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Dengue viremia kinetics in asymptomatic and symptomatic infection

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ABSTRACT

Background: Dengue infection is a global health threat. While symptomatic cases contribute to morbidity and mortality, the majority of infected people are asymptomatic but serve as an important reservoir. However, the kinetics of viremia in asymptomatic infections remains unknown.
Methods: We enrolled 279 hospital-based symptomatic index cases and quantified dengue virus (DENV) RNA at enrollment and at the day of defervescence. To identify asymptomatic cases, 175 household members of index cases were monitored for clinical symptoms during follow-up, and blood was taken twice weekly to test for and quantify DENV RNA until cleared.
Results: We detected DENV in thirteen asymptomatic household members (7.43%). Their DENV serotypes were primarily the same as those of their family index cases. The median peak DENV viremia in asymptomatic subjects was lower than that of symptomatic individuals during the febrile phase, and the viral decay rate was slower in asymptomatic infections.
Conclusions: DENV level and kinetics in asymptomatic individuals differed significantly from those of symptomatic cases. Despite the lower viremia, the slower decay rate in asymptomatic infections could lead to their prolonging the infectious reservoir. The improvement of transmission control to prevent such long-lived asymptomatic infections from transmitting the DENV is needed.

Introduction

Dengue is the most important arthropod-borne viral infection worldwide, infecting an estimated 390 million people annually (Bhatt et al., 2013). The incidence of dengue virus (DENV) infection has been rising over the last five decades (Gubler, 2020). The outcome of DENV infection ranges from asymptomatic/inapparent infection, mild self-limited dengue fever (DF) to severe dengue hemorrhagic fever (DHF), with the potential development of life-threatening dengue shock syndrome (DSS). However, the majority of infected individuals have no or insufficient symptoms to result in a clinical presentation;

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nevertheless, they could serve as a significant reservoir for DENV transmission (Endy, 2002; Endy et al., 2011; Grange et al., 2014; ten Bosch et al., 2018).

The level of DENV viremia is one of the most important determinants of human infectiousness to mosquitoes (Nguyen et al., 2013; Duong et al., 2015), and the duration of infection is a crucial parameter determining the epidemiological dynamics of any pathogen and the subsequent R_0 (Anderson and May, 1991). In symptomatic DENV infections, classical experimental infection studies showed that DENV was infectious from two days before and five days after illness onset (Siler et al., 1926; Simmons et al., 1931; Nishiura and Halstead, 2007). The duration of infectiousness, estimated via the success of transmission to mosquitoes or viral isolation, was found to range from between 1–7 days with a mean of 4–5 days and with longer durations in primary than secondary infections (Siler et al., 1926; Duyen et al., 2020; Kuberski et al., 1977). More recent studies using molecular detection of the virus have revealed a similar duration of infection, with a range lasting up to six days post-onset of fever and with some variation according to serotype, disease severity, and 1° vs. 2° infections (Duyen et al., 2020). In experimental non-human primate sylvatic DENV infections, viremia duration ranged from three to five days, depending on the DENV serotype (Althouse et al., 2014). To date, the kinetics of DENV in individuals with asymptomatic DENV infections remains unknown.

We conducted a cohort study to identify and follow asymptomatic DENV infected individuals, DF, and DHF patients prospectively to characterize their viremia kinetics. Asymptomatic DENV infected individuals were identified by investigating the presence of DENV by Reverse transcription PCR (RT-PCR) in household members (HHM) of symptomatic DENV infected patients (index cases) (Dussart et al., 2012). Once identified, we followed these individuals prospectively, monitored their symptoms, and measured the level of DENV in blood samples twice a week until DENV was cleared from the circulation. This knowledge of DENV kinetics in asymptomatic individuals is crucial for predicting the level and duration of infectiousness in this important DENV reservoir.

Materials and methods

Ethics statement

The study was approved by the Institutional Review Board of Faculty of Medicine Vajira Hospital (No.015/12) and the Faculty of Tropical Medicine Mahidol University (TMEC 13−041). All subjects or their legal guardians signed written informed consent before study participation.

Study population

Subjects were recruited from two study sites in Bangkok (Vajira hospital and the Faculty of Tropical Medicine, Mahidol University) and one study site in Tak province (Tasongyang hospital). Subjects were recruited from Vajira hospital in 2012–2015, and the Faculty of Tropical Medicine and Tak sites were added in 2015. A total of 279 index cases were enrolled

Index case sample and data collection

Demographic and clinical data of index cases were collected with standardized case report forms. Blood samples were collected for RT-PCR twice: on the day of enrollment (D1) and on the day fever subsided (Day of defervescence, Ddef). Additional blood samples were collected once a day during the febrile phase between D1 and Ddef and at 2-week and 2-month follow-up time points for a heme-agglutination inhibition (HI) test and other immunological studies. Index cases were classified into DF and DHF according to WHO criteria (World Health Organization, 1997) (Figure 1).

Household member samples and data collection

Once the index cases were confirmed for DENV infection, their HHM were enrolled within 1–2 days. Their body temperature and clinical symptoms were recorded on standardized case report forms throughout the follow-up period. Blood samples were taken

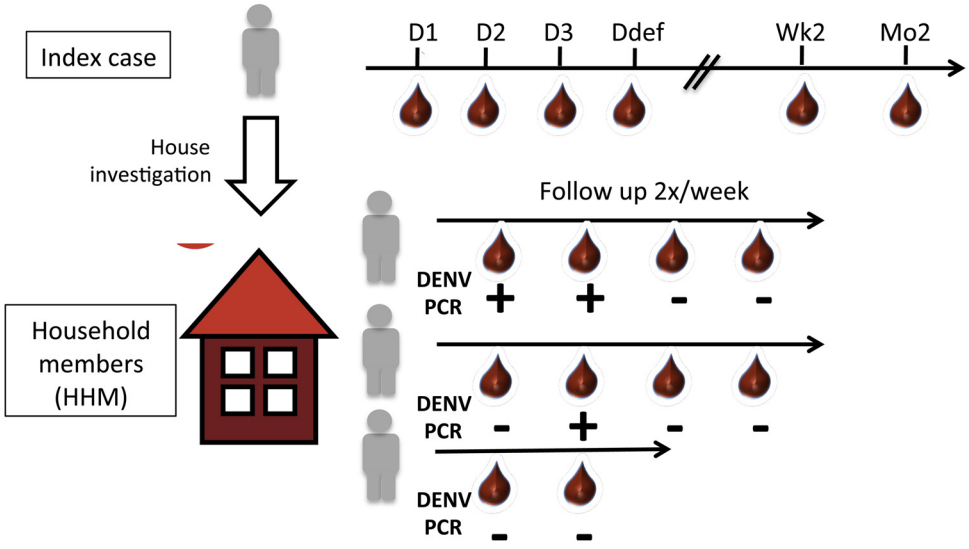

Figure 1. Study design for index cases and household members investigation. D1 (day of enrollment); Ddef (Day of defervescence); Wk2 (two weeks after enrollment); Mo2 (two months after enrollment); DENV PCR (RT-PCR of DENV result).

for DENV RT-PCR on the first day of enrollment (D1) and then between 24–72 hours later. The result of DENV RT-PCR became available within 24 h after blood collection and dictated subsequent investigation and follow-up. If DENV was detectable by RT-PCR, two drops of blood were taken onto filter paper (Aubry et al., 2012) every 2–4 days until DENV become undetectable twice in a row (or the subject was lost to follow-up). The clinical follow-up also ended when DENV was undetectable twice consecutively by RT-PCR (Figure 1).

DENV detection, quantification and serotype determination by PCR

Serotype-specific nested RT-PCR was performed on all samples to detect the presence of DENV RNA and determine the DENV serotype. Quantitative RT-PCR (qRT-PCR) was used to quantify the DENV viral load when possible. First, viral RNA was extracted from blood samples using the QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's instructions and stored at −80 °C until used. Serotype-specific nested RT-PCR was performed according to the published protocol (Lanciotti et al., 1992). The serotype and quantity of DENV RNA were determined by the qRT-PCR assay, as previously described (Duong et al., 2015) (supplementary Table 1).

Primary/secondary DENV infection determination

In-house IgM capture ELISA (Duong et al., 2015) and HI assays were performed as previously described (Clarke, 1958). Paired plasma during acute infection and two weeks or two months follow-up time points (when available) were used for HI. Primary and secondary infections were determined using WHO/TDR (1997) criteria (World Health Organization, 1997).

Statistical analysis

DENV decay rate in index cases was calculated based on the DENV viral load on D1 subtracted by that on Ddef and divided by the number of days between D1 and Ddef. Decay rates in asymptomatic HHM were also calculated for every time point when RT-PCR was performed on an individual.

For risk factor analyses of viral load, decay rate, day to defervescence, and day to viral clearance, a Generalized Linear Model with Poisson error structure was fitted to test the association with the following explanatory variables: age (continuous), gender (M/F), year, site, infection severity (DHF vs. DF) and serotype and where indicated, log10 transformed viral load at D1. The number of individuals included in each analysis varied: all viral load samples were analyzed irrespective of whether they were subsequently lost to follow-up; decay rate included only individuals who were not lost to follow-up and who showed a decrease in viremia from D1 to Ddef (only eight individuals had an increase in viremia from D1 to Ddef); days to defervescence included all individuals not lost to follow-up irrespective of whether viremia increased from D1 to Ddef; day to viral clearance included only those with zero viremia at Ddef.

To assess whether there was a difference in the decay rate between asymptomatic household members and their index cases, a Generalized linear mixed model with Poisson error structure was fitted to decay rate with age, gender, serotype (DENV-3 vs. DENV-4), infection type (Index case vs. Asymptomatic HHM) as explanatory variables and household ID as the random factor. The viral load at D1 and Ddef of the index cases and the peak viral load of the asymptomatic HHM were compared by the Mann-Whitney *U* test For all analyses, a dispersion parameter was estimated and used to account for overdispersion in the data. Analyses were performed in Genstat version 15 (VSN International, 2017).

Results

Index cases characteristics

290 index cases were initially enrolled in the study, of which 279 cases had confirmed DENV infection from 138 households. DENV was detected by RT-PCR in 262 of these 279 cases (94.27%). Of those 17 with negative RT-PCR, eleven had anti-DENV IgM, four had detectable NS1, and two had both anti-DENV IgM and NS1. The majority of index cases had DF (63.44%), All four DENV serotypes circulated during the study years, with DENV-4 (34.40%) and DENV-3 (31.90%) being more prevalent than the other serotypes. An almost equal number of male (48.75%) and female (51.25%) subjects were enrolled, and the majority were adults (68.46%) (Table 1).

DENV kinetics in index cases

Overall, the mean viral load in DENV positive individuals on the day of enrollment (D1) was 7.24×10^9 viral copies/mL (SEM: 3.45×10^9). The time to defervescence ranged from 1 to 7 days post day of recruitment (the average time was 2.28 days, SEM 0.08). Of those individuals that cleared their viral load completely by the day of defervescence, the mean time to clearance was 2.77 days (SEM 0.15).

DENV viral load on D1 was higher in DHF than DF (Relative Risk (RR) = 5.88, $P < 0.001$), decreased with age (RR = 0.92, P = 0.003), was lower in male than female gender (RR = 0.31, $P < 0.001$) and DENV-1 (RR = 0.25, P = 0.001), DENV-2 (RR = 0.08, P = 0.002), and DENV-4 (RR = 0.06, P = 0.006) as compared to DENV-3 (Table 2, top). The full minimum adequate model explained 29.2% of the variation in viral load.

DENV decay rate (from D1 to Ddef) was faster in DHF than DF (RR = 1.44, P = 0.004) and DENV-1 (RR = 1.43, P = 0.014) as compared to DENV-3 (Table 2, middle) and with an increased viral

Table 1
Index cases characteristics.

		Number of cases (percentage)
Severity	DF	177 (63.44%)
	DHF	102 (36.55%)
Year	2012	31 (11.11%)
	2013	118 (42.29%)
	2014	16 (5.73%)
	2015	114 (40.86%)
Serotype	1	39 (13.98%)
	2	38 (13.62%)
	3	89 (31.90%)
	4	96 (34.40%)
	indeterminate[a]	17 (6.09%)
Primary/secondary infection	Primary	22 (8.24%)
	Secondary	208 (74.55%)
	Indeterminate	49 (17.5%)
Gender	Male	136 (48.75%)
	Female	143 (51.25%)
Age	Children(<=15)	88 (31.54%)
	Adults (>15)	191 (68.46%)

[a] These subjects had a positive result for NS1 or IgM, but DENV RNA undetectable by PCR and, therefore, unable to determine serotype.

Table 2
Factors associated with index case DENV kinetics.

A. Day 1 Viral load					
		N	Mean Log_{10} Viremia (SEM)	RR (95% CI)	P-value
Age (years)		222	7.31 (0.14)	0.92 (0.87–0.97)	0.003
Gender	F	113	7.12 (0.21)	Ref	
	M	109	7.51 (0.18)	0.31 (0.16–0.62)	<0.001
Severity	DF	142	7.34 (0.17)	Ref	
	DHF	80	7.27 (0.23)	5.88 (2.92–11.82)	<0.001
Serotype	1	38	7.11 (0.40)	0.25 (0.11–0.57)	0.001
	2	31	6.82 (0.40)	0.08 (0.02–0.40)	0.002
	3	80	7.54 (0.24)	Ref	
	4	73	7.39 (0.17)	0.06 (0.01–0.43)	0.006

B. Decay Rate					
		N	Mean (SEM) decay rate/day	RR	P-value
Severity	DF	114	7.47 (6.83)	Ref	
	DHF	69	7.63 (7.03)	1.44 (1.12–1.84)	0.004
Serotype	1	30	7.71 (7.13)	1.43 (1.08–1.90)	0.014
	2	25	7.66 (7.13)	0.32 (0.79–2.08)	0.319
	3	69	7.55 (6.97)	Ref	
	4	59	7.30 (6.92)	0.18 (0.24–1.32)	0.182
Log10 Viral load D 1		183	7.54 (6.90)	6.34 (5.49–7.32)	<0.001

C. Days to Defervescence					
		N	Mean (SEM) days	RR	P-value
Age (years)		225	2.38 (0.08)	0.990 (0.986–0.997)	0.003
Serotype	1	32	2.24 (0.18)	1.13 (0.93–1.37)	0.21
	2	35	2.07 (0.19)	1.04 (0.84–1.29)	0.69
	3	79	1.98 (0.11)	Ref	
	4	79	3.03 (0.16)	1.53 (1.31–1.78)	<.001
Log10 Viral load D 1		225	2.38 (0.08)	1.12 (1.08–1.16)	<.001

Shown in this table are the number of samples analyzed for each of the significant risk factors, the Relative Risk (RR) and associated P-value and the dependent variable estimate with standard error from the final fit in the multivariate log-linear regression (see Methods).

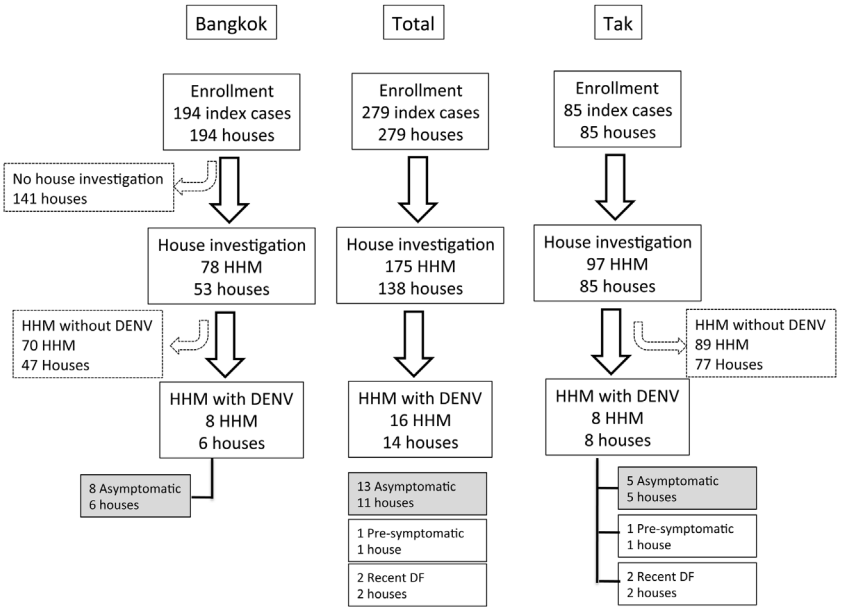

Figure 2. Numbers of index cases and household members investigated and numbers of asymptomatic dengue viremia in Bangkok and Tak study sites.

load on D1 (RR = 6.34, P < 0.001). The full minimum adequate model explained 94.7% of the variation in the decay rate.

Time taken to defervescence decreased with age (RR = 0.99, P = 0.003) and increased with viral load at D1 (RR = 1.12, P < 0.001) and for DENV-4 (RR = 1.53, P < 0.001) as compared to DENV-3 (Table 2, bottom). The full minimum adequate model explained 27.4% of the variation in days to defervescence. A similar result was found when using only those individuals who had a completely cleared viremia on the day of defervescence.

Rate of asymptomatic DENV infections in household members of dengue index cases

Overall, thirteen subjects of 175 HHM (7.43%) from the 138 households investigated had asymptomatic DENV infections (hereon called "asymptomatic HHM") as determined by the presence of DENV RNA by RT-PCR and absence of symptoms during the follow-up period. These thirteen asymptomatic HHM were from eleven households out of the 138 households investigated (7.97%). There was an additional one HHM with DENV viremia that subsequently developed symptoms (pre-symptomatic) in Tak. A further two HHM had had a recent clinical dengue infection within two weeks before their family index cases were diagnosed. Overall, the attack rate with more than one infection in a household (including asymptomatic, pre-symptomatic, and recent DENV infection) was 14/138 households (10.14%). The proportion of households with asymptomatic HHM was 6/53 (11.32%) in Bangkok and 5/85 (5.88%) in Tak (Figure 2).

Characteristics of subjects with asymptomatic dengue infection and their family index cases

The characteristics of each of the thirteen asymptomatic HHM and their family index cases are shown in Supplementary Table 2. There were two households from the Bangkok site that had two asymptomatic HHM in the same house. Most but not all of the asymptomatic HHM 9/13 (69.23%) harbored the same DENV serotypes as their family index cases. Interestingly, two asymptomatic HHM (H1 and H2) had co-infection with two DENV serotypes (DENV-3 and -4), while their family index case was infected with only DENV-4. An additional asymptomatic HHM (H9) had a different DENV serotype from the family index case; there was one index case (family of H10) for whom we do not know the DENV serotype. Most family index cases were male 9/11 (81.82%), while 5/13 (38.46%) of asymptomatic HHM were male. Most, 10/11 (90.90%), family index cases had DF, and only one had DHF.

The kinetics of DENV viremia in asymptomatic infections

Overall, the DENV kinetics varied widely among individuals (Supplementary Fig. 1). While some asymptomatic HHM had high viremia but rapidly cleared (H1, H2, H6, H11), others had lower viremia that lasted longer (H3, H4). H9 likely had a very low viral load below the detection limit of qRT-PCR, but detectable by nested PCR. Unfortunately, several subjects were lost to follow-up before the virus was cleared from the circulation. Taken together, the DENV kinetics in asymptomatic HHM are variable, but DENV viremia could persist up to 1–2 weeks after the detection of index cases in the household.

Factors affecting DENV kinetics in asymptomatic infections

Excluding the single instance of a DENV-1 infection, we assessed risk factors associated with viral load, decay, and clearance rate. The mean maximum viral load was 3.89×10^6 viral copies/mL (SEM 1.35×10^6); there was no association with any explanatory variables (age, gender, serotype, mixed or single serotype infection: P > 0.05). The time needed for DENV clearance from the maximum (measured) viral load was found to decrease with increasing maximum viral load ($\chi^2_1 = 5.54$, P = 0.019) and to be also faster in mixed serotype infections (Single serotype infections:

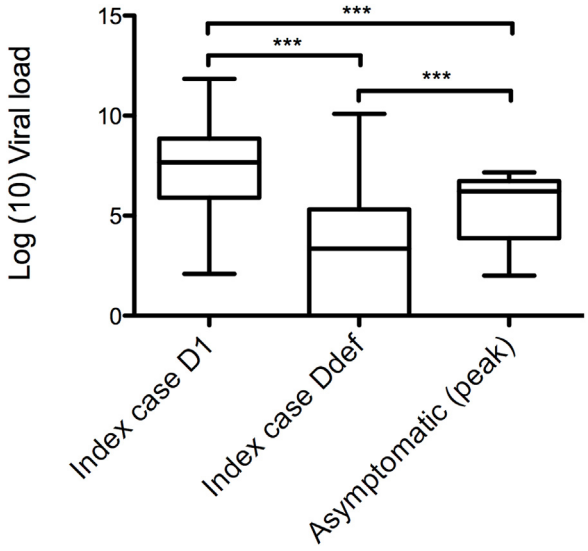

Figure 3. Dengue viral load in asymptomatic dengue infected HHM compared to index cases. The peak viral load of asymptomatic dengue infected HHM (asymptomatic (peak)) was compared to the viral load of index cases at the day of enrollment (D1) and day defervescence (Ddef). Mann-Whitney was used to compare two groups indicated, *p < 0.05, **p < 0.01, ***p < 0.001.

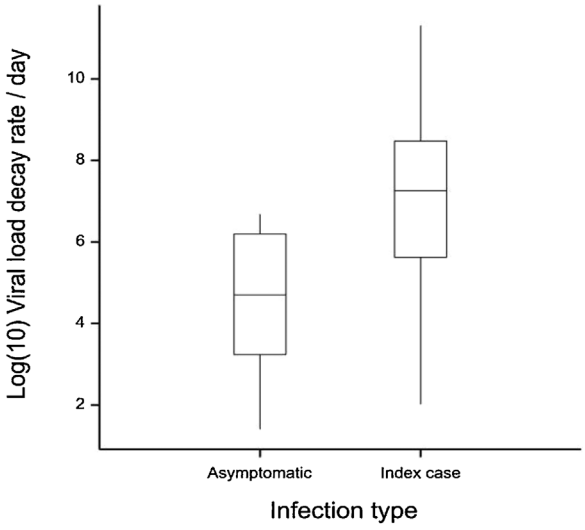

Figure 4. Dengue viral load decay rate by day for asymptomatic HHM infections and index cases from the day of recruitment (D1) to the day of defervescence (Ddef). The decay rate is log (10) transformed for visual clarity.

Mean 6.4 days SEM 1.5; Mixed serotype infections: 3.0 days SEM 0.7. χ^2_1 = 5.02, P = 0.025).

Comparing asymptomatic HHM and index case viral kinetics

The peak viremias of asymptomatic DENV infections were lower than those of index cases at the D1 when patients were still febrile. At Ddef, index cases' viral loads dropped markedly from D1 and were even lower than those of asymptomatic HHM peak viral load (Figure 3).

Asymptomatic infections were associated with a slower decay rate than index cases (Index case: mean 4.31 $\times$ 10^8 / day SEM: 2.91 $\times$ 10^8; Asymptomatic HHM: 5.32 $\times$ 10^5 / day, SEM 2.89 x 10^5. χ21 =72.0, P=0.003) (Figure 4).

Discussion

A DENFREE cohort of DENV infected patients with their household members in Thailand was established to identify asymptomatic DENV infections to provide the first description of viral kinetics within asymptomatic infections and compare them with that in symptomatic clinical presentations from the vicinity and/or the same household. In the symptomatic index cases with a measurable virus at enrollment (D1), viremias were generally higher than those previously reported (Duyen et al., 2020; Yeh et al., 2020), but time to defervescence and/or viral clearance significantly faster (Duyen et al., 2020; Yeh et al., 2020; Gubler et al., 1978; Gubler et al., 1979; Vaughn et al., 1997; Libraty et al., 2002). Significant variation within viral load and time to both defervescence and viral clearance was observed. Notably, patients with DHF and DENV-3 had a higher viral load, and those who were male and older had lower viral loads. A trend for increased viremia with disease severity has been observed previously (Perdomo-Celis et al., 2017), and variation amongst serotypes has also been noted (Duyen et al., 2020). Previous studies have repeatedly found shorter durations of infection in secondary infections as compared to primary infections but which also had higher viremias (Kuberski et al., 1977; Duyen et al., 2020; Yeh et al., 2020; Libraty et al., 2002). In our study, we had too few primary infections to make a reasonable comparison. The viral decay rate from enrollment to defervescence was faster in patients with DHF than those with a higher viral load at enrollment. Despite this, time to defervescence was still longer with higher enrollment viral load.

From index case houses, 7.43% of HHM of index cases had DENV viremia without any symptoms. Most of these asymptomatic HHM had the same DENV serotypes as their family index cases. The kinetics of DENV of these asymptomatic HHM were highly variable among individuals. There were no factors associated with the maximum measured viral load. A higher decay rate was found in DENV-4 infections, and time to viral clearance was faster with increasing viral load and mixed serotype infections. Thus, in both index cases and asymptomatic infections, high viral load was associated with more rapid viral decay, although this did not lead to clearance at defervescence in symptomatic cases. Significant among serotype differences in viral kinetics were observed here as often before (Duyen et al., 2020), and both among and within-serotype differences in the immune response have been recently highlighted (Katzelnick et al., 2015), albeit not in the context of asymptomatic infections.

Although the exact duration of viremia is difficult to estimate, the viremia lasted up to two weeks in some of the asymptomatic infections, and the decay rate was slower than that of index cases. Similarly, compared with their index cases, the maximum level of viremia in asymptomatic HHM was lower than that of index cases at enrollment but higher than that on the day of defervescence, consistent with the observed slower overall decay rate in asymptomatic infections. This observation supports previous studies that found lower viremia in asymptomatic infections than those of symptomatic infections (Duong et al., 2015; Dussart et al., 2012; Sowath et al., 2019

Overall, the kinetics of asymptomatic infections differ from symptomatic infections in the magnitude of the viremia and the rate of clearance, suggesting these infections last longer but with a lower viremia. We and others have previously described fundamental immunological differences in the immune responses associated with symptomatic and asymptomatic infections (García et al., 2010; Simon-Lorière et al., 2017; Halstead and O'Rourke, 1977). A polymorphism in the FcγRIIA was found to be associated with inapparent infection *vs*. DF or DHF in the Cuban population (García et al., 2010). Asymptomatic DENV infected individuals have been found to have increased T cell responses with feedback regulation when compared to symptomatic counterparts (Simon-Lorière et al., 2017). Classically secondary infections are associated with more severe disease due to the phenomenon of ADE and/or cross-reactive T cells (Halstead and O'Rourke, 1977), but whether or not this leads to a decreased risk of an infection being inapparent remains moot (Grange et al., 2014; Clapham and Cummings, 2020). Post-secondary infections will likely induce different immune responsiveness and have also been found to impact upon inapparent rates (Olkowski et al., 2013), which could introduce additional variability in the viral kinetics in both asymptomatic and symptomatic infections. In our study, however, the vast majority of infections were secondary, although it is notable that increasing age had a significant impact on viral clearance rates, suggesting a potential impact of post-secondary infections. More recently, the importance of the interplay between viral genotype within serotype and the interaction with the immune response has been found to be significant (Katzelnick et al., 2015; OhAinle et al., 2020), thereby introducing additional variability that we can not capture in our study.

We have previously shown that asymptomatic infections are as, if not more, infectious to mosquitoes than symptomatic infections (Duong et al., 2015). The likelihood that such asymptomatic infections also last longer does suggest that their epidemiological contribution is even more important. In symptomatic dengue infections, the level of dengue viremia was shown to be the most critical factor for transmission to mosquitoes (Duong et al., 2015). Thus, both those asymptomatic cases with higher but shorter-lived viremia and those with lower but longer-lasting viremia could contribute to transmitting the disease.

A wide range of different epidemiological studies has attempted to ascertain the extent of asymptomatic infections and associated risk factors (Endy, 2002; Endy et al., 2011; Grange et al., 2014; Gordon et al., 2013; Balmaseda et al., 2010; Montoya et al., 2013; Morrison et al., 2010). It is widely agreed that inapparent, sub-clinical infections are prevalent, and thus this silent infectious reservoir will be a significant contributor to transmission. That we observed asymptomatic infections lasting for two weeks will prove particularly problematic in preventing their role as a reservoir through traditional fumigating approaches around index cases: mosquito re-invasion of a fumigated neighborhood occurs far quicker than such slow-decaying infections. A more pro-active program encouraging individual level protection of household members of index cases from being bitten by mosquitoes is likely the only reasonable approach to reduce any epidemiological contribution of potentially infected asymptomatic HHM. The relatively low percentage of infected HHM found in this study is likely underestimated, further underscoring the futility of single time point surveys to see whether HHM of index cases are infected: the variable intrinsic incubation period undermines the utility of active "case" detection.

In conclusion, his work not only the significant role that asymptomatic infections can play in DENV epidemiology but also emphasizes the need for alternative strategies to prevent infected individuals from spreading the virus in the course of their daily mobility.

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Authors contribution

RP, AS, PS, PM designed the study; KM, ST performed experiments; NP clinical data collection and cohort management; PM, VD supervised experiments; PM, RP analyzed data; SS, TY oversaw patient recruitment and clinical data collection; PM oversaw cohort sample collection; RP, AS coordinated the multinational DENFREE project; PS, PM managed DENFREE: Thailand cohort; PM, RP wrote the manuscript; PM, VD, RP edited the manuscript, and all authors read and approved the final manuscript.

Conflict of interest statement

All authors declared no conflict of interest.

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Appendix A. Supplementary data

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