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Asymptomatic humans transmit dengue virus to mosquitoes

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Three-quarters of the estimated 390 million dengue virus (DENV) infections each year are clinically inapparent. People with inapparent dengue virus infections are generally considered dead-end hosts for transmission because they do not reach sufficiently high viremia levels to infect mosquitoes. Here, we show that, despite their lower average level of viremia, asymptomatic people can be infectious to mosquitoes. Moreover, at a given level of viremia, DENV-infected people with no detectable symptoms or before the onset of symptoms are significantly more infectious to mosquitoes than people with symptomatic infections. Because DENV viremic people without clinical symptoms may be exposed to more mosquitoes through their undisrupted daily routines than sick people and represent the bulk of DENV infections, our data indicate that they have the potential to contribute significantly more to virus transmission to mosquitoes than previously recognized.

mosquito experimental infection | Cambodia | *Aedes aegypti* | human-to-mosquito transmission | dengue

With 3.97 billion people living in 128 countries currently at risk for infection, dengue viruses (DENV-1 to -4) cause more human morbidity and mortality worldwide than any other arthropod-borne virus (1, 2). *Aedes aegypti* mosquitoes are the primary vectors of DENV throughout the tropics (3). Dengue prevention relies on the control of *Ae. aegypti* populations, which is failing in most parts of the world due to lack of resources, lack of political will, and/or ineffective implementation (4).

Virus transmission from infected humans to mosquitoes is a critical step in dengue epidemiology, but due to logistical constraints it has been directly examined only in a handful of studies to date (5). In initial experimental infections of human volunteers during the 1920s (6, 7), the onset of clinical symptoms occurred 4–9 d after virus inoculation by mosquito bite (8). DENV-infected humans were infectious to mosquitoes from 2 d before to 2 d after the onset of symptoms, and *Ae. aegypti* fed on viremic people were able to transmit virus to another person after at least 11 d of extrinsic incubation (8). Results from later studies indicated that, for naturally infected people with clinically apparent dengue, the duration of detectable viremia was on average 4–5 d after the onset of symptoms, but could range from 2 to 12 d (9, 10). Investigators in Vietnam fed *Ae. aegypti* directly on 208 symptomatic, hospitalized dengue patients and reported that the probability of successful human-to-mosquito DENV transmission was coincident with the kinetics of viremia (11). Dengue patients were infectious up to 5 d after the onset of symptoms, which generally corresponded with "defervescence" (11).

All previous studies on human-to-mosquito DENV transmission were limited to people with overt illness and did not consider subclinical infections. An estimated 300 million of the total 390 million DENV infections per year are clinically inapparent or mildly symptomatic, i.e., no illness that disrupted a person's daily routine (1). Following Grange et al. (12), we use "inapparent" or "subclinical" interchangeably to denote infections confirmed by virus detection or seroconversion, but with insufficient symptoms to be detected by existing surveillance systems and health care providers. "Asymptomatic" refers to a confirmed DENV infection in the complete absence of reported or detected symptoms. Inapparent human DENV infections are a potentially important component of the overall burden of dengue because they can serve as a previously unrecognized source of mosquito infection (12). Epidemic transmission of DENV associated with low viremia levels and mild illness has been reported (13). It has long been assumed, but not empirically verified, that people with inapparent infections fail to infect mosquitoes because they do not reach sufficiently high viremia levels (5). This assumption is based on the observation that disease severity is positively correlated with the magnitude of DENV viremia (10, 11, 14). To our knowledge, the only study that quantified viral RNA levels in a limited number of asymptomatic DENV infections in humans did not detect a significantly lower viremia (15), but infectiousness to mosquitoes was not evaluated. The aim of the present study was to document variation in DENV infectiousness of naturally infected humans across the spectrum of disease manifestations, including fully asymptomatic infections, and to verify the assumption that people with inapparent infections are not infectious to mosquitoes.

Significance

Our work provides evidence that people who are infected with dengue virus without developing detectable clinical symptoms or prior to the onset of symptoms are infectious to mosquitoes. At a given level of viremia, symptom-free people were markedly more infectious to mosquitoes than clinically symptomatic patients. Our results fundamentally change the current paradigm for dengue epidemiology and control, based on detection of dengue virus-infected cases with apparent illness.

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Conflict of interest statement: P.B. is currently an employee of GlaxoSmithKline Vaccines, but the research presented does not have any relation with his current position.

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Results

Before the development of studies based on geographic cluster sampling around index cases (16, 17), inapparent DENV infections could only be detected in very small numbers or retrospectively in cohort studies after the viremic period had ended (18, 19). We overcame this obstacle by capturing DENV-infected people across the continuum of disease manifestations while they were viremic using a comprehensive catchment system combining passive, hospital-based surveillance and cluster investigations in and around the households of hospitalized index cases (*Materials and Methods*). Following informed consent, wild-type but laboratory-reared *Ae. aegypti* mosquitoes were allowed to feed on the blood of study participants through direct (biting the person) and indirect (artificial feeder) methods (*Materials and Methods*). Legs and wings of blood-engorged mosquitoes that survived a 2-wk extrinsic incubation period (EIP) were tested by serotype-specific, quantitative reverse transcription–PCR (qRT-PCR) to detect and quantify DENV RNA. Mosquitoes with DENV-positive wings and legs indicated that virus had disseminated from their midguts, which is a recognized proxy for their mosquito-to-human transmission potential (20).

Our analyses included a total of 181 study participants who were either viremic at the time of mosquito feeding ($n = 176$) or were viremic at the time of inclusion, but had already reached an undetectable level of viremia at the time of experimental mosquito exposure and were nevertheless subsequently found to be infectious to mosquitoes ($n = 5$). Most study participants (89%) were children under 16 y of age [mean, 9.32; median, 8; interquartile range (IQR), 5–12 y] with a slight male-biased sex ratio (1.2). All four DENV serotypes were represented with a marked predominance of DENV-1 (51.4%), followed by DENV-2 and DENV-4 (24.9% and 22.7%, respectively), and two DENV-3–infected participants (1.1%). There were more secondary (46.3%) than primary DENV infections (33.1%); immune status was undetermined for 20.6% of the infected people. Overall, 126 (69.6%) participants had symptomatic dengue when experimental mosquito feeding took place, 42 (23.2%) developed symptoms after mosquitoes had fed, and 13 (7.2%) did not report and/or exhibit any detectable symptom at recruitment and throughout the 10-d follow-up period. Relative frequencies of DENV serotypes did not differ between asymptomatic, presymptomatic, and symptomatic disease categories ($P = 0.639$). Although DENV transmission to mosquitoes from naturally infected people was recently studied in a symptomatic cohort in Vietnam (11), by extending the present study design to include people with presymptomatic and asymptomatic infections we addressed an unexplored knowledge gap in dengue epidemiology.

Most participants ($n = 156$; 86.2%) underwent both direct and indirect mosquito feedings; a small subset participated in only direct ($n = 8$; 4.4%) or only indirect ($n = 17$; 9.4%) mosquito feedings. Direct mosquito feedings were only performed with children above 4 y of age. A total of 3,163 individual *Ae. aegypti* was assayed, of which 1,645 were fed directly on the infected person (mean per participant, 10.0; median, 9; IQR, 5–14) and 1,518 indirectly on viremic blood (mean per participant, 8.8; median, 7; IQR, 5–11). The proportion of positive mosquitoes was positively and significantly correlated between direct and indirect feedings on the same participant, although indirect feedings generally resulted in a significantly lower proportion of infected mosquitoes (weighted linear regression of infection percentage: indirect = 1.05 + 0.768*direct; $r^2 = 0.69$; *SI Appendix*, Fig. S1). This result indicates that indirect feeding is a less sensitive method for assessing human-to-mosquito transmission than direct feeding, but may nevertheless represent a reasonable alternative when direct feeding is not possible.

First, we examined the relationship between illness and infectiousness to mosquitoes in people who developed symptoms during the course of their DENV infection. Our study design allowed us to identify viremic people before the onset of fever and associated symptoms. Across the presymptomatic and symptomatic cohorts, participants were exposed to mosquitoes from 3 d before to 8 d after the onset of symptoms. Collectively, viremia peaked around 0–2 d after the onset of symptoms and then gradually decreased, but viral RNA remained detectable in some participants on their eighth day of illness (Fig. 1*A*). Infectiousness to mosquitoes closely matched the kinetics of viremia (Fig. 1 *B* and *C*). There was no successful DENV transmission to *Ae. aegypti* beyond the sixth day of illness. One participant's blood did successfully infect mosquitoes by indirect feeding on the seventh day of illness (Fig. 1*C*), but this was not confirmed by direct feeding (Fig. 1*B*). Some participants were infectious as early as 2 d ($n = 3$) or 1 d ($n = 8$) before the onset of symptoms (Fig. 1*B*), confirming results previously reported by Siler et al. (6). Although duration of infectiousness is best measured with longitudinal profiles of the same individuals, our composite time course at the population level suggests that for the viruses we studied humans naturally infected with DENV can be infectious to mosquitoes from 2 d before to 6 d after the onset of illness.

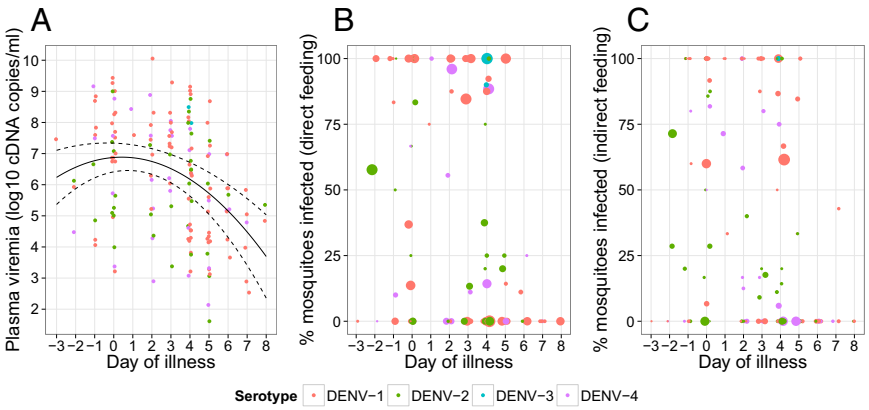


Fig. 1. Profiles of plasma viremia and infectiousness by day of illness. Plasma viremia expressed as DENV cDNA copy number per milliliter (*A*) and percentage of infected mosquitoes following direct (*B*) and indirect feeding (*C*) are shown as a function of day of illness for presymptomatic/symptomatic participants. Day 0 is the onset of clinical symptoms. Each dot corresponds to an individual participant and is color-coded by DENV serotype. *A*, *B*, and *C* include 163, 142, and 144 participants who developed symptoms, respectively. In *A*, solid lines indicate the quadratic regression and dotted lines represent the SE of regression parameter estimates. In *B* and *C*, the size of the dots is proportional to the number of mosquitoes tested per participant.

Next, we examined the human, virological, and entomological factors that were associated with infectiousness to mosquitoes. In multivariate regression analyses, both viremia level and disease category were consistently and significantly associated with the likelihood of mosquito infection (Table 1). Conversely, infection immune status (primary, secondary, or indeterminate status), age, and EIP were not significant predictors of successful mosquito infection. A significant effect of serotype and gender was only detected for one mosquito feeding method, but not both, which may be due to the fact that the cohorts of individuals involved in each mosquito feeding method were not exactly the same.

Our data confirmed that the level of viremia is one of the most important determinants of human infectiousness to mosquitoes (11). The amount of detectable DENV RNA in participants' plasma (expressed in cDNA copies per milliliter) ranged from 1.61 to 10.1 $\log_{10}$ cDNA copies per mL of plasma across all study participants (mean ± SE: 6.17 ± 0.14). The observed magnitude of viremia was not associated with age, gender, DENV serotype, or immune status. It was, however, significantly different between disease categories (*SI Appendix*, Table S1). Viremia level measured in people with asymptomatic infections (mean ± SE: 4.75 ± 0.39 $\log_{10}$ cDNA copies per mL) was lower on average than in people with presymptomatic (mean ± SE: 6.74 ± 0.25 $\log_{10}$ cDNA copies per mL) or symptomatic (mean ± SE: 6.12 ± 0.17 $\log_{10}$ cDNA copies per mL) infections. Because viremia level depends on when it was measured during the course of infection (Fig. 1*A*), we also compared the average viremia level of asymptomatic infections with that of people who developed symptoms, stratified by day of illness. The average viremia level of asymptomatic infections did not differ from viremia levels observed 2–3 d before or 5–8 d after the onset of symptoms. It was, however, significantly lower than between −1 and 4 d of illness. Thus, the average DENV viremia level of asymptomatic infections corresponded to the magnitude or viremia observed during the early and late viremic period of people who develop symptoms. To confirm that the dynamic nature of the viremic phase (Fig. 1*A*) did not confound our time-independent analysis, we repeated the multivariate regression analysis with temporal stratification of disease categories. Although the effect of viremia level decreased, overall results did not change when symptomatic people were split into groups of 0–2, 3–4, and 5–8 d of illness (*SI Appendix*, Table S5). Asymptomatic DENV-infected people were always significantly more infectious than symptomatic people, with the exception of group 0–2 d of illness, which was marginally insignificant for indirect feeding. Presymptomatic DENV infections did not differ significantly from asymptomatic infections (*SI Appendix*, Table S5).

Viremia levels measured by qRT-PCR and expressed, like in this study, as cDNA copy numbers are generally well correlated with infectious titers, but should be interpreted with caution. The number of infectious virus particles measured by the mosquito inoculation technique can be 2–5 logs lower, depending on the virus strain, cell type used, level of viremia, host immune response, and several other factors such as handling and storage conditions of viremic blood sample (21). RT-PCR detects RNA from infectious viruses, but also from immature virions and defective particles. On the other hand, five participants with undetectable DENV viremia by qRT-PCR were infectious to mosquitoes. This further highlights the limited correlation between infectious titer and concentration of viral RNA in plasma. It also suggests that the amount of virus might differ between venous blood (used to measure viremia) and capillary or venule blood (imbibed by mosquitoes) and/or between plasma and whole blood. As previously reported, it is also possible that the high sensitivity of the mosquito infection model may help to detect low levels of viremia that are below the limit of detection by qRT-PCR, but are actually sufficient to infect mosquitoes (21). In our multivariate analysis (Table 1), factors other than the amount of viral RNA in plasma (e.g., serotype and disease category) contributed to differences in participant infectiousness.

Strikingly, asymptomatic and presymptomatic infections were associated with increased overall probability of mosquito infection, independently of viremia level measured as cDNA copy numbers. Asymptomatic and presymptomatic DENV infections did not differ significantly from each other (direct: $P = 0.430$; indirect: $P = 0.496$). Dose–response scatterplots revealed that asymptomatic and presymptomatic infections were more infectious to mosquitoes than symptomatic infections at any given viremia level (Fig. 2). By direct feeding, the 50% mosquito infectious doses [95% confidence interval (CI)] were 5.31 (4.81–5.80), 5.68 (5.30–6.00), and 7.21 (7.05–7.36) $\log_{10}$ viral cDNA copies per mL of plasma in the asymptomatic, presymptomatic, and symptomatic categories, respectively. By indirect feeding, the corresponding estimates were 5.68 (5.14–6.55), 5.97 (5.55–6.33), and 7.78 (7.59–7.99), respectively.

We analyzed viral loads in the wings and legs of infected mosquitoes because they are positively correlated with DENV in *Ae. aegypti* saliva and thus mosquito transmission potential (20). The strongest predictor of viral loads in positive mosquitoes was human viremia, followed by disease category and DENV serotype

Table 1. Multivariate regression analysis of successful human-to-mosquito DENV transmission

	Direct feeding		Indirect feeding	
Factor	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Serotype	N.S.			
DENV-1			3.66 (1.76–7.63)	<0.001
DENV-2			1.74 (0.72–4.19)	0.213
DENV-4			Ref.	
Gender			N.S.	
Male	2.08 (1.07–4.04)	0.032		
Female	Ref.			
Viremia, +1 $\log_{10}$ copies/mL	2.05 (1.64–2.56)	<0.001	1.81 (1.52–2.16)	<0.001
Disease category				
Asymptomatic	10.05 (1.76–57.51)	0.010	6.72 (1.90–23.9)	0.003
Presymptomatic	4.84 (2.02–11.58)	<0.001	4.19 (1.94–9.05)	<0.001
Symptomatic	Ref.		Ref.	

Minimal adequate model based on marginal logistic regression of mosquito infection status. DENV-3 was excluded due to small sample size. Direct and indirect feedings are analyzed separately as indicated in the table heading. Test statistics for the full model are in *SI Appendix*, Table S2. CI, confidence interval; N.S., not significant; OR, odds ratio; Ref., reference level.

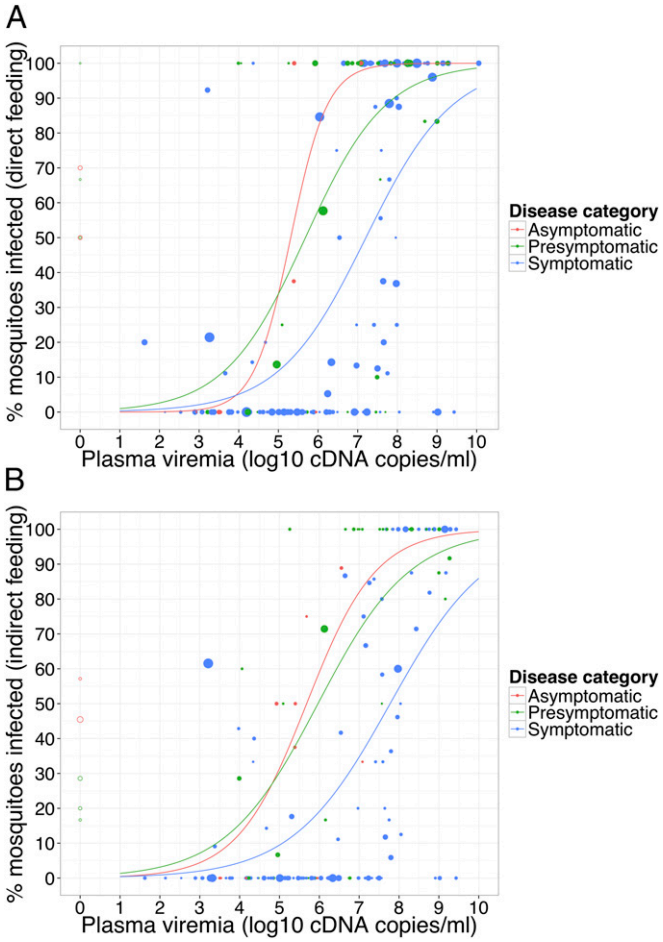


Fig. 2. Dose–response profiles by disease category. The percentage of infected mosquitoes is shown as a function of viremia (measured as cDNA copies per milliliter of plasma) for each study participant involved in direct (*A*) and indirect (*B*) mosquito feedings. Solid symbols represent positive viremia levels. Open symbols correspond to undetectable viremia levels in five participants that resulted in mosquito infection. The size of the dot is proportional to the number of mosquitoes tested per participant (direct feeding: mean, 10.0; median, 9; IQR, 5–14; indirect feeding: mean, 8.8; median, 7; IQR, 5–11). *A* includes 8 asymptomatic, 37 presymptomatic, and 117 symptomatic participants. *B* includes 10 asymptomatic, 41 presymptomatic, and 120 symptomatic participants. Curves are logistic regressions of the data (excluding participants with undetectable viremia). The minimum adequate model for direct feedings explained 34.2% of the variation in the proportion of mosquitoes infected, and disease category alone accounted for 8% of the overall variation. For the indirect feedings, the percentage of variation explained by the model was 37.4% and 7.8% for disease category alone.

(*SI Appendix*, Tables S3 and S4). For both direct and indirect feedings, asymptomatic and presymptomatic infections resulted in a significantly higher viral load in infected mosquitoes than symptomatic infections ($P < 0.001$). This conclusion was confirmed with temporal stratification of symptomatic infections as a function of day of illness (*SI Appendix*, Table S6). Following direct feeding, the mean viral loads (±SE) of infected mosquitoes expressed in $\log_{10}$ cDNA copies per milliliter were higher for mosquitoes that fed on asymptomatic people (6.52 ± 0.26) and presymptomatic people (5.29 ± 0.16) than on symptomatic patients in the 0–2 d (4.91 ± 0.18), 3–4 d (5.20 ± 0.13), and 5–8 d of illness (5.14 ± 0.16). Following indirect feeding, the mean viral loads (±SE) of infected mosquitoes were also higher after feeding on the blood drawn from asymptomatic people (6.06 ± 0.32) and presymptomatic people (5.06 ± 0.22) than on blood from symptomatic patients in the 0–2 d (4.33 ± 0.25), 3–4 d (4.86 ± 0.18), and 5–8 d of illness (3.43 ± 0.21). Thus, not only a larger proportion of mosquitoes became infected when they fed on the blood of participants without symptoms, but mosquitoes that became infected had significantly more viral genome copies in their bodies than those infected by feeding on the blood of symptomatic participants.

Discussion

Our results show that people with natural DENV infections and no clinical symptoms can contribute to virus transmission dynamics by efficiently infecting mosquito vectors. Moreover, people with asymptomatic and presymptomatic infections had an ∼100-fold lower 50% mosquito infectious dose and resulted in larger viral loads in infected mosquitoes, which we interpret as increased transmission potential (20). The dynamic nature of changes in human infectiousness to mosquitoes could reflect a shift in the ratio of noninfectious to infectious viral particles (undetected by qRT-PCR alone) through the course of infection and/or the influence of differential cofactors in asymptomatic compared with symptomatic infections. The strong immune response and high cytokine levels developed during illness may play a role in reducing human infectiousness to mosquitoes (22). Consistent with this hypothesis, reduced risk of human-to-mosquito transmission has been associated with increasing day of illness and rising IgG and IgM titers (11). Understanding and characterizing the mechanistic basis of variation in infectiousness among DENV-infected people should be a priority for future research.

Regardless of the underlying mechanism(s), our results are consistent with the hypothesis that DENV transmission from humans to mosquitoes can be "silent" because it occurs before the onset of symptoms or in the absence of apparent illness. Quantitative estimates of the relative contribution to DENV transmission among disease categories remain to be determined by combining assessments of magnitude and duration of infectiousness across the infectious period of different people with measures of various people's exposure to biting mosquitoes during the course of their infection (23). Larger sample sizes than in the present study and sequential mosquito feedings on the same person will help to further clarify the role of presymptomatic and asymptomatic infections in DENV transmission. Asymptomatic infections had a lower average level of viremia, which is thought to be associated with a shorter time window of infectiousness (11). On the other hand, people without symptoms may be more likely to visit multiple locations during their daily routines where they are cumulatively bitten by more mosquitoes than sick people who are hospitalized or who stay at home and are exposed to only their resident mosquitoes (24). Additional sources of heterogeneity to consider include duration of the mosquito's EIP (25), genetic variation among DENV strains, and intrinsic differences among human and mosquito populations (26, 27).

The observation that clinically inapparent human DENV infections can contribute to the population of infected mosquitoes supports the hypothesis that they are not dead-end hosts for transmission (5). Inapparent infections could contribute to DENV persistent circulation during interepidemic periods and may provide new insights and approaches into outbreak detection and response. For example, inapparent cholera infections play a central role in driving the shape of epidemic curves; people with severe disease are relatively minor contributors (28). Inapparent infections force a more rapid rise and fall of cholera epidemics, shifting the peak of the epidemic curve to an earlier time than previously recognized, and accelerating the process of pathogen epidemic transmission and geographic spread. This has important public health implications for dengue because it reemphasizes the need to rapidly detect and effectively respond to an outbreak, which will depend on the amount of transmission occurring before detection of an increase in disease (29). It also encourages measurement of infection, not just disease, in dengue vaccine and vector control trials. Evaluating the risk of transmission in the absence of symptoms could be important for

other arboviruses with a high proportion of inapparent infections such as yellow fever virus (30).

Global efforts to reverse the increasing burden of dengue disease will require more than overcoming the lack of resources, lack of political will, or ineffective intervention implementation. Equally important will be an improved understanding and application of influential features of virus transmission dynamics. It is our hope that changing views about how people with inapparent and mild DENV infections contribute to transmission will improve the theory of DENV transmission dynamics, lead to innovation in outbreak detection and response, and refine assessment of new prevention strategies.

Materials and Methods

Ethics Statement. This study was approved by the Cambodian National Ethics Committee for Health Research (Protocol 063NECHR) and by the Institut Pasteur Ethics Board for European Projects. A participant's enrollment was subject to obtaining written consent signed by the participant or by a legal representative for participants under 16 y of age.

Study Participants. Patients presenting with acute dengue-like illness between June and October of 2012 and 2013 were enrolled at Kampong Cham City Provincial Hospital and at two district hospitals of the Kampong Cham province (*SI Appendix*, Fig. S2). This province is ∼120 km northeast from Phnom Penh, the capital of Cambodia, where the Institut Pasteur in Cambodia (IPC) is located. Participant inclusion criteria were as follows: (*i*) age ≥2 y (age ≥4 y for direct mosquito feeding); (*ii*) axillary temperature >38.0 °C; (*iii*) two or more of the following symptoms: headache, retroorbital pain, muscle pain, joint pain, rash, and any bleeding; and (*iv*) written informed consent from the participant or a legal representative for participants under 16 y of age.

DENV infection of hospitalized patients was confirmed by NS1 antigen detection using a commercial rapid diagnostic test (for details, see *Dengue Diagnosis and Classification* below) followed by qRT-PCR on the plasma sample obtained during the acute febrile phase of disease (*SI Appendix*, Fig. S3) (31). Patients with a confirmed DENV infection by NS1 antigen detection and/or qRT-PCR were considered dengue index cases (DICs) that initiated geographic cluster investigations. Cluster participants were enrolled from family members at the DIC's household as well as from people living in houses within a 200-m radius of the DIC's home or in the 20 closest houses when the population density of the area exceeded the logistical capacity of the field team. Cluster participants were aged from 2 to 40 y and lived in villages located within 30 km from the provincial hospital (*SI Appendix*, Fig. S2). Cluster participants or representative for the participants under 16 y of age provided a written informed consent before enrollment. Exclusion criteria were as follows: (*i*) pregnancy or breastfeeding; (*ii*) symptoms inconsistent with dengue and obvious nondengue acute infection (e.g., otitis media, pneumonia, meningitis); or (*iii*) known chronic illness.

Clinical Data and Blood Collection. DICs and cluster participants were examined during sequential visits as shown in *SI Appendix*, Figs. S3 and S4. At each visit, data were collected using a standardized questionnaire designed for either hospitalized patients or cluster participants.

For hospitalized patients, three blood samples were collected: shortly after hospital admission during the febrile acute phase (visit 1), at the time of defervescence (visit 2), and during the convalescent phase at hospital discharge (visit 3), which in general corresponded to days 3–5 of hospitalization. The severity of the disease was assessed according to the 1997 World Health Organization (WHO) criteria using clinical, biological, and paraclinical examination data recorded at admission and throughout the entire hospitalization period (32).

To screen cluster participants for DENV infection, home visits were conducted within 24 h after DIC identification [home visit 0 (HV0)] and then again at day 2 (HV2) and day 7 (HV7) during 2012. In 2013, only HV0 and then a visit 4 d later (HV4) were conducted (*SI Appendix*, Fig. S4).

Blood samples drawn during visits were sent with ice packs to IPC within 5–10 h for laboratory diagnosis. Blood samples were collected in tubes with EDTA anticoagulant, and volume drawn was adapted according to participant weight: 3.0 mL for children below 20 kg of weight and 5.0 mL for individuals over 20 kg.

Cluster Investigation Participant Follow-Up. Once confirmed positive for DENV infection, cluster investigation participants were visited at different time points depending on their clinical presentation. Cluster participants with any dengue-like clinical symptoms described above, at any point in their observation period, were considered as symptomatic cases and those who did not report or present any symptom during examination in the follow-up period were classified as asymptomatic individuals. The participant was classified as symptomatic if any symptoms appeared or were reported by the participant or their caregiver during the follow-up period or if the participant or their caregiver reported antipyretic drug intake.

Symptomatic cluster participants were visited immediately after confirmation of DENV infection at three time points: day 0 (D0), D2, and D7. A longer and closer monitoring was conducted in the asymptomatic group at D0, D1, D2, D3, D4, D5, D6, D7, and D10 (*SI Appendix*, Fig. S4). During each daily visit, a blood sample was collected and a standard questionnaire was administered regarding current symptoms or history of symptoms during at least 2 consecutive days for the last 7 d including dengue-like symptoms (headache, retroorbital pain, muscle pain, joint pain, rash, and any bleeding), body temperature, and intake of an antipyretic drug. D0 of the cluster investigation follow-up usually corresponded to the second day after the participant was enrolled (at HV0) due to the time required for blood sample transportation to IPC and subsequent laboratory confirmation.

Dengue Diagnosis and Classification. Blood samples collected at admission (visit 1) of hospitalized patients were tested for DENV NS1 antigen by SD BIOLINE Dengue Duo rapid diagnostic kit (Standard Diagnostic) according to the manufacturer's recommendations. This test was performed by well-trained nurses at the patient's bedside and the result was confirmed at IPC by DENV RNA detection in the same blood sample using a serotype-specific multiplex qRT-PCR method (31). The qRT-PCR had a limit of detection of 0.5–3 plaque-forming units (PFU) and 5–10 cDNA copies per 20-μL reaction, depending on the serotype (31). To screen for additional DENV infections among household members and neighbors, blood samples that were collected at HV0, HV2, HV7 (2012), or HV0 and HV4 (2013) were tested by qRT-PCR at IPC. Results were communicated to the field team on a daily basis to initiate cluster investigations, perform mosquito feedings when required, and ensure the follow-up of cluster participants. DENV viremia was expressed as cDNA copy number per milliliter of plasma.

Serological tests were performed on sera collected during the acute and convalescent phase of the infection in both symptomatic and asymptomatic groups for detection of antibodies against DENV. Because of potential cross-reactivity among flaviviruses, all specimens were tested for both anti-DENV and anti-Japanese encephalitis virus (JEV) using an in-house IgM capture ELISA (MAC-ELISA) and hemagglutination inhibition (HI) assay as previously described (33, 34). Primary or secondary immune status of DENV infections was determined by HI test according to WHO criteria (32).

***Aedes aegypti* for Human-to-Mosquito Transmission Experiments.** Wild immature stages of *Ae. aegypti* were repeatedly collected at ∼2-mo intervals at different locations in Kampong Cham province from areas where the study participants resided. All *Ae. aegypti* females that were used in human-to-mosquito DENV transmission experiments were from laboratory-reared F_2 or F_3 generations derived from F_1 parental females that tested negative for DENV, JEV, and chikungunya virus (CHIKV) by virus-specific RT-PCR and for other potential flaviviruses by pan-flavivirus RT-PCR (31, 35–37).

Briefly, field-caught *Ae. aegypti* larvae (F_0) were pooled, reared in distilled water, and fed on commercial dry fish food. Following emergence, adult mosquitoes were fed twice weekly directly on immobilized mice and maintained on cotton soaked with 10% (wt/vol) sugar solution in an environmental chamber with 12:12 light/dark hours, at 27 °C, and 70% relative humidity. F_1 eggs were collected on paper towels lining oviposition cups placed in the cages. The F_1 generation was reared as described above, and F_1 adults were kept in cages containing males and females. F_1 females were provided blood meals for multiple gonotrophic cycles over a period of 30 d. At the end of this period, surviving F_1 adults were removed and freeze-killed. F_1 adults that died before 30 d were also collected for testing. All F_1 mosquitoes were sorted by sex in a Petri dish placed on ice, pooled into vials of 10 mosquitoes (male and female) per vial, and homogenized. Extracted RNA was tested for DENV, JEV, CHIKV, and other flaviviruses (31, 35–37).

Eggs from cages that included only F_1 females and that tested negative for all tested viruses were hatched and reared to produce F_2 and F_3 adults for experimental feeding as described above. Nulliparous female *Ae. aegypti* of the F_2 and F_3 generations were used in human-to-mosquito transmission experiments.

Experimental Exposure of *Ae. aegypti* to DENV Viremic Blood. Following confirmation of DENV infection, each study participant was asked, or through his/her legal guardian, to participate in human-to-mosquito transmission experiments by direct and/or artificial membrane (indirect) feeding methods (*SI Appendix*, Fig. S5). Written informed consent was provided for all enrolled participants. Both hospitalized patients (DICs) and cluster participants were involved in mosquito feedings (*SI Appendix*, Fig. S6).

For direct feeding, two mesh-covered 500-mL paper cups, each containing 25 female 5- to 9-d-old *Ae. aegypti* that had been starved for 24 h, were placed on

the participant's legs for 5 min. A nurse monitored participants for 30 min for any side effects and was prepared to advise the participant to seek treatment if fever or any other symptoms occurred in the following 2 wk. Antihistamine cream was applied to the sites of mosquito bites to relieve itchiness.

For indirect feeding, venous blood was drawn from the participant into a tube with EDTA anticoagulant within 30 min after direct mosquito feeding. Briefly, 3.0 mL of whole blood was thoroughly mixed with 30 μL of 10 mM ATP used as a phagostimulant, and the remaining blood was kept to quantify DENV RNA by qRT-PCR. Approximately 1.5 mL of mixture was loaded into two glass feeders maintained at 37 °C by a connected heated water source. Two mesh-covered 500-mL paper cups, each containing 25 female 5- to 9-d-old *Ae. aegypti* that had been starved for 24 h, were placed under the feeders for 30 min to allow blood feeding through a piece of desalted porcine intestine used as a membrane.

After direct or indirect feeding, mosquitoes were cold anesthetized at 4 °C for 10–15 min and sorted on a Petri dish placed on ice. Unfed or partially fed females were discarded. Only fully engorged females were transferred to a new 500-mL paper cup and maintained in an environmental chamber maintained in a biosafety level 2+ facility with 12:12 light/dark hours, at 27 °C, and 70% relative humidity for ∼2 wk. EIP ranged from 10 to 19 d, but for most participants (92%) surviving females were harvested between 13 and 16 d after blood feeding. Variation in EIP was taken into account in the statistical analyses.

DENV Detection in Mosquitoes. When harvested for processing, mosquitoes were freeze-killed, and their legs and wings were separated from the rest of the body with sterile forceps and, for each mosquito, placed individually in a vial containing 500 μL of sterile PBS with 10% (vol/vol) FCS and 15–20 ceramic beads. The mixture was homogenized using a MagNA Lyser Instrument (Roche Life Science Thailand, catalog no. 03358976001) at 6,500 rpm for 50 s. Viral load in individual mosquito body parts was measured by qRT-PCR and the results expressed as cDNA copy number per milliliter of legs and wings suspension (31).

Statistical Analyses. All analyses were based on the final cohort of 181 infected human participants including 13 asymptomatic, 42 presymptomatic, and 126 symptomatic people. Analyses of mosquito infection data were performed separately for direct and indirect feeding methods. Detectable plasma viremia levels were $\log_{10}$-transformed and analyzed with an analysis of variance. The proportion of infected mosquitoes was analyzed as a function of the covariates using marginal logistic regression models. Dose–response curves and 50% mosquito infectious dose estimates were derived from the logistic regression coefficients. Because of the small size of the asymptomatic cohort, dose–response curves could not be further stratified within each disease category. Viral load in infected mosquitoes was $\log_{10}$-transformed and analyzed with a generalized linear mixed model that included the random effect of the participant and fixed effects of other covariates. Full models were reduced to minimal adequate models by backward elimination of nonsignificant terms in a stepwise fashion. Dispersion was estimated from the data, and *P* values were obtained with *F* statistics. All statistical analyses were performed in GenStat (38).

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