phylogenetic analysis of the 23
394 isolates of our study was described previously (48). In their study, Zhang *et al*. (48)
395 observed that the DENV-1 clade replacement was associated with a decline in DENV-1
396 prevalence and a concomitant rise of DENV-4 in Thailand. They speculated that clade
397 replacement events might result from differential susceptibility to cross-reactive immune
398 responses in the human host. Here, we provide a non-mutually exclusive, alternative
399 explanation. Our data suggest that this clade replacement event may have been driven, at
400 least in part, by adaptation to mosquito vectors. Even though it occurred in a context of
401 declining DENV-1 incidence, the new clade may have outcompeted the early clade
402 through enhanced mosquito transmission. Isolates in the new clade had a higher
403 dissemination titer, which is expected to result in higher viral fitness because the

404 probability of transmission by a mosquito is positively correlated with the quantity of

405 virus that circulates in the insect's hemocoel.

406

407 Our estimate of DENV transmission potential relied on an experiment demonstrating a

408 positive correlation between the titer of disseminated virus and the probability of virus

409 detection in the mosquito's saliva (Fig. 1). Virus presence in the saliva was determined

410 using an established *in vitro* method (2) that likely underestimates transmission

411 probability because it may fail to detect small, but transmissible amounts of virus. Indeed,

412 infectious virus was only detected in the saliva of 7.5% of females with a disseminated

413 infection. Our analysis showed, however, that the probability to detect infectious virus in

414 saliva samples was positively correlated with the infectious titer of disseminated virus,

415 across two different *Ae. aegypti* populations and four different DENV-1 isolates. In

416 particular, there was a lower threshold titer below which none of the mosquitoes with a

417 disseminated infection had a positive saliva sample. Although the actual threshold may be

418 lower than we estimate due to the relatively low sensitivity of the method, our data

419 established that the titer of disseminated virus is a strong predictor of transmission

420 probability in this system.

421

422 It is worth noting that we did not consider other entomological parameters underlying

423 DENV transmission such as vector survival or biting rate, which could potentially vary

424 among viruses. Future studies will determine whether variation of these traits contribute

425 to fitness differences among viruses. Another limitation of the study was that adaptation

426 to cell culture could have occurred during the 3-4 passages of virus amplification prior to

427 their use in experimental infections of mosquitoes. The selection regime, however, was
428 the same for all isolates, thus comparisons among vector competence phenotypes
429 remained meaningful. The mechanisms underlying the phenotypic differences we
430 observed between the two clades remain unknown. Authors of a recent study suggested
431 that variation in vector competence among DENV-2 lineages does not depend on
432 mosquito midgut binding affinity, but rather on replication ability (12).
433
434 Investigators who preceded us and studied DENV-2 and DENV-3 indicated that vector-
435 driven selection could play an important role in DENV evolution. By comparing the
436 ability of three DENV-3 isolates of a native lineage and three DENV-3 isolates of an
437 invasive lineage to be transmitted by *Ae. aegypti*, Hanley *et al*. (19) showed that a clade
438 replacement event in Sri Lanka during the 1980s was associated with enhanced mosquito
439 transmission potential for the invasive lineage. Enhanced transmissibility was associated
440 with a higher body titer and an increased proportion of *Ae. aegypti* females with a
441 disseminated infection (19). At a larger geographic scale, Rico-Hesse and others
442 suggested in a series of studies that displacement of a native DENV-2 lineage in the
443 Americas by Southeast Asian DENV-2 lineages during the early 1990s was driven in part
444 by adaptation to mosquitoes (3-5, 11). In both the DENV-3 and DENV-2 cases, invasive
445 lineages were associated with more severe disease in humans, which highlights the
446 critical need to more fully explore the connection between DENV evolutionary biology
447 and dengue epidemiology.
448

449 Our study helps to understand the respective contributions of stochastic and adaptive
450 processes in DENV evolution. Frequent introductions of new lineages (gene flow) are
451 known to maintain substantial DENV genetic diversity even at a very local scale (23). In
452 contrast, elimination of deleterious mutations (purifying selection) strongly limits *in situ*
453 evolution of lineages (23). Purifying selection is likely the dominant evolutionary force in
454 DENV evolution (20), and nucleotide fixation events are due primarily to genetic drift
455 (13, 20, 26). Although stochastic processes are undoubtedly important in DENV
456 evolution, the present study indicates that more dramatic major clade replacement events
457 may have an adaptive basis (43). That extinction of the early DENV-1 clade was
458 concomitant with rise of the new clade, with an overlapping period in the early 1990s
459 (48), was suggestive of a fitness difference between the two clades. The relatively modest
460 average fitness difference between the two clades (a 0.5-log difference in head titer) may
461 explain why they co-circulated for at least four years before the early clade went extinct.
462 It is worth noting that we also observed significant variation in vector competence indices
463 within each clade (Fig. 4), indicating that other evolutionary forces than vector-driven
464 selection promote DENV genetic diversity. In particular, the temporal pattern within each
465 clade did not provide a clear indication that adaptation to mosquitoes occurs continuously
466 through time (Fig. 4). We speculate that the temporal trends observed within clades
467 resulted from uncontrolled variation in experimental blood meal titers (Fig. 3), but this
468 merits further investigation.
469
470 Although other selective forces may be important drivers of DENV evolution, especially
471 those occurring in the human host that we did not examine, we provide evidence for the

472 possible role of adaptation to mosquito vectors in a major DENV-1 clade replacement
473 event in Thailand in the early 1990s. Our results reinforce the idea that vector-virus
474 interactions can play a critical role in DENV epidemiological and evolutionary dynamics,
475 and suggest that the role of vector-mediated selection be investigated in other instances of
476 clade replacement.
477

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L.L. conceived and coordinated the study, designed the research, analyzed the data and wrote the manuscript. T.F. and A.P. performed the field collection of mosquitoes and vector competence assays. B.T. supervised the isolation, amplification and titration of viruses. C.K. supervised the molecular detection and sequencing of viruses, and performed the phylogenetic analyses. J.H.R. and A.P. coordinated the field collection of mosquitoes and vector competence assays. R.G.J. coordinated the collection of virus isolates, virological assays, and phylogenetic analyses. T.W.S. conceived and coordinated

501 the study, and helped to design the research and write the manuscript. All authors read

502 and approved the final manuscript.

503

504 **REFERENCES**

505

506 1. **Adams, B., E. C. Holmes, C. Zhang, M. P. Mammen, Jr., S. Nimmannitya, S.
507 Kalayanarooj, and M. Boots.** 2006. Cross-protective immunity can account for
508 the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok.
509 Proc Natl Acad Sci U S A **103:**14234-14239.

510 2. **Aitken, T. H. G.** 1977. An *in vitro* feeding technique for artificially
511 demonstrating virus transmission by mosquitoes. Mosq News **37:**130-133.

512 3. **Anderson, J. R., and R. Rico-Hesse.** 2006. *Aedes aegypti* vectorial capacity is
513 determined by the infecting genotype of dengue virus. Am J Trop Med Hyg
514 **75:**886-892.

515 4. **Armstrong, P. M., and R. Rico-Hesse.** 2001. Differential susceptibility of *Aedes*
516 *aegypti* to infection by the American and Southeast Asian genotypes of dengue
517 type 2 virus. Vector Borne Zoonotic Dis **1:**159-168.

518 5. **Armstrong, P. M., and R. Rico-Hesse.** 2003. Efficiency of dengue serotype 2
519 virus strains to infect and disseminate in *Aedes aegypti*. Am J Trop Med Hyg
520 **68:**539-544.

521 6. **Bennett, S. N., A. J. Drummond, D. D. Kapan, M. A. Suchard, J. L. Munoz-
522 Jordan, O. G. Pybus, E. C. Holmes, and D. J. Gubler.** 2010. Epidemic
523 dynamics revealed in dengue evolution. Mol Biol Evol **27:**811-818.

524 7. **Bennett, S. N., E. C. Holmes, M. Chirivella, D. M. Rodriguez, M. Beltran, V.
525 Vorndam, D. J. Gubler, and W. O. McMillan.** 2006. Molecular evolution of

526 dengue 2 virus in Puerto Rico: positive selection in the viral envelope
527 accompanies clade reintroduction. J Gen Virol **87:**885-893.
528 8. **Bennett, S. N., E. C. Holmes, M. Chirivella, D. M. Rodriguez, M. Beltran, V.**
529 **Vorndam, D. J. Gubler, and W. O. McMillan.** 2003. Selection-driven evolution
530 of emergent dengue virus. Mol Biol Evol **20:**1650-1658.
531 9. **Carrillo-Valenzo, E., R. Danis-Lozano, J. X. Velasco-Hernandez, G. Sanchez-**
532 **Burgos, C. Alpuche, I. Lopez, C. Rosales, C. Baronti, X. de Lamballerie, E.**
533 **C. Holmes, and J. Ramos-Castaneda.** 2010. Evolution of dengue virus in
534 Mexico is characterized by frequent lineage replacement. Arch Virol **155:**1401-
535 1412.
536 10. **Cazelles, B., M. Chavez, A. J. McMichael, and S. Hales.** 2005. Nonstationary
537 influence of El Nino on the synchronous dengue epidemics in Thailand. PLoS
538 Med **2:**e106.
539 11. **Cologna, R., P. M. Armstrong, and R. Rico-Hesse.** 2005. Selection for virulent
540 dengue viruses occurs in humans and mosquitoes. J Virol **79:**853-859.
541 12. **Cox, J., H. E. Brown, and R. Rico-Hesse.** 2011. Variation in vector competence
542 for dengue viruses does not depend on mosquito midgut binding affinity. PLoS
543 Negl Trop Dis **5:**e1172.
544 13. **Descloux, E., V. M. Cao-Lormeau, C. Roche, and X. De Lamballerie. 2009.**
545 Dengue 1 diversity and microevolution, French Polynesia 2001-2006: connection
546 with epidemiology and clinics. PLoS Negl Trop Dis **3:**e493.
547 14. **Duong, V., C. Simmons, L. Gavotte, A. Viari, S. Ong, N. Chantha, N. J.**
548 **Lennon, B. W. Birren, S. Vong, J. J. Farrar, M. R. Henn, V. Deubel, R.**

549 **Frutos, and P. Buchy.** 2011. Genetic diversity and lineage dynamic of dengue
550 virus serotype 1 (DENV-1) in Cambodia. Infect Genet Evol, in press.
551 15. **Endy, T. P., K. B. Anderson, A. Nisalak, I. K. Yoon, S. Green, A. L.**
552 **Rothman, S. J. Thomas, R. G. Jarman, D. H. Libraty, and R. V. Gibbons.**
553 2011. Determinants of inapparent and symptomatic dengue infection in a
554 prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS
555 Negl Trop Dis **5:**e975.
556 16. **Gubler, D. J., D. Reed, L. Rosen, and J. R. Hitchcock, Jr.** 1978.
557 Epidemiologic, clinical, and virologic observations on dengue in the Kingdom of
558 Tonga. Am J Trop Med Hyg **27:**581-589.
559 17. **Gubler, D. J., W. Suharyono, I. Lubis, S. Eram, and S. Gunarso.** 1981.
560 Epidemic dengue 3 in central Java, associated with low viremia in man. Am J
561 Trop Med Hyg **30:**1094-1099.
562 18. **Guzman, M. G., S. B. Halstead, H. Artsob, P. Buchy, J. Farrar, D. J. Gubler,**
563 **E. Hunsperger, A. Kroeger, H. S. Margolis, E. Martinez, M. B. Nathan, J. L.**
564 **Pelegrino, C. Simmons, S. Yoksan, and R. W. Peeling.** 2010. Dengue: a
565 continuing global threat. Nat Rev Microbiol **8:**S7-16.
566 19. **Hanley, K. A., J. T. Nelson, E. E. Schirtzinger, S. S. Whitehead, and C. T.**
567 **Hanson.** 2008. Superior infectivity for mosquito vectors contributes to
568 competitive displacement among strains of dengue virus. BMC Ecol **8:**1.
569 20. **Holmes, E. C.** 2003. Patterns of intra- and interhost nonsynonymous variation
570 reveal strong purifying selection in dengue virus. J Virol **77:**11296-11298.

571 21. **Holmes, E. C., and S. S. Burch.** 2000. The causes and consequences of genetic
572 variation in dengue virus. Trends Microbiol **8:**74-77.
573 22. **Holmes, E. C., and S. S. Twiddy.** 2003. The origin, emergence and evolutionary
574 genetics of dengue virus. Infect Genet Evol **3:**19-28.
575 23. **Jarman, R. G., E. C. Holmes, P. Rodpradit, C. Klungthong, R. V. Gibbons,**
576 **A. Nisalak, A. L. Rothman, D. H. Libraty, F. A. Ennis, M. P. Mammen, Jr.,**
577 **and T. P. Endy.** 2008. Microevolution of Dengue viruses circulating among
578 primary school children in Kamphaeng Phet, Thailand. J Virol **82:**5494-5500.
579 24. **Johansson, M. A., F. Dominici, and G. E. Glass.** 2009. Local and global effects
580 of climate on dengue transmission in puerto rico. PLoS Negl Trop Dis **3:**e382.
581 25. **Klungthong, C., R. V. Gibbons, B. Thaisomboonsuk, A. Nisalak, S.**
582 **Kalayanarooj, V. Thirawuth, N. Nutkumhang, M. P. Mammen, Jr., and R.**
583 **G. Jarman.** 2007. Dengue virus detection using whole blood for reverse
584 transcriptase PCR and virus isolation. J Clin Microbiol **45:**2480-2485.
585 26. **Klungthong, C., C. Zhang, M. P. Mammen, Jr., S. Ubol, and E. C. Holmes.**
586 2004. The molecular epidemiology of dengue virus serotype 4 in Bangkok,
587 Thailand. Virology **329:**168-179.
588 27. **Lambrechts, L., C. Chevillon, R. G. Albright, B. Thaisomboonsuk, J. H.**
589 **Richardson, R. G. Jarman, and T. W. Scott.** 2009. Genetic specificity and
590 potential for local adaptation between dengue viruses and mosquito vectors. BMC
591 Evol Biol **9:**160.
592 28. **Lambrechts, L., K. P. Paaijmans, T. Fansiri, L. B. Carrington, L. D. Kramer,**
593 **M. B. Thomas, and T. W. Scott.** 2011. Impact of daily temperature fluctuations

594 on dengue virus transmission by *Aedes aegypti*. Proc Natl Acad Sci U S A
595 **108:**7460-7465.
596 29. **Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V.**
597 **Vorndam.** 1992. Rapid detection and typing of dengue viruses from clinical
598 samples by using reverse transcriptase-polymerase chain reaction. J Clin
599 Microbiol **30:**545-551.
600 30. **Messer, W. B., D. J. Gubler, E. Harris, K. Sivananthan, and A. M. de Silva.**
601 2003. Emergence and global spread of a dengue serotype 3, subtype III virus.
602 Emerg Infect Dis **9:**800-809.
603 31. **Myat Thu, H., K. Lowry, L. Jiang, T. Hlaing, E. C. Holmes, and J. Aaskov.**
604 2005. Lineage extinction and replacement in dengue type 1 virus populations are
605 due to stochastic events rather than to natural selection. Virology **336:**163-172.
606 32. **Nisalak, A., T. P. Endy, S. Nimmannitya, S. Kalayanarooj, U. Thisayakorn,**
607 **R. M. Scott, D. S. Burke, C. H. Hoke, B. L. Innis, and D. W. Vaughn.** 2003.
608 Serotype-specific dengue virus circulation and dengue disease in Bangkok,
609 Thailand from 1973 to 1999. Am J Trop Med Hyg **68:**191-202.
610 33. **Posada, D.** 2008. jModelTest: phylogenetic model averaging. Mol Biol Evol
611 **25:**1253-1256.
612 34. **Posada, D., and T. R. Buckley.** 2004. Model selection and model averaging in
613 phylogenetics: advantages of akaike information criterion and bayesian
614 approaches over likelihood ratio tests. Syst Biol **53:**793-808.
615 35. **Pybus, O. G., and A. Rambaut.** 2009. Evolutionary analysis of the dynamics of
616 viral infectious disease. Nat Rev Genet **10:**540-550.

617 36. **Recker, M., K. B. Blyuss, C. P. Simmons, T. T. Hien, B. Wills, J. Farrar, and**
618 **S. Gupta.** 2009. Immunological serotype interactions and their effect on the
619 epidemiological pattern of dengue. Proc Biol Sci **276:**2541-2548.
620 37. **Rico-Hesse, R.** 2003. Microevolution and virulence of dengue viruses. Adv Virus
621 Res **59:**315-341.
622 38. **Rico-Hesse, R., L. M. Harrison, R. A. Salas, D. Tovar, A. Nisalak, C. Ramos,**
623 **J. Boshell, M. T. de Mesa, R. M. Nogueira, and A. T. da Rosa.** 1997. Origins
624 of dengue type 2 viruses associated with increased pathogenicity in the Americas.
625 Virology **230:**244-251.
626 39. **Shrestha, B., J. D. Brien, S. Sukupolvi-Petty, S. K. Austin, M. A. Edeling, T.**
627 **Kim, K. M. O'Brien, C. A. Nelson, S. Johnson, D. H. Fremont, and M. S.**
628 **Diamond.** 2010. The development of therapeutic antibodies that neutralize
629 homologous and heterologous genotypes of dengue virus type 1. PLoS Pathog
630 **6:**e1000823.
631 40. **Steel, A., D. J. Gubler, and S. N. Bennett.** 2010. Natural attenuation of dengue
632 virus type-2 after a series of island outbreaks: a retrospective phylogenetic study
633 of events in the South Pacific three decades ago. Virology **405:**505-512.
634 41. **Swofford, D. L.** 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and
635 Other Methods), 4.0 ed. Sinauer Associates, Inc., Sunderland, MA.
636 42. **Thomas, S. J., A. Nisalak, K. B. Anderson, D. H. Libraty, S. Kalayanarooj, D.**
637 **W. Vaughn, R. Putnak, R. V. Gibbons, R. Jarman, and T. P. Endy.** 2009.
638 Dengue plaque reduction neutralization test (PRNT) in primary and secondary

639 dengue virus infections: How alterations in assay conditions impact performance.
640 Am J Trop Med Hyg **81:**825-833.
641 43. **Vu, T. T., E. C. Holmes, V. Duong, T. Q. Nguyen, T. H. Tran, M. Quail, C.**
642 **Churcher, J. Parkhill, J. Cardosa, J. Farrar, B. Wills, N. J. Lennon, B. W.**
643 **Birren, P. Buchy, M. R. Henn, and C. P. Simmons.** 2010. Emergence of the
644 Asian 1 genotype of dengue virus serotype 2 in Vietnam: *in vivo* fitness advantage
645 and lineage replacement in South-East Asia. PLoS Negl Trop Dis **4:**e757.
646 44. **Wahala, W. M., E. F. Donaldson, R. de Alwis, M. A. Accavitti-Loper, R. S.**
647 **Baric, and A. M. de Silva.** 2010. Natural strain variation and antibody
648 neutralization of dengue serotype 3 viruses. PLoS Pathog **6:**e1000821.
649 45. **Weaver, S. C., and N. Vasilakis.** 2009. Molecular evolution of dengue viruses:
650 contributions of phylogenetics to understanding the history and epidemiology of
651 the preeminent arboviral disease. Infect Genet Evol **9:**523-540.
652 46. **Wilder-Smith, A., E. E. Ooi, S. G. Vasudevan, and D. J. Gubler.** 2010. Update
653 on dengue: epidemiology, virus evolution, antiviral drugs, and vaccine
654 development. Curr Infect Dis Rep **12:**157-164.
655 47. **Wittke, V., T. E. Robb, H. M. Thu, A. Nisalak, S. Nimmannitya, S.**
656 **Kalayanrooj, D. W. Vaughn, T. P. Endy, E. C. Holmes, and J. G. Aaskov.**
657 2002. Extinction and rapid emergence of strains of dengue 3 virus during an
658 interepidemic period. Virology **301:**148-156.
659 48. **Zhang, C., M. P. Mammen, Jr., P. Chinnawirotpisan, C. Klungthong, P.**
660 **Rodpradit, P. Monkongdee, S. Nimmannitya, S. Kalayanarooj, and E. C.**

661 **Holmes.** 2005. Clade replacements in dengue virus serotypes 1 and 3 are
662 associated with changing serotype prevalence. J Virol **79:**15123-15130.
663
664

665 **Table 1. DENV-1 isolates used in experimental mosquito infections.** For each isolate,
666 the year of virus isolation, location of origin, number of passages in cell culture, and
667 phylogenetic group (clade) are indicated. KPPH: Kamphaeng Phet Provincial Hospital;
668 QSNICH: Queen Sirikit National Institute for Child Health (Bangkok); TS:
669 *Toxorhynchites splendens* mosquitoes; C6/36: *Aedes albopictus* cells.
670

Experiment	Isolation year	Isolate ID	Source	Passage History	Clade
1	1986	KD86-035	KPPH	TS-1, C6/36-3	Early
	1990	KD90-157	KPPH	TS-1, C6/36-2	Early
	1992	KD92-080	KPPH	TS-1, C6/36-2	Early
	1995	30399/95	KPPH	C6/36-4	New
	1997	30231/97	KPPH	C6/36-4	New
	2000	30529/00	KPPH	C6/36-4	New
	2003	30247/03	KPPH	C6/36-4	New
	2005	30230/05	KPPH	C6/36-4	New
	2006	30118/06	KPPH	C6/36-4	New
	2009	30015/09	KPPH	C6/36-4	New
	2009	30025/09	KPPH	C6/36-4	New
2	1985	D85-372	QSNICH	TS-1, C6/36-3	Early
	1986	D86-412	QSNICH	TS-1, C6/36-3	Early
	1987	D87-116	QSNICH	TS-1, C6/36-3	Early
	1990	D90-1197	QSNICH	TS-1, C6/36-3	Early
	1992	03881/92	QSNICH	TS-1, C6/36-3	New

	1995	00407/95	QSNICH	TS-1, C6/36-3	Early
	1997	00616/97	QSNICH	TS-1, C6/36-3	New
	2000	04805/00	QSNICH	TS-1, C6/36-3	New
	2003	01417/03	QSNICH	TS-1, C6/36-3	New
	2005	00442/05	QSNICH	TS-1, C6/36-3	New
	2007	02128/07	QSNICH	C6/36-4	New
	2009	00132/09	QSNICH	C6/36-4	New

671

672

673 **Table 2. Logistic regression analysis of dissemination prevalence 7 and 14 days post-**

674 **blood meal among mosquitoes experimentally exposed to different DENV-1 isolates**.

675 df: degrees of freedom; L-R: likelihood ratio. **P*<0.05; ***P*<0.01.

676

		Dissemination 7 days pbm		**Dissemination 14 days pbm**	
Source	**df**	**L-R χ^2**	***P*-value**	**L-R χ^2**	***P*-value**
Blood meal titer	1	1.451	0.228	0.749	0.387
Experiment	1	1.058	0.304	11.45	0.001**
Clade	1	0.877	0.349	3.619	0.057
Experiment*Clade	1	0.443	0.506	0.461	0.497
Block [within Experiment]	2	1.604	0.448	11.34	0.003**
Block*Clade [within Experiment]	2	0.080	0.961	4.236	0.120
Isolate [within Experiment, Clade]	19	34.82	0.015*	46.21	0.001**

677

678

679 **Table 3. Analysis of variance of head titers at 7 and 14 days post-blood meal among**
680 **mosquitoes experimentally exposed to different DENV-1 isolates**. Titers were log-
681 transformed to satisfy statistical assumptions. The analysis is performed on individual
682 females with a disseminated infection (i.e., excluding those that did not develop a
683 disseminated infection). df: degrees of freedom; SS: sum of squares. *$P<0.05$; **$P<0.01$.
684

	Head titer 7 days pbm				**Head titer 14 days pbm**			
Source	**df**	**SS**	**F ratio**	***P*-value**	**df**	**SS**	**F ratio**	***P*-value**
Blood meal titer	1	2.005	4.086	0.046*	1	0.027	0.048	0.828
Experiment	1	2.651	5.403	0.022*	1	0.353	0.633	0.427
Clade	1	1.898	3.869	0.052	1	5.494	9.838	0.002**
Experiment*Clade	1	0.235	0.479	0.491	1	0.007	0.013	0.908
Block [within Experiment]	2	0.787	0.802	0.451	2	0.483	0.433	0.649
Block*Clade [within Experiment]	2	3.215	3.276	0.042*	2	3.153	2.823	0.062
Isolate [within Experiment, Clade]	19	15.60	1.673	0.054	19	26.76	2.522	0.001**
Error	100	49.06			215	120.1		

685
686

687 **FIGURE LEGENDS**

688

689 **Figure 1. Mosquito transmission potential strongly correlates with infectious titer of**
690 **disseminated DENV-1**. The percentage of female *Ae. aegypti* tested positive for
691 infectious virus in saliva samples collected *in vitro* is shown as a function of infectious
692 virus titers in their legs at either 8 or 14 days post-blood meal. Bars represent individuals
693 with a leg liter > the lower value of the corresponding interval on the *x*-axis. Data shown
694 is pooled from vector competence assays involving two mosquito populations exposed to
695 four different DENV-1 isolates (see Materials and Methods for details). Dotted, vertical
696 lines indicate the 95% confidence intervals of percentages. Sample sizes are indicated
697 above the bars.

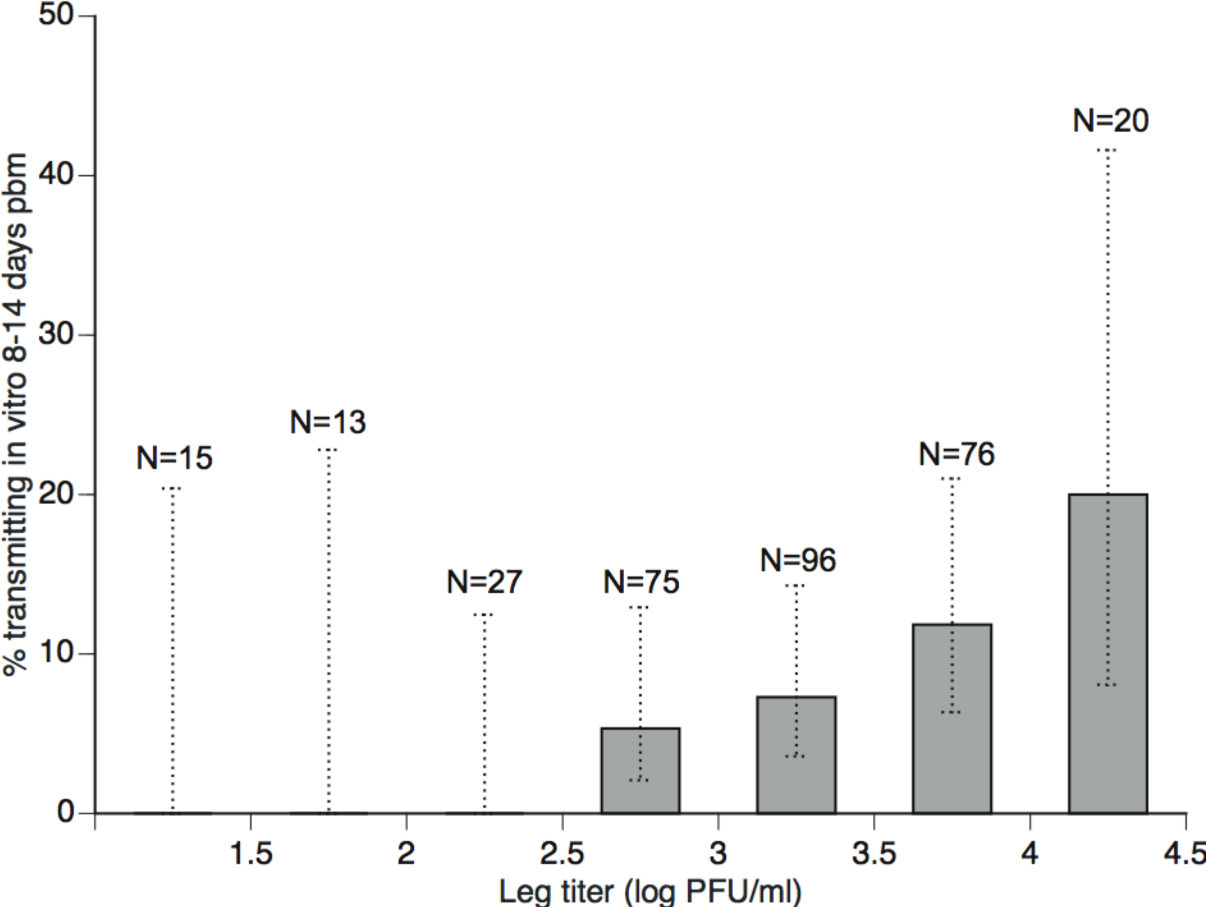
698

699 **Figure 2. Phylogenetic relationships among DENV-1 isolates**. Maximum likelihood
700 trees based on *E* gene (A) or complete genome (B) sequences. Sequences from GenBank
701 are in black font and sequences generated in this study are in color. Blue and red fonts
702 indicate isolates used in experiment 1 and 2, respectively. All horizontal branch lengths
703 are drawn to scale, with bootstrap support values shown next to relevant nodes.

704

705 **Figure 3. Variation of artificial DENV-1 blood meal titers in vector competence**
706 **assays.** Infectious titers of the artificial blood meals measured by plaque assay are shown
707 for each isolate as a function of their date of isolation and phylogenetic group. Each
708 isolate was tested twice in two experimental blocks (for details see Materials and
709 Methods). Lines represents linear y=f(x) regressions to give a graphical indication of

710 temporal trends. Lower lines correspond to experimental block 1; upper lines correspond
711 to experimental block 2.
712
713 **Figure 4. Variation in vector competence indices among DENV-1 isolates**. In the
714 upper panels, the percentage of females with a disseminated infection is indicated at 7
715 days post-blood meal (pbm) (A) and 14 days pbm (B) as a function of the year of virus
716 isolation and phylogenetic group (clade). In the lower panels, mean infectious titer in
717 heads of females with a disseminated infection is indicated for each isolate at 7 days pbm
718 (C) and 14 days pbm (D) as a function of virus isolation year and phylogenetic group.
719 Each isolate is represented by the mean of two experimental blocks (for details see
720 Materials and Methods). Lines represents linear y=f(x) regressions to give a graphical
721 indication of temporal trends.
722
723 **Figure 5. DENV-1 isolates in the new clade disseminate at higher titers in orally**
724 **challenged *Ae. aegypti***. Bars show the mean head titer of isolates belonging to the early
725 and new clades at 7 and 14 days post-blood meal. Error bars indicate the standard errors
726 of the means. Statistical significance of difference between clades is given by *P*-values
727 derived from a complete multifactorial analysis of variance accounting for the effects of
728 infectious dose (blood meal titer), experiment, block, clade and isolates; the analysis only
729 includes mosquitoes with a disseminated infection.


% transmitting in vitro 8-14 days pbm
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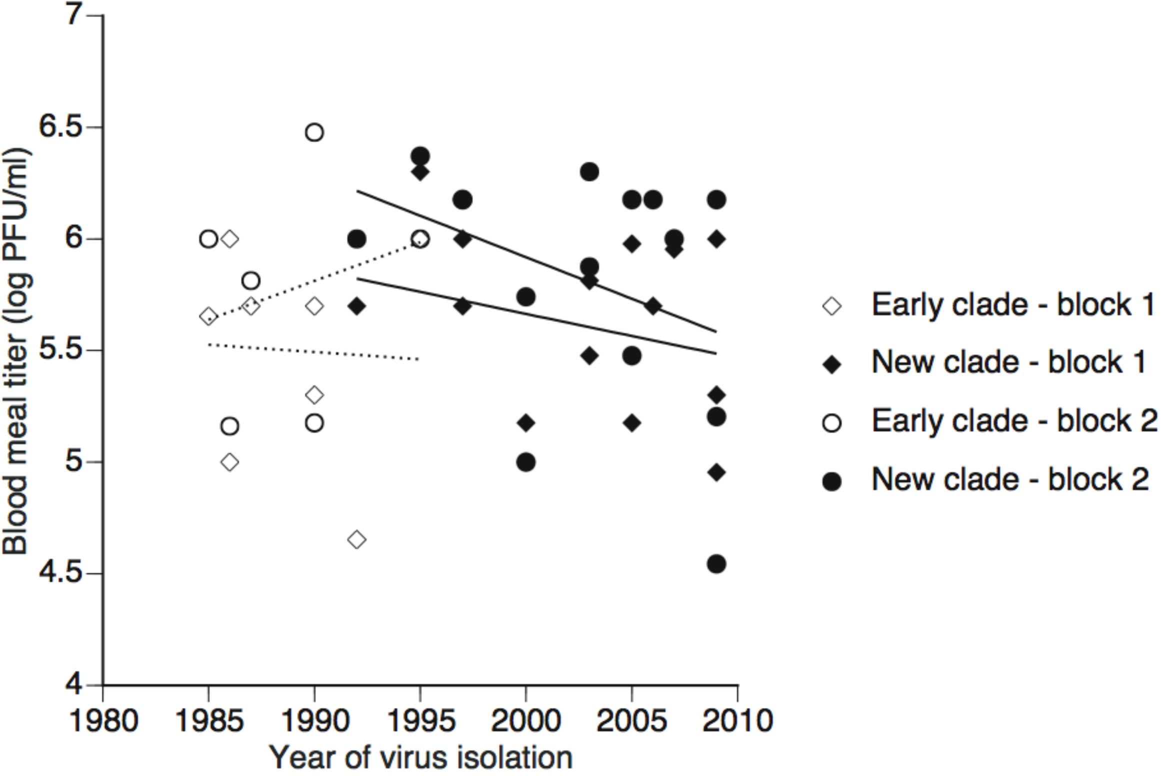
20
10
0
N=15
N=13
N=27
N=75
N=96
N=76
N=20
1.5
2
2.5
3
3.5
4
4.5
Leg titer (log PFU/ml)

A

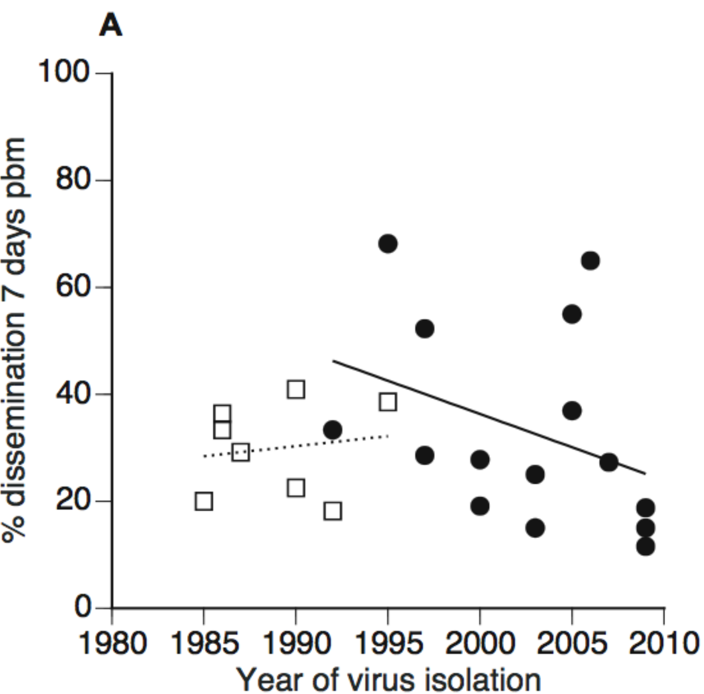
30247/03
00616/97
30529/00
AF309641 Cambodia 1998
04805/00
30118/06
30025/09
01417/03
00442/05
AY732482 ThD1-0049-01
AY732479 ThD1-0102-01
30230/05
00132/09
02128/07
30015/09
AY732480 ThD1-0097-94
AY732475 ThD1-0488-94
03881/92
30399/95
30231/97
New Clade
87
100
Genotype I
AY732483 ThD1-0008-81
AY732481 ThD1-0081-82
99
AF350498 China 1980
D87-116
D85-372
D90-1197
AY732478 ThD1-0323-91
D86-412
AY732477 ThD1-0336-91
KD86-035
KD90-157
00407/95
KD92-080
Early Clade
100
AB189121 Indonesia 1998
DQ285560 Reunion 2004
AB204803 Japan 2004
Genotype IV
AF180817 Thailand 1964
Genotype II
100
AF298807 Ivory Coast 1999
Genotype V
100
AY732474 ThD1-0673-80
AY732476 ThD1-0442-80
Genotype III
0.01 substitution/site

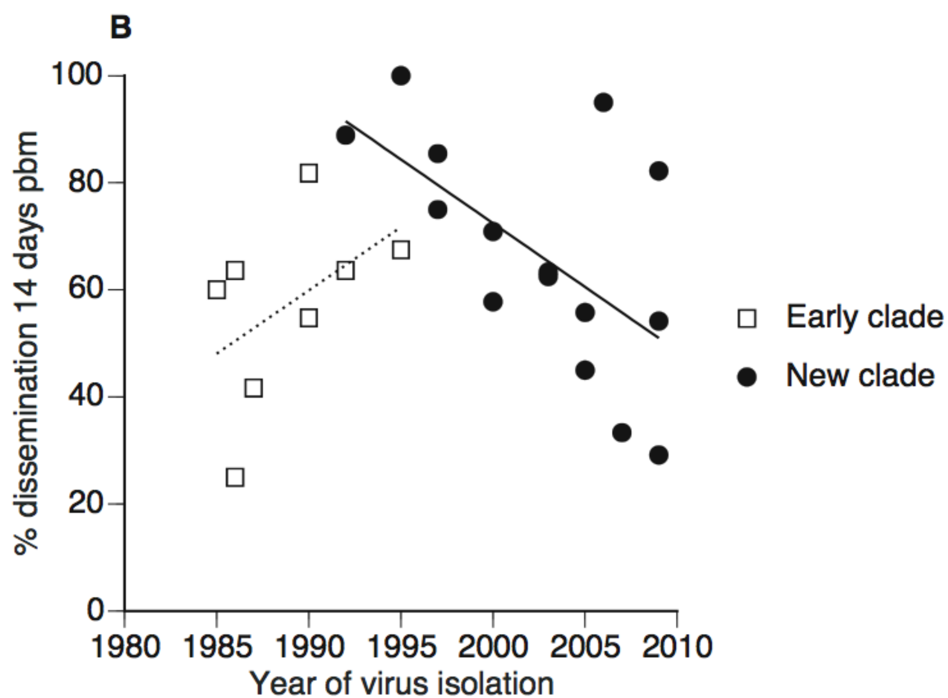
B

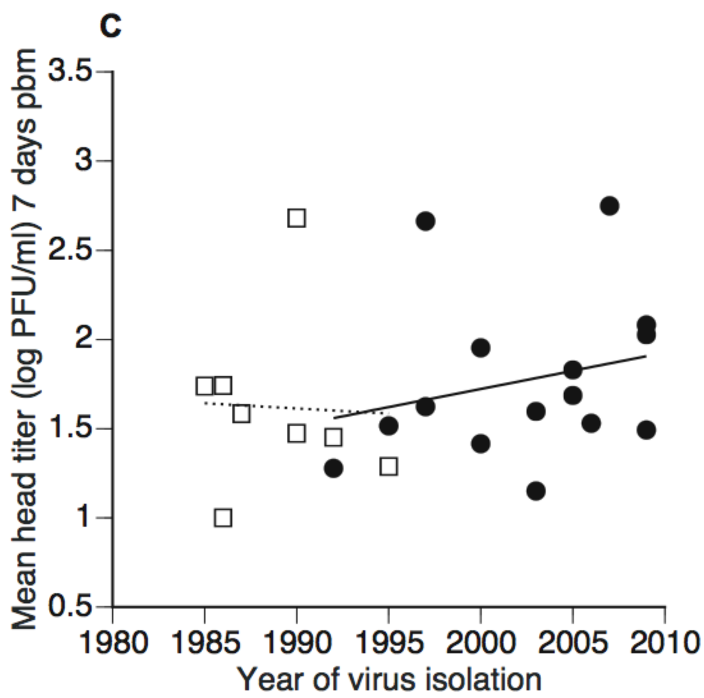
AF309641 Cambodia 1998
AY732482 ThD1-0049-01
AY732479 ThD1-0102-01
100
AY732475 ThD1-0488-94
AY732480 ThD1-0488-94
New clade
03881/92
30231/97
30399/95
100
Genotype I
AF350498 China 1980
D87-116
D90-1197
KD86-035
AY732477 ThD1-0336-91
100
AY732478 ThD1-0323-91
Early clade
KD90-157
KD92-080
00407/95
AY732483 ThD1-0008-81
AY732481 ThD1-0081-82
AF180817 Thailand 1964
Genotype II
AF298807 Ivory Coast 1999
Genotype V
100
100
AY732474 ThD1-0673-80
AY732476 ThD1-0442-80
Genotype III
100
AB189121 Indonesia 1998
DQ285560 Reunion 2004
Genotype IV
AB204803 Japan 2004
0.01 substitution/site

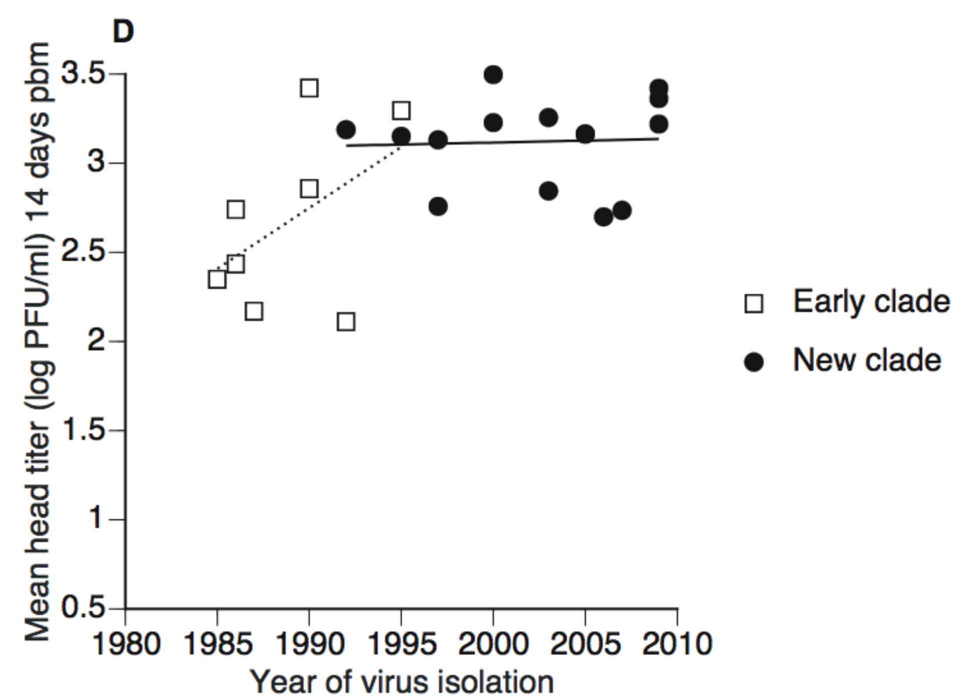


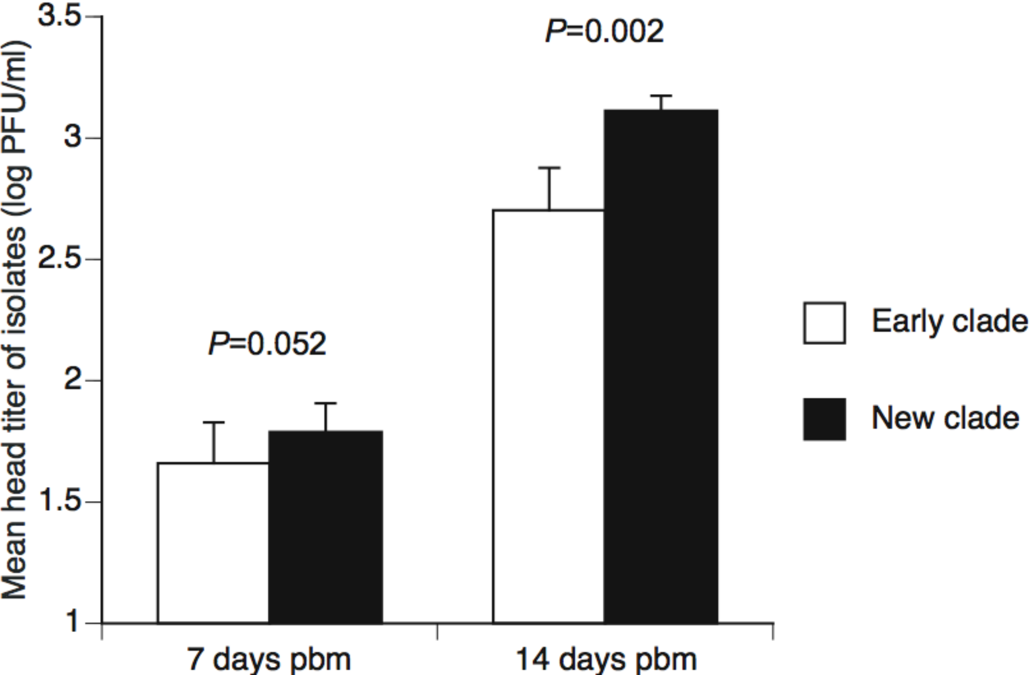
Blood meal titer (log PFU/ml)
7
6.5
6
5.5
5
4.5
4
1980
1985
1990
1995
2000
2005
2010
Year of virus isolation
Early clade - block 1
New clade - block 1
Early clade - block 2
New clade - block 2


A
% dissemination 7 days pbm
Year of virus isolation
0
20
40
60
80
100
1980
1985
1990
1995
2000
2005
2010


B
% dissemination 14 days pbm
Year of virus isolation
0
20
40
60
80
100
1980
1985
1990
1995
2000
2005
2010
Early clade
New clade


C
Mean head titer (log PFU/ml) 7 days pbm
Year of virus isolation
0.5
1
1.5
2
2.5
3
3.5
1980
1985
1990
1995
2000
2005
2010


D
Mean head titer (log PFU/ml) 14 days pbm
Year of virus isolation
0.5
1
1.5
2
2.5
3
3.5
1980
1985
1990
1995
2000
2005
2010
Early clade
New clade


Mean head titer of isolates (log PFU/ml)
3.5
3
2.5
2
1.5
1
P=0.052
P=0.002
7 days pbm
14 days pbm
Early clade
New clade