Improving dengue virus capture rates in humans and vectors in Kamphaeng Phet Province, Thailand, using an enhanced spatiotemporal surveillance strategy

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5   Phet Province, Thailand, Using an Enhanced Spatiotemporal Surveillance Strategy

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34 **Abstract:**

35 Dengue is of public health importance in tropical and sub-tropical regions. DENV transmission

36 dynamics was studied in Kamphaeng Phet Province, Thailand using an enhanced spatiotemporal

37 surveillance of 93 hospitalized subjects with confirmed dengue (initiates) and associated cluster

38 individuals (associates) with entomologic sampling. A total of 438 associates were enrolled from

39 208 houses with household members with fever history located within a 200 meter radius of an

40 initiate case. Of 409 associates, 86 (21%) had laboratory-confirmed DENV infection. A total of

41 63 (1.8%) of the 3,565 mosquitoes collected were dengue PCR+. There was a significant

42 relationship between spatial proximity to the initiate case and likelihood of detecting DENV

43 from associate cases and *Aedes* mosquitoes. The viral detection rate from human hosts and

44 mosquito vectors in this study was higher than previously observed by the study team in the

45 same geographic area using different methodologies. We propose that the sampling strategy used

46 in this study could support surveillance of DENV transmission and vector interactions.

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Introduction

Dengue illness is a disease of increasing public health importance [1]. Available data and modeling estimate that there are 390 million dengue virus (DENV) infections annually with 96 million manifesting clinically [2]. International travel, population growth, increasing urbanization, and a changing global ecology foster an increasingly favorable environment for the expanding dengue endemic areas and the peridomestic *Aedes aegypti* which transmit the viruses [3]. There are currently no licensed drugs or vaccines to treat or prevent dengue. When applied properly, vector control and personal protective measures have successfully disrupted epidemic and endemic DENV transmission [4,5]. Unfortunately, successful vector control programs have been the exception and difficult to sustain [6]. The strategic use of safe and efficacious dengue vaccines in combination with appropriately targeted and sustained vector control measures is increasingly being considered as the optimal approach to produce a sustained reduction in dengue's global burden [7].

Once a vaccine is available, numerous questions will remain about how to most effectively target and co-implement vaccination and vector control programs. The prospect of implementing large-scale, control programs raises a number of questions:

1) How will vaccination and vector control affect the complex, dynamic, and evolving interactions between vector, virus, and host occurring at the macro (i.e., country or region) and micro (i.e., province, district, village or neighborhood) spatial scales?

2) How will vaccination and vector control affect the complex, dynamic, and evolving interactions between vector, virus, and host at the population (i.e., *Aedes* species, DENV serotypes and genotypes, and people of various ethnic backgrounds) level?

3) How will "herd immunity" be affected and how will this influence DENV evolution at the micro and macro population levels and the associated observed clinical phenotypes?

4) What effect, if any, will existing herd immunity (due to vaccination or natural infection) to non-dengue flaviviruses (e.g. Japanese encephalitis and yellow fever viruses) have on DENV transmission and the observed clinical phenotypes following infection?

The overarching study objective was to explore DENV transmission dynamics and virus-vector-host interactions prior to, during, and following the introduction of dengue vaccines into central Thailand. The authors pursued this objective by building upon observations from previous prospective studies such as the focality of DENV transmission, presence or history of fever increasing the likelihood of isolating virus from a household, and the significance of year-round DENV transmission [8-10]. Study methods were modified in an effort to maximize DENV isolation rates from human hosts and mosquito vectors and further explore earlier observations, which included:

1) Enrolling initiate and associate cases throughout the year (i.e., high dengue season and low dengue season) to explore trends in seasonal and spatial DENV transmission;

2) Only enrolling DENV PCR+ initiate cases to increase the likelihood of capturing active transmission;

3) Only enrolling associates with fever or a history of fever within the last 7 days or sharing a household with someone meeting these criteria to increase the likelihood of identifying associates with recent infection;

4) Reassessing associates for the occurrence of fever between the acute and convalescent blood collection to capture additional viremic cases;

5

94   5) Expanding the age of enrollment to include children above the age of 6 months and adults

95   to improve understanding of transmission inside the home; and

96   6) Extending the enrollment of associates and mosquito collection from a 100 to 200m

97   (meter) radius around the initiate.

98 In this report the authors describe initiation of the baseline phase (i.e. prior to vaccine

99 introduction) and include detailed accounts of study methodology and the entomologic, clinical,

100 epidemiologic, virologic, serologic, and molecular characterization of human cases captured by

101 active and passive surveillance methods between November 2009 and December 2010.

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Methods:

Ethics Statement

      The study protocol was approved by the Institutional Review Boards (IRB) of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR), and the State University of New York (SUNY), Upstate Medical University. The IRB's of the University of California, Davis (UCD), University of Rhode Island (URI) and University at Buffalo established relying agreements with WRAIR IRB.

      All study subjects engaged in documented informed consent or assent process, as applicable, prior to participating in any study activities. In the event the subject was unable to participate in the informed consent/assent process, a recognized health care proxy represented them in the process and documented consent. From this point forward, when the authors discuss consent, assent is also implied as applicable.

Role of the Funding Source

      Funding sources for this project included National Institutes of Health grants R01 GM083224-01 and P01 AI034533. Additional funding was provided by the US Military Infectious Diseases Research Program. The funding sources had no involvement in study design, data collection, analysis or interpretation, report writing, or publication submission. The corresponding author had full access to all study data and final responsibility for the decision for publication.

Study Location

      The study was conducted in Kamphaeng Phet (KPP) province in north-central Thailand. There were 725,846 registered residents of KPP in 2009 (Thailand, Department of Provincial Administration, 2010). The KPP Provincial Hospital (KPPPH) is located in the province's

127   central district. The Armed Forces Research Institute of Medical Sciences (AFRIMS),

128   Department of Virology field site (KPP AFRIMS Virology Research Unit [KAVRU]) is located

129   on the KPPPH grounds. The Department of Entomology, AFRIMS field site is located a short

130   distance from KPPPH [11].

131   Demographics of house residents were collected and house spatial coordinates were

132   identified using a Geographic Positioning System hand held unit (Trimble(r) GeoXH(tm),

133   GeoExplorer(r) 2008 series, Trimble Navigation Limited, CO, USA) and geo-coded into a

134   Geographic Information System (GIS) database (CorporationArcMap^{TM}, version 9.1, ESRI, CA,

135   USA).

136   <u>Study Definitions</u>

137   The authors recognize the potential confusion using terms such as, "index case,"

138   "contacts," and "cluster investigations." As the index case may not be the true first infection in

139   space and time and the contact may not be a true infection resulting from DENV transmission

140   from the initiating case we have attempted to more accurately define the relationships

141   investigated in this study without making claims of causation. The hospitalized dengue cases

142   serving as the initiator of community transmission investigations are referred to as the "initiating

143   case," or "initiate". The enrolled individuals residing within the initiate's home or within 200

144   meters of the initiate are referred to as "associates" and if found to have DENV infection, as

145   "associate cases".   The combination of the initiate, associate and associate cases is referred to as

146   a "spatiotemporal group."

147   Symptomatic DENV infection in either initiating or associated cases are defined as any

148   febrile illness (reported or measured fever) paired with a confirmatory molecular or serologic

149   assay run on the acute or acute and convalescent blood sample pair, respectively. Subclinical

150   DENV infections are defined as associated cases (all initiating cases were symptomatic and

151   hospitalized) with a positive molecular or serologic result, but without reported or measured fever

152   during the period between the acute and convalescent blood sample collection (0 to 14 days).

153   Subjects are serologically classified as having an "acute" DENV infection if their acute

154   and/or convalescent blood sample pair (or day 0 and/or day 14 blood samples in associates) was

155   dengue IgM positive; or, if IgM is negative, IgG is positive with rising titer. Subjects are

156   classified as "recent" DENV infection if IgM was negative and IgG was positive with declining

157   titer. These are further categorized as "primary" infection if the IgM to IgG ratio is $\geq 1 \cdot 8$ and

158   "secondary" infection if the ratio is $< 1 \cdot 8$ [12].

159   <u>Initiate Case Identification and Evaluation</u>

160   KAVRU provides the KPPPH dengue diagnostic research assays for patients presenting

161   with fever or history of fever and dengue-like symptoms as outlined in the World Health

162   Organization (WHO) guidelines for the diagnosis and treatment of dengue [13]. Hospital staff

163   members identified suspected dengue cases, completed and documented the informed consent

164   process allowing the testing of a blood sample, and then sent the sample to KAVRU for RT-PCR

165   testing. Virus RNA was extracted from human serum or mosquito suspension using Qiagen Viral

166   RNA Extraction kits. Serotype-specific DNA fragments from each unknown sample were

167   amplified by TaqDNA polymerase through RT-PCR performed at KAVRU following

168   modifications to the Lanciotti protocol [14]. Suspected cases >6 months of age, that provided a

169   sample collected within the prior 24 hours, and had detectable DENV RNA by RT-PCR were

170   provided the opportunity for study enrollment as initiate cases. Following acquisition of informed

171   consent, the acute blood sample was accessed and served as the baseline sample. Demographic

172   and clinical laboratory information was collected. Initiate case house spatial coordinates were

173 recorded and geo-coded into a GIS database. Mosquitoes inside and outside of the initiate home

174 were collected by aspiration and processed as described in preparation for DENV RNA detection

175 RT-PCR [10]. In approximately 14 days a second blood sample was collected from the initiating

176 case for serologic testing by in-house IgM/IgG EIA.

177 <u>Associate Case Identification and Evaluation</u>

178 Residents >6 months of age sharing the initiate household as their primary residence were

179 provided the opportunity for study enrollment. Residents previously enrolled as an associate

180 within the past 6 months were excluded in an attempt to improve geographic diversity and the

181 likelihood of isolating DENV (i.e. reduce chances of recent infection and lingering homo- or

182 heterotypic immunity). Following the informed consent process, clinical information and a blood

183 sample were collected. In approximately 14 days a second blood sample was collected for

184 serologic testing. General well-being of the associates was tracked by active surveillance (i.e.,

185 combination of self-reporting and outreach by staff via phone or daily home visit by village

186 health worker over the 14 day period). If, between the day of enrollment and the day of

187 convalescent blood sampling, the associate develops fever, a second acute blood sample was

188 collected and the 14 day "clock" started again. Therefore, it was possible an associate might have

189 two acute samples, one triggered as an associate of the initiating case (acute sample #1) and one

190 triggered by the development of illness (acute sample #2), as well as one convalescent sample.

191 Individuals not residing within the same house as the initiate case, but living in a home

192 within a 200m radius of the initiate case were also considered for enrollment as associates. If

193 anyone within the home reported an active fever or history of fever (temperature ≥38ºC) within

194 the past 7 days, all residents of the home >6 months of age were eligible for enrollment.

195   Following the informed consent process, residents had clinical and demographic information and

196   an acute blood sample collected. Associates were followed and blood collected as detailed above.

197   House Mapping, Associate Case Home Identification, and Entomologic Sampling

198   GPS mapped initiate households were used to construct a digital map, enabling the team

199   to precisely identify houses located within a 200m radius of the initiate case [10,15]. Study

200   nurses visited households starting closest to the initiate house and moving in sequential fashion

201   to the periphery of the area and back to the initiate house, then back out again along a different

202   line until the circle was complete or 25 eligible associates were enrolled, whichever occurred

203   first (Figure 1).

204   On day 1 of each initiate/associate case investigation, adult *Ae aegypti* were collected

205   using standard backpack aspirators from inside and within the immediate vicinity of each

206   potential associate's house. The end of each aspirator tube was fitted with a 1-pint cardboard

207   cage. After completing the collection for each home, the cage was labeled and stored on dry ice

208   for transportation to the laboratory where the chilled mosquitoes were examined, speciated, and

209   processed for DENV detection. A thorough adult aspiration collection usually requires ~10-15

210   minutes per house. It was estimated that approximately 25% of the adult *Ae. aegypti* were

211   captured in a single pass through the house (TW Scott, unpublished data). All specimens were

212   identified to species by entomology field supervisors to ensure speciation accuracy and quality

213   control. As previously mentioned, mosquitoes inside and outside of the initiate home were

214   collected by aspiration and processed as described with serotype–specific DENV RNA detection

215   RT-PCR performed on each individual mosquito [10].

216   Statistical Analyses

217   Data were analyzed using SPSS (SPSS for Windows version 19) and R (The R Project for

218   Statistical Computing 2.12). Demographic, clinical, and laboratory parameters were analyzed at

219   the initiate, associate, and house levels. Student's *t*-test and analysis of variance (ANOVA) were

220   used to test for differences in continuous variables. Fisher's exact test and Pearson's chi-squared

221   were used to examine associations between categorical variables.

222   **Results**

223   Enrollment of Initiates and Associates

224   Figure 2 depicts enrollment of initiates and associates and results of serologic and

225   molecular testing for DENV infection. Approximately 49% of patients hospitalized with

226   suspected dengue were PCR+ and of those 62% were enrolled in the study. Of the 4,345

227   households within a 200m radius of the 93 initiate homes, 124 (2.8%) had someone with active

228   fever or history of fever; 115 of these households had volunteers who consented to enroll. Initiate

229   and associate households contained 793 individuals meeting enrollment criteria, and 438 (55%)

230   consented to enroll as associates. Complete serologic data was available on 93% of the enrolled

231   associates and of these 21% had a positive dengue serology. Of the 86 associates with positive

232   serology, 42% also were PCR+. All PCR+ associates had fever at the time of enrollment. Eleven

233   associates who enrolled without fever developed fever following their first acute blood sampling;

234   of these 8 were serology positive and 7 PCR+.

235   Of note logistic limitations guided enrollment on days with heavy dengue patient census

236   accounting for the difference between the 149 PCR+ cases meeting inclusion criteria and the 93

237   ultimately enrolled. In these instances the study team randomly selected which initiate cases

238   would lead to an associate case investigation using a random numbers table.

239   Characteristics of the Initiating Cases

240   Each of the 93 initiate cases was a hospitalized acute, PCR+ DENV infection (Table 1).

241 Infections with DENV-2 were most numerous followed by DENV-3 and then DENV-1; there

242 were no DENV-4 infections. Most cases were acute secondary DENV infections (85%).

243 Approximately half of all cases were DF (48%) and half DHF (52%); there were no deaths among

244 the initiates.

245 Associated Cases Available for Enrollment

246   The number of associate houses within 200m of an initiating case ranged from 1-232 with

247 a mean of 47.7 (SD 42 houses). The number of households enrolled ranged from 1-9 with a mean

248 of 2.2 (SD 1.5 households). For each spatiotemporal group there was a range of associates

249 enrolled from 0-18 people with a mean of 4.7 (SD 4.0 people).

250 Characteristics of the Associated Cases

251   The majority (79%) of 409 enrolled associates had no serologic evidence of infection,

252 20% had evidence of an acute DENV infection, and there was 1 case with evidence of JE

253 infection (IgM positive, no encephalitis; Table 1). There was complete concordance between the

254 DENV serotype of the initiating case and the DENV serotypes detected in associate cases in that

255 spatiotemporal unit. Nested PCR results among associates revealed that DENV-3 was the most

256 common infection followed by DENV-2 and then DENV-1.

257   There was a significant difference in the reporting of nausea between primary and

258 secondary DENV infections and significant variation in reporting of headache, rhinorrhea, cough,

259 and retro-orbital pain among the age groups (Tables 2 and 3). There was a statistically significant

260 difference in the probability of an associate experiencing a DENV infection based on the DENV

261 type infecting the initiate. DENV-3 infection in the initiate carried the highest probability of

262 associate infection (Table 4).

263   Spatial Distribution of Associate Case Households

264   Closer proximity of a household to the initiate was correlated with an increased

265   associate household enrollment (Table 5). Households further from the initiate household

266   had a lower rate of DENV infection among associate household residents (Table 6). This

267   pattern of transmission was not gradual with distance. In an associate from a household with

268   fever for example, the rate of infection was similarly high (around 0.3) within 120 meters

269   and very low in households with fever beyond 120 meters. This would indicate that

270   spatiotemporal transmission in this investigation was primarily limited to 120 meters and

271   extending the radius from 100 to 200 meters did not substantially increase the efficiency of

272   detecting dengue cases or viremia.

273   DENV Infection in *Ae. aegypti* Collected in the Homes of Human Dengue Cases

274   A total of 3,565 *Ae. aegypti* were collected from 4,438 households (i.e. all households).

275   A total of 233 (6.5%) mosquitoes were from initiate households, of which 23 (9.9%) were

276   DENV PCR+. There was a greater likelihood one or more collected mosquitoes was DENV

277   PCR+ within the initiate house or a house closer to the initiate's house (Table 7).

278   There were 162 initiate households with at least one associate resident who had complete

279   serology and 212 associate households with at least one associate with complete serology. Of the

280   initiate households, female *Ae. aegypti* were collected in 54.9% and 58.5% of the associate

281   households had female *Ae. aegypti* collected. Of the female mosquitoes collected in initiate

282   households 29% of households had a DENV PCR+ mosquito while 6.5% of associate

283   households had a DENV PCR+ mosquito. There was a higher likelihood of finding a DENV

284   PCR+ mosquito in an initiate household (Table 8). Of the 31 initiate households with a DENV

285   serology and an associate resident, 54.8% of the houses had female *Ae. aegypti* captured and

286   64.7% of these households had a DENV PCR+ mosquito. Of the 42 associate households with a

287   DENV serology + associate, 57.1% of households had a female *Ae. aegypti* mosquito and one

288   (4.1%) of these households had a DENV PCR+ mosquito. Despite a similar likelihood of

289   finding female *Ae. aegypti* in households with a DENV+ serology resident, there was a higher

290   likelihood of finding DENV PCR+ mosquitoes in the initiate household (Table 8). In total, there

291   were 46 households with a DENV PCR+ mosquito and an associate residing with complete

292   serology; 80.4% were initiate households (Table 8.). There was a high degree of concordance

293   between the isolated DENV serotypes from mosquitoes within a cluster and the infecting

294   serotype of the index case (Table 9). In year 2010, only one cluster (10-077) was found

295   discordant from the mosquito (DENV-2) as compared to the index case (DENV-1). In 2012,

296   one cluster (12-025) was found DENV-1 in index case while PCR results were DENV-2 and

297   DENV-4 in 2 mosquito samples. The rest of the clusters were concordant.

298

299   **Discussion**

300   Our current study further characterizes the complex transmission dynamics and virus-

301   vector-host interactions in a well characterized spatial area around an infected viremic inpatient

302   in Central Thailand. We demonstrated a significant relationship between spatial proximity to the

303   initiate case and likelihood of detecting DENV from associate cases and *Ae aegypti* with higher

304   than anticipated virus detection from both human hosts and mosquito vectors. We propose that

305   the sampling strategy described is valuable for ongoing surveillance of DENV transmission

306   during and after field studies and the introduction of dengue vaccines.

307   The design of this study was built upon observations from previous prospective cohort

308   studies of DENV transmission conducted with and without initiate case and associate

309 (contact/cluster) investigations [8-10,16,17]. Modifications were made to the study design in an

310 attempt to increase the detection of virus, symptomatic and subclinical associates of initiate cases,

311 and infected mosquito vectors. The result was a demographically diverse group of DENV

312 infected people representing a broad virologic, serologic, and clinical spectrum. Substantial virus

313 detection rates were observed in both human associate cases and mosquitoes.

314   The age range of initiate cases was surprisingly wide (2.6-56 years), and the mean age

315 was higher than expected at 18.7 years [18]. This observation is consistent with unpublished data

316 from the authors across additional dengue seasons and data from other sources. For example, in

317 2010, the Thailand MOPH reported that the highest case rate for DHF was in the 10-14 year age

318 group, and that the 5-9 and 15-24 year age groups had the second and third highest rates,

319 respectively (Thailand, Ministry of Public Health). These findings are in contrast to decades of

320 data where the mean age of hospitalized dengue was in the age range of 5-9 years [19-24]. One

321 explanation for the shift is that smaller birth cohorts had reduced number of susceptibles, thereby

322 impacting the force of infection and the time in a person's life when they acquire their first and

323 second DENV infection, the latter being more often associated with clinically significant disease

324 in Thailand [18].

325   We also enrolled associates across a wide age range (7 months – 94.2 years) with a mean

326 age almost double the initiate cases (31.4 years vs. 18.7 years). Serologic evidence of DENV

327 infection and symptomatic DENV infection was observed in a number of subjects aged 40 years

328 and older. Most of these were secondary (i.e., post-primary) DENV infections, but we also

329 detected primary DENV infections, defined serologically, in the 50-59 and 85+ year age groups.

330 Dengue occurs throughout the year with all four serotypes circulating with high rates of infection

331 and transmission (hyperendemic) in KPP. Pediatric cohort studies have demonstrated high

332 DENV infection attack rates (combined symptomatic and subclinical) in the range of 2.2% to

333 7.9% per year (average incidence 5.8%) over at least the preceding 10 years [9]. Based on these

334 observations, it was assumed that lifelong KPP residents have experienced multiple DENV

335 infections by their late 20's. A modified plaque reduction neutralization assay (single dilution

336 neutralization assay, 1:30 dilution, 70% viral plaque reduction) completed on a cohort of children

337 enrolled in a KPP prospective study from 1998 to 2002 revealed a gradually increasing

338 prevalence of neutralizing antibodies to at least one DENV type from 45% among 4 year olds to

339 91% among 13 year olds (Yoon, IK, unpublished data) [10,16,25]. Our finding that all PCR+

340 associate cases had fever at the time of enrollment may also underscore the increasing infection

341 burden in adults as they are more likely to experience symptomatic DENV infections compared to

342 primary infection in children. Our observation of symptomatic dengue in older individuals merits

343 reconsideration of traditional views on the force of infection in hyperendemic areas and the

344 durability of homotypic and heterotypic immunity.

345   There was complete concordance between the infecting DENV serotypes in the initiate

346 case and the corresponding associate cases. This finding supports the concept that DENV

347 transmission is focal and serotype-conserved in space and time.[17,26] The authors'

348 acknowledge that the assumption that each initiating case represents the true index (i.e., first)

349 infection in each spatiotemporal group may be incorrect. Because most DENV infections are

350 asymptomatic or not clinically severe enough to drive health care-seeking behavior it is possible

351 the initiating case simply represents the first clinically overt infection in that defined geographic

352 area.

353   The ratio of symptomatic to subclinical infection among primary infections was 1:0.2; the

354 ratio in secondary infections was 1:0.4 and similar (1:0.4) among the 86 serologically positive

17

355   associates. These ratios are consistent with previous work by our group and others, which

356   reported symptomatic to subclinical ratios ranging from 1:0.9 to 1:18 [9,10,27-30]. The

357   differences in results are most likely associated with the study's surveillance focus in and around

358   symptomatic, PCR+ initiating cases. Previous studies conducted routine biologic sampling to

359   capture subclinical cases and cluster studies exploring houses with or without active or a recent

360   history of fever [9].

361   The probability of an associate becoming infected was associated with a number of factors

362   [31-33]. As mentioned above, a significant association existed between the DENV serotype

363   infecting the initiating case and the proportion of associates subsequently infected with the same

364   DENV serotype (p-value <0.001). Associates in samples initiated by a DENV-3 infection had a

365   30% chance of being serologically positive. The likelihood of infection was 19% for associates

366   in DENV-2 samples and 9% in DENV-1 samples. Taken by itself, this may point to viral

367   properties allowing for more efficient transmission, such as higher titer viremia in the human host

368   or mosquito vector, a longer duration of viremia increasing the potential window for transmission

369   to vectors and / or shorter incubation period in the mosquito. Another significant association was

370   between the distance a potential associate lived from an initiating case and the likelihood of

371   becoming infected (negative correlation, p-value <0.001). It is reasonable to assume the factors

372   culminating in an initiate infection (i.e., convergence of susceptible host and infected vector in

373   space and time) would also drive efficiency of transmission and associate infections until a

374   geographic barrier was introduced (i.e. next susceptible beyond the dispersal distance of the

375   vector) or other factors limited transmission (i.e., protective herd immunity of associates). We

376   did not define the relative roles of humans and mosquitoes in moving virus from one house to

377 another within clusters, although in general, humans tend to move more often and greater

378 distances than *Ae. Aegypti* [31-33].

379   Approximately 29% of all households had female *Ae. aegypti* and 1.8% of the females

380 collected were infected with DENV. Although initiating case houses and associate houses with a

381 PCR+ contact had similar rates of female *Ae. aegypti* infestation (55% and 59%, respectively),

382 the percentage of PCR+ mosquitoes was higher in the initiate case households (9.9%) compared

383 to non-initiate households with and without a DENV positive contact, 1.2% and 1.1%

384 respectively. There was a significant association (p-value < 0.001) between the distance of a

385 home from the initiating case and the proportion of PCR+ mosquitoes found within the house.

386 These observations are concordant with previous reports and intuitive; once a clinical case of

387 dengue is identified there is a high likelihood of finding infected vector(s) co-residing in the same

388 geographic location as the ill human.

389   Initiate cases represented the full spectrum of DENV infections clinically, serologically,

390 and virologically. We consider the finding of more than 1 in 5 associates of an initiating case

391 having serologic and, in some cases, clinical evidence of an acute DENV infection significant

392 from an epidemiologic and transmission dynamics standpoint. These results represent the initial

393 phase of a multi-year prospective study. The study design employed improved the efficiency of

394 capturing DENV in associates and vector populations compared to previous efforts by focusing

395 surveillance in areas and households where DENV transmission was most likely to be occurring.

396 Our observations emphasize the focal spread of DENV and its spatial restriction associated with

397 the mosquito vector and suggests that initiate cases were important in this study (ill and PCR+) in

398 transmitting DENV to mosquitoes and thus to others living near the initiate. Previous studies

399 support the observation that patient DENV viremia was a marker of human infectiousness and

400 blood meals containing high concentrations of DENV were positively associated with the

401 prevalence of infectious mosquitoes.[34] Our results and study design may be useful when

402 designing experiments to study fine scale patterns of DENV transmission and to increase

403 detection of case contacts with inapparent infections.

415 **Conflicts of Interest**

416 None of the authors on this manuscript have a conflict of interest with the manuscript and data

417 presented.

418 **Disclaimer**

419 The opinions or assertions contained herein are the private views of the authors and are not to be

420 construed as reflecting the official views of the United States Army, the United States

421 Department of Defense, or the National Institutes of Health.

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Tables and Figures:

449   Figure 1. Method of identifying and enrolling associates around an initiating case. The clinical

450   research team would begin at the initiate's home, identify a collection of homes within 200

451   meters of the initiate's home, identify homes with an occupant having active fever or history of

452   fever within the past 7 days, and enroll associates only from those homes while bypassing homes

453   without active fever or history of fever. The entomology research team, meanwhile, collected

454   mosquitoes inside and outside the home from all homes within a 200-meter radius of the initiating

455   case home regardless of fever history. Once investigations and enrollment of associates were

456   completed in one grouping, the teams would return to the initiating case's home and then identify,

457   using the aerial map, the next grouping of homes moving in a clockwise manner.
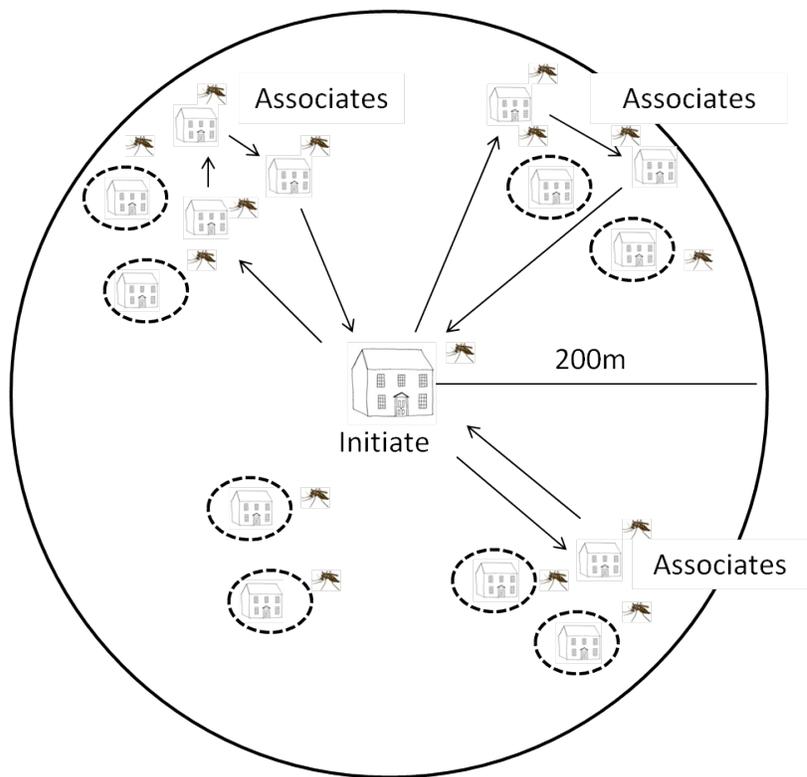
458   Circled home has no active fever or history of fever and is therefore not included in the investigation of associates.

23

459 Table 1. Initiate and Associate Group Demographic Data and Infection Characterization.

		Initiate (N = 93)	Associate		
			Available Serology (N = 409)	Available Serology + Fever (N = 156)	Dengue + Serology (N = 86)
Age (years)					
	minimum	2.6	0.58	0.58	0.83
	maximum	56.0	94.2	77.0	82.0
	mean	18.7	31.4	20.1	23.1
Sex (%)					
	Female	48	54	57	55
	Male	52	46	43	45
Infecting DENV type (% of total typed cases)		Total case count = 93	Acute sample not available, no clinical symptoms.		Total case count = 86

	DENV-1	16			1
	DENV-2	60			16
	DENV-3	24			24
	DENV-4	0			0
	Not detected				58
Serology Results (% of total results)					
	Acute Primary	2	3	6	16
	Acute Secondary	85	17	25	80
	Recent Secondary	2	1	1	3
	No Serologic Diagnosis	8	79	67	
	JE Infection		0.2		

460   Figure 2. Flow Diagram of Initiate and Associate Enrollment and Testing Outcome
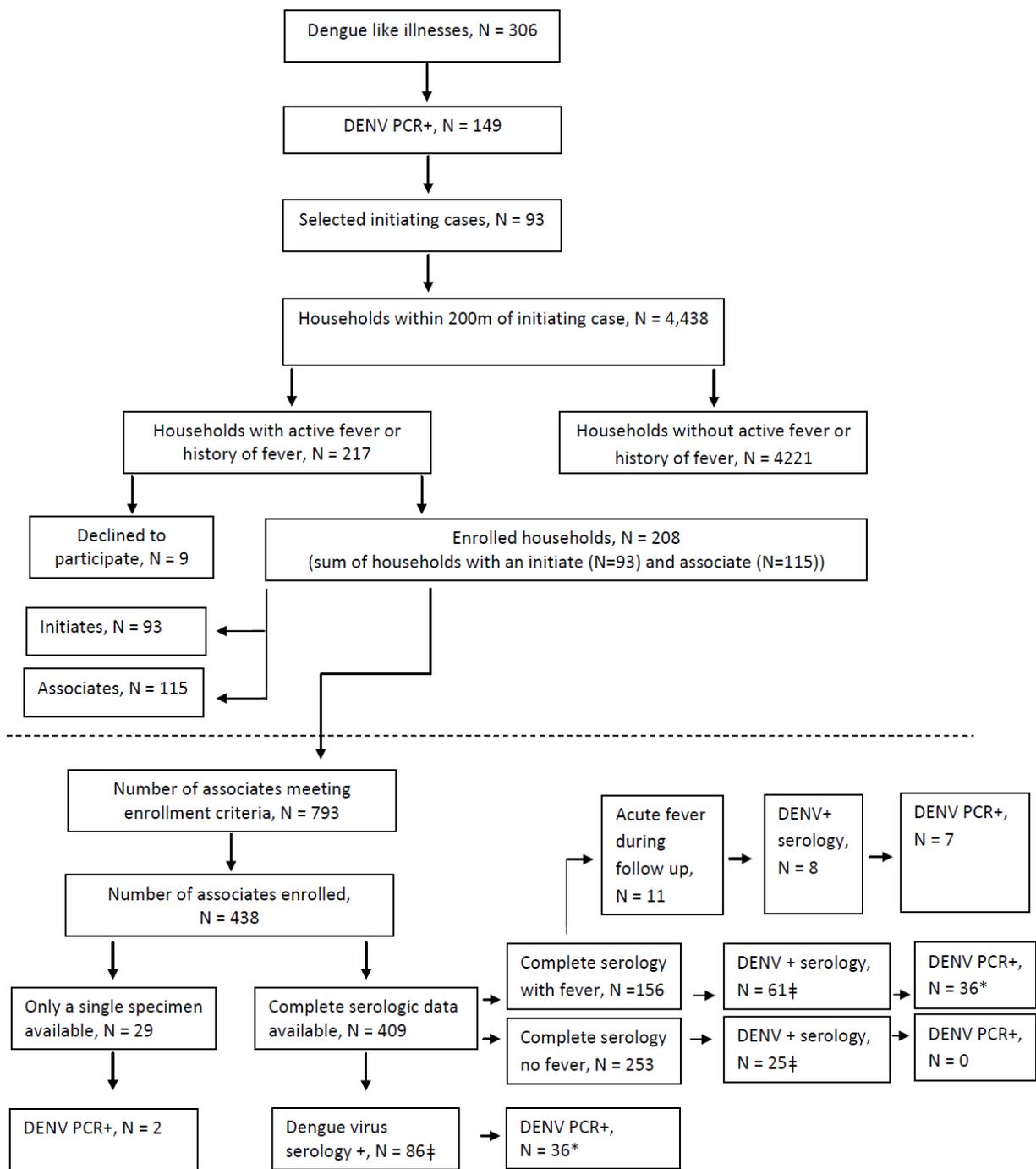
463   Table 2. Symptom Complex in Primary vs. Secondary Associate Infections

Symptom	Primary (N=14)	Secondary (N=72)	p-value
Fever	11 (0.79)	50 (0.69)	0.749
Headache	5 (0.36)	37 (0.51)	0.384
Rhinorrhea	3 (0.21)	16 (0.22)	1.000
Anorexia	5 (0.36)	27 (0.38)	1.000
Cough	4 (0.29)	27 (0.38)	0.762
Nausea	0 (0.00)	25 (0.35)	0.008
Drowsiness	0 (0.00)	12 (0.17)	0.202
Muscle or Joint Pain	3 (0.21)	36 (0.50)	0.077
Abdominal Pain	2 (0.14)	15 (0.21)	0.727
Retro-orbital Pain	3 (0.21)	22 (0.31)	0.749
Rash	4 (0.29)	10 (0.14)	0.231
Diarrhea	2 (0.14)	14 (0.19)	1.000
Bleeding	0 (0.00)	5 (0.07)	0.586
Hospitalized	1 (0.07)	6 (0.05)	1.000

464   Table 3. Clinical Spectrum of EIA Positive Associates by Age Groups

Symptom	0-9 Years (N=22)	10-19 Years (N=31)	20-29 Years (N=10)	>30 Years (N=23)	p-value
Fever	18 (0.82)	24 (0.77)	8 (0.80)	11 (0.48)	0.052
Headache	6 (0.27)	20 (0.65)	7 (0.70)	9 (0.39)	0.021
Rhinorrhea	8 (0.36)	7 (0.23)	3 (0.30)	1 (0.04)	0.043
Anorexia	9 (0.41)	11 (0.35)	6 (0.60)	6 (0.26)	0.325
Cough	13 (0.59)	11 (0.35)	4 (0.40)	3 (0.13)	0.012
Nausea	4 (0.18)	12 (0.39)	5 (0.50)	4 (0.17)	0.101
Drowsiness	2 (0.09)	7 (0.23)	1 (0.10)	2 (0.09)	0.519
Muscle or Joint Pain	7 (0.32)	16 (0.52)	7 (0.70)	9 (0.39)	0.189
Abdominal Pain	3 (0.14)	8 (0.26)	2 (0.20)	4 (0.17)	0.741
Retro-orbital Pain	1 (0.05)	15 (0.48)	4 (0.40)	5 (0.22)	0.002
Rash	5 (0.23)	6 (0.19)	2 (0.20)	1 (0.04)	0.282
Diarrhea	2 (0.09)	8 (0.26)	3 (0.30)	3 (0.13)	0.285
Bleeding	2 (0.09)	2 (0.06)	1 (0.10)	0 (0.00)	0.440
Hospitalized	1 (0.05)	3 (0.10)	2 (0.20)	1 (0.04)	0.432

465   Table 4. Probability of Dengue Virus Infection in Associates According to the Initiating Case

466   Infecting DENV Serotype.

467

	Infecting DENV of Initiate		
Serologic Diagnosis	DENV-1	DENV-2	DENV-3
EIA Negative	43	191	88
EIA Positive	4 (0.09)	44 (0.19)	38 (0.30)

468   Footnote: Numbers in parentheses are the proportions EIA positive. The infecting DENV type is

469   taken from the initiating case. The differences are significant (Fisher's exact test, p-value =

470   0.003).

471   Table 5. Spatial Distribution of Households and Enrolled Households

472

	Distance from Initiate Household (meters)				
	>0-40	>40-80	>80-120	>120-160	>160-200
Total Households	539	814	971	1051	1063
Enrolled Households	103 (0.191)	53 (0.065)	32 (0.033)	13 (0.012)	7 (0.007)

473   Footnote: Numbers in parentheses indicate proportions of houses enrolled for each distance

474   category. Differences are significant, p-value < 0.001 by Fisher's exact test –.

475 Table 6. Spatial Distribution of Associates and Associates with DENV Infection by Serology

	Initiate Household	Distance from Initiate Household				
		>0-40	>40-80	>80-120	>120-160	>160-200
Total Associates with Available Serology	187	41	67	51	38	25
EIA Positive Associates	42 (0.225)	14 (0.341)	12 (0.179)	16 (0.314)	0 (0.000)	2 (0.080)

476 Footnote: Numbers in parentheses indicate proportions of associates that were EIA positive

477 within the initiate house and for each distance category. Differences are significant, p-value <

478 0.001 by Fisher's exact test.

479

480   Table 7. Spatial Distribution of Female *Ae. aegypti* within Houses and PCR+ Mosquitoes within

481   Houses

482

	Initiate Household	Distance to Initiate Household				
		>0-40	>40-80	>80-120	>120-160	>160-200
Total Mosquitoes	233	328	545	733	745	981
PCR+ Mosquitoes	23 (0.099)	10 (0.030)	7 (0.013)	13 (0.018)	7 (0.009)	3 (0.003)

483   Footnote: Numbers in parentheses indicate proportions of mosquitoes that were PCR+ for each

484   distance category. Differences are significant, p-value < 0.001 by Fisher's exact test.

485

486 Table 8. Associate Infection Rates by Infection Status of Female *Ae. aegypti* Captured in

487 Households.

488

	Initiate Houses			Associate Houses		
	PCR Positive Female *Ae.aegypti*	Females Captured *Ae.aegypti*	No Females Captured *Ae.aegypti*	PCR Positive Females *Ae.aegypti*	Females Captured *Ae.aegypti*	No Females Captured *Ae.aegypti*
Total Associates with Available Serology	26	88	71	8	125	90
Serology Positive Associates	11 (0.423)	17 (0.193)	14 (0.197)	1 (0.125)	25 (0.200)	18 (0.200)

489 Footnote: Numbers in parentheses indicate proportions of associates that were serology positive

490 within the initiate house and for each category.

491

492   Table Nine. Isolated DENV serotypes from clusters.

493

Year	2009-2010	2009-2012
Number of Ae. Samples	3545	9322
DENV-1	5	13
DENV-2	36	87
DENV-3	22	28
DENV-4	0	3

494
495   Year 2010, one cluster (10-077) was found discordant mosquito result (DENV-2) from the index
496   case (DENV-1)
497
498   Year 2012, one cluster (12-025) was found DENV-1 in index case while PCR result were
499   DENV-2 and DENV-4 in 2 mosquito samples.
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References

526 1. Morens DM, Fauci AS (2008) Dengue and hemorrhagic fever: a potential threat to public health in the United
527   States. JAMA 299: 214-216.

528 2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of
529   dengue. Nature 496: 504-507.

530 3. Diaz FJ, Farfan-Ale JA, Olson KE, Lorono-Pino MA, Gubler DJ, et al. (2002) Genetic variation within the
531   premembrane coding region of dengue viruses from the Yucatan peninsula of Mexico. Am J Trop Med Hyg
532   67: 93-101.

533 4. Ooi EE, Goh KT, Gubler DJ (2006) Dengue prevention and 35 years of vector control in Singapore. Emerg Infect
534   Dis 12: 887-893.

535 5. Goh KT (1997) Dengue--a re-emerging infectious disease in Singapore. Ann Acad Med Singapore 26: 664-670.

536 6. Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R (2008) Defining challenges and proposing solutions for
537   control of the virus vector Aedes aegypti. PLoS medicine 5: e68.

538 7. Thomas SJ (2011) The necessity and quandaries of dengue vaccine development. J Infect Dis 203: 299-303.

539 8. Endy TP, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, et al. (2002) Spatial and temporal circulation of dengue
540   virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J
541   Epidemiol 156: 52-59.

542   9. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, et al. (2002) Epidemiology of inapparent and

543   symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng

544   Phet, Thailand. Am J Epidemiol 156: 40-51.

545   10. Mammen MP, Pimgate C, Koenraadt CJ, Rothman AL, Aldstadt J, et al. (2008) Spatial and temporal clustering of

546   dengue virus transmission in Thai villages. PLoS Med 5: e205.

547   11. Gibbons RV, Nisalak A, Yoon IK, Tannitisupawong D, Rungsimunpaiboon K, et al. (2013) A model international

548   partnership for community-based research on vaccine-preventable diseases: the Kamphaeng Phet-

549   AFRIMS Virology Research Unit (KAVRU). Vaccine 31: 4487-4500.

550   12. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, et al. (1989) An enzyme-linked

551   immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-

552   circulate. Am J Trop Med Hyg 40: 418-427.

553   13. Anonymous (1997) Dengue haemorrhagic fever: diagnosis, treatment, prevention and control, 2nd edition.

554   Geneva: World Health Organization. 84 p.

555   14. Klungthong C, Gibbons RV, Thaisomboonsuk B, Nisalak A, Kalayanarooj S, et al. (2007) Dengue virus detection

556   using whole blood for reverse transcriptase PCR and virus isolation. J Clin Microbiol 45: 2480-2485.

557   15. Paul A, Harrington LC, Zhang L, Scott JG (2005) Insecticide resistance in Culex pipiens from New York. J Am

558   Mosq Control Assoc 21: 305-309.

559   16. Yoon IK, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, et al. (2012) Fine scale spatiotemporal clustering

560   of dengue virus transmission in children and Aedes aegypti in rural Thai villages. PLoS Negl Trop Dis 6:

561   e1730.

562   17. Jarman RG, Holmes EC, Rodpradit P, Klungthong C, Gibbons RV, et al. (2008) Microevolution of Dengue viruses

563   circulating among primary school children in Kamphaeng Phet, Thailand. J Virol 82: 5494-5500.

564   18. Cummings DA, Iamsirithaworn S, Lessler JT, McDermott A, Prasanthong R, et al. (2009) The impact of the

565   demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical

566   modeling. PLoS medicine 6: e1000139.

567   19. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. The dengue hemorrhagic fever project:

568   viremia and antibody responses early in dengue; 1997 14 -17 April; Papeete, Tahiti.

569   20. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (1997) Dengue in the early febrile phase:

570   viremia and antibody responses. J Infect Dis 176: 322-330.

571   21. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. Antibody response patterns and disease

572   severity by dengue virus serotype; 1997 7-11 December; Orlando, FL.

573   22. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (2000) Dengue viremia titer, antibody

574   response pattern, and virus serotype correlate with disease severity. J Infect Dis 181: 2-9.

575   23. Nimmannitya S (1987) Clinical spectrum and management of dengue haemorrhagic fever. Southeast Asian

576   JTropMedpublic Health 18: 392-397.

577   24. Nimmannitya S (1987) Dengue haemorrhagic fever in Thailand. Southeast Asian JTropMedpublic Health 18:

578   291-294.

579   25. Yoon IK, Rothman AL, Tannitisupawong D, Srikiatkhachorn A, Jarman RG, et al. (2012) Underrecognized mildly

580   symptomatic viremic dengue virus infections in rural Thai schools and villages. J Infect Dis 206: 389-398.

581   26. Rabaa MA, Klungthong C, Yoon IK, Holmes EC, Chinnawirotpisan P, et al. (2013) Frequent in-migration and

582   highly focal transmission of dengue viruses among children in Kamphaeng Phet, Thailand. PLoS Negl Trop

583   Dis 7: e1990.

584   27. Burke DS, Nisalak A, Johnson DE, Scott RM (1988) A prospective study of dengue infections in Bangkok.

585   AmJTropMedHyg 38: 172-180.

586   28. Balmaseda A, Standish K, Mercado JC, Matute JC, Tellez Y, et al. (2010) Trends in patterns of dengue

587   transmission over 4 years in a pediatric cohort study in Nicaragua. J Infect Dis 201: 5-14.

588   29. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, et al. (2005) Epidemiology of dengue and dengue

589   hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72:

590   60-66.

591   30. Endy TP, Yoon IK, Mammen MP (2010) Prospective cohort studies of dengue viral transmission and severity of

592   disease. Curr Top Microbiol Immunol 338: 1-13.

593   31. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector

594   Aedes aegypti within and between rural communities. Am J Trop Med Hyg 72: 209-220.

595   32. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz Soldan V, Kochel TJ, et al. (2009) The role of human

596   movement in the transmission of vector-borne pathogens. PLoS neglected tropical diseases 3: e481.

597   33. Stoddard ST, Forshey BM, Morrison AC, Paz-Soldan VA, Vazquez-Prokopec GM, et al. (2013) House-to-house

598   human movement drives dengue virus transmission. Proc Natl Acad Sci U S A 110: 994-999.

599   34. Nguyet MN, Duong TH, Trung VT, Nguyen TH, Tran CN, et al. (2013) Host and viral features of human dengue

600   cases shape the population of infected and infectious Aedes aegypti mosquitoes. Proc Natl Acad Sci U S A

601   110: 9072-9077.

602