Parasites & Vectors

BRIEF REPORT

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*Wolbachia w*Mel strain-mediated effects on dengue virus vertical transmission from *Aedes aegypti* to their offspring

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Abstract

Background Dengue virus serotypes (DENV-1 to -4) can be transmitted vertically in *Aedes aegpti* mosquitoes. Whether infection with the *w*Mel strain of the endosymbiont *Wolbachia* can reduce the incidence of vertical transmission of DENV from infected females to their offspring is not well understood.

Methods A laboratory colony of Vietnamese *Ae. aegypti*, both with and without *w*Mel infection, were infected with DENV-1 by intrathoracic injection (IT) to estimate the rate of vertical transmission (VT) of the virus. VT in the DENV-infected mosquitoes was calculated via the infection rate estimation from mosquito pool data using maximum likelihood estimation (MLE).

Results In 6047 F1 Vietnamese wild-type *Ae. aegypti*, the MLE of DENV-1 infection was 1.49 per 1000 mosquitoes (95% confidence interval [CI] 0.73–2.74). In 5500 *w*Mel-infected *Ae. aegypti*, the MLE infection rate was 0 (95% CI 0–0.69). The VT rates between mosquito lines showed a statistically significant difference.

Conclusions The results reinforce the view that VT is a rare event in wild-type mosquitoes and that infection with *w*Mel is effective in reducing VT.

Keywords Vertical transmission, Dengue virus, Mosquitoes, *Aedes aegypti*, *Wolbachia*, *w*Mel

Background

Dengue is a mosquito-borne viral infection caused by one of four dengue virus serotypes (DENV-1 to -4) that is endemic in many tropical and sub-tropical countries [1]. The global incidence of dengue has increased dramatically in the last 50 years, with approximately 50–100 million symptomatic infections and 20,000 deaths reported annually in over 125 countries [2, 3]. DENV is transmitted between humans through the bite of an infected female *Aedes* sp. mosquito (*Ae. aegypti* or *Ae. albopictus*). However, DENV can also be transmitted vertically, from the DENV-infected female mosquito to her offspring during follicle development or oviposition [4–6]. This latter transmission mode is hypothesized to contribute to DENV persistence in the mosquito population [7]. A sign of vertical transmission (VT) of DENV in a mosquito populations is the presence of infected male mosquitoes (which do not blood feed) and

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the presence of viruses in immature forms of mosquitoes of any sex. VT is a rare event in nature [8–11] but it has been observed in laboratory studies in both *Ae. aegypti* and *Ae. albopictus* [12, 13]. Several studies have also shown that VT is influenced by a number of factors, including mosquito-rearing temperature, mosquito strain and virus strain [14–18].

Although there are two licensed dengue vaccines, vector control has been the mainstay of dengue control efforts for decades. However, it is obvious that existing vector control methods have not removed the public health burden of dengue in any endemic country. A new approach that involves the insect endosymbiont *Wolbachia* (*w*Mel or *w*AlbB strains) is being applied to render *Ae. aegypti* populations much less competent at transmitting DENV between humans [19–21]. *Wolbachia* inhibits virus replication in mosquito cells via multiple mechanisms, including altering the intracellular environment, activating the innate immune system and interacting with the cell machinery involved in RNA virus infection [22]. Multiple epidemiological studies, including a cluster randomized trial, have shown a large decrease in dengue cases in communities with *w*Mel-*Wolbachia*-treated mosquitoes, demonstrating that *w*Mel introgression is an effective disease control measure [23, 24].

We previously found a very low DENV VT rate (0.23%) among wild-type (Wt) *Ae. aegypti* orally infected with DENV after feeding on viremic blood from dengue patients [9]. To assess whether *w*Mel would eliminate VT of DENV, we established an experimental model system to measure VT in the presence and absence of *w*Mel infection.

Methods

Mosquito lines and rearing

The *w*Mel-infected *Ae. aegypti* population (*w*Mel-*Ae. aegypti*; Vietnamese genetic background) was generated by backcrossing, as previously described [25]. Generations G59 to G61 of colonized *w*Mel-*Ae. aegypti* and generations F54 and F55 of colonized Wt *Ae. aegypti* were used in this study, as previously described [9, 26]. The presence of *w*Mel in each *w*Mel-*Ae. agypti* generation was confirmed by testing [27]. The mosquitoes were reared and maintained under laboratory conditions, at 26–28 °C, 65–85% relative humidity, and a 12:12-h light/dark cycle, with access to 10% sucrose solution ad libitum.

Generating DENV-infected F0 mosquitoes

Both Wt and *w*Met-infected adult female mosquitoes, aged 2–3 days, were injected intrathoracically with 1 µl of solution containing DENV-1 grown in cell culture (10^5 pfu/ml; Genbank Accession Number: FJ432735). DENV-1 was used to establish infection based on the findings of our previous study which indicated that this serotype was less inhibited by *w*Mel than the other three DENV serotypes [28]. Ten injected females were then kept in cups containing 10 male mosquitoes (female:male ratio = 1:1) where they were maintained on sucrose for 10 days.

A human blood meal (non-infectious blood provided via a membrane feeder) was provided to surviving F0 females on day 10 post-injection [28]. After 30 min of blood feeding, fully engorged females were isolated and placed into separate cups (isofemales) containing wet cotton balls for oviposition. Sugar (a piece of cotton soaked in 10% sucrose) was provided for 14 days. On day 14 post non-infectious blood meal, individual F0 females were harvested for testing of their DENV infection status.

Hatching and harvesting F1 mosquitoes

F1 eggs were collected at 5–7 days after the F0 females had taken their non-infectious blood meal and placed in trays filled with fresh water; the trays were kept in incubators at 28 °C under a 12/12-h light/dark cycle. Each tray was provided with one 100 mg tablet of fish food. The larval density was maintained at approximately 3300–3500 larvae per 1.5 l of water. F1 mosquitoes were individually stored and sorted by sex within cohorts originating from the same mother.

F0 and F1 mosquitoes homogenized in a TissueLyser II instrument (Qiagen, Hilden, Germany) at 30 Hz for 2–5 min. Each F0 mosquito homogenate was stored individually, while F1 mosquito homogenates (50 µl from each sample) were pooled before conducting the reverse transcriptase PCR (RT-PCR) assays to detect the presence of *Wolbachia* [27] and DENV [29]. The positive pooled samples were then un-pooled to determine the number of infected individuals using the remaining volume of mosquito homogenate.

Estimating VT of DENV-1 in colonized *Ae. aegypti* with and without *w*Mel infection

To estimate the VT of DENV-1 in a large number of F1 mosquitoes, we utilized the maximum likelihood estimate (MLE) method. This method estimated the proportion of DENV-infected individuals in pooled samples, defined as the infection rate most likely observed given the test results and an assumed probabilistic model (binomial distribution of infected individuals in a positive pool) [30–35]. To account for biases, we utilized bias-corrected likelihood methods and calculated a skew-corrected score confidence interval (95% CI) [36]. The infection rate was reported as the number of infected mosquitoes per 1000 individuals. In addition, to perform comparisons of

CIs, we utilized the Wilson score-based interval of the Newcombe method, which relies on exact calculations of coverage probabilities [37]. This approach allowed us to assess the statistical significance of the differences in infection rates between populations.

To accurately estimate the proportion of infected individuals in a population using MLE, determining the appropriate pool size is crucial to minimize the likelihood of false-negative results. In this study, the pool size was examined using a sample-media pooling approach, adapted from the Clinical and Laboratory Standards Institute (CLSI) document EP12 (CLSI EP12 [38]). The positive percent agreement (PPA) and negative percent agreement (NPA) values were calculated for different pool sizes. The highest PPA value (96%) was obtained with a pool size of four mosquitoes, whereas a pool size of eight mosquitoes showed a PPA of 89% (95% CI 0.8–0.9) and a pool size of sixteen showed only 68% agreement [39]. All three sizes showed 100% agreement fir NPA values. Therefore, a pool size of eight was selected [39].

Results and discussion

Our study aimed to measure whether VT was less likely to occur in *w*Mel-infected *Ae. aegypti* mosquitoes (*w*Mel-*Ae. aegypti*) versus their Wt counterparts. The estimation of VT was conducted in 480 *w*Mel-*Ae. aegypti* females and in 480 Wt females (Fig. 1). Ten days after intrathoracic (IT) inoculation of DENV-1, 420 (87.50%, $N=480$) Wt and 389 (81.04%, $N=480$) *w*Mel-*Ae. aegypti* mosquitoes survived and were given a non-infectious blood meal. Between 5 and 7 days later, approximately 10,328 eggs were collected from 343 (89.09%, $n=385$) virus-infected Wt F0 females (out of 385 Wt F0 females that survived and laid eggs), and approximately 12,027 F1 eggs were collected from 304 (91.84%, $n=331$) virus-infected *w*Mel-*Ae. aegypti* F0 females (out of 331 *w*Mel-*Ae. aegypti* F0 females that survived and laid eggs). In both mosquito lines, high parental infection rates of DENV-1 were observed, with 89.09% (343/385, surviving mosquitoes only) of Wt mosquitoes infected and 91.84% (304/331, surviving mosquitoes only) of *w*Mel-*Ae. aegypti* mosquitoes infected, respectively (Fig. 1). Consistent with previous findings, IT injection resulted in a higher prevalence of DENV-1 compared to oral feeding [9, 40]. DENV-1 RNA concentrations in whole bodies of F0 Wt and *w*Mel-*Ae. aegypti* were comparable and high: 7.7 (95% CI 7.63–7.72) and 7.6 (95% CI 7.57–7.67) $\log_{10}$ copies/ml, respectively (Fig. 2). The DENV copy numbers detected in the whole bodies of each mosquito line were not significantly different (Mann–Whitney test: $U=48216$, $P=0.09$, 95% CI = -0.01-0.13), possibly due to the IT inoculation with a large inoculum of the virus.

A total of 6047 F1 adults emerged from the approximately 10,328 eggs collected from 343 virus-infected Wt F0 females. Similarly, the approximately 12,027 F1 eggs from 304 virus-infected *w*Mel-*Ae. aegypti* F0 females completed their development, providing 5500 *w*Mel-*Ae. aegypti* F1 adults (Fig. 1). All Wt F1 adult mosquitoes were grouped into 785 pools, and 5500 *w*Mel-*Ae. aegypti* F1 adult mosquitoes were grouped into 712 pools (for details, see Additional file 1: Table S1). These pools were tested for DENV-1, and nine positive pools were detected from the Wt group, while no positive pools were found in the *w*Mel-*Ae. aegypti* group. The MLE for the VT rate of DENV-1 in Wt mosquitoes was estimated to be 1.49 (95% CI 0.73–2.74) per 1000 adults. However, in *w*Mel-*Ae. aegypti* mosquitoes, the transmission rate was estimated to be zero (95% CI 0–0.69). The observed difference in the infection rates between the Wt group and the *w*Mel-*Ae. aegypti* group is 1.49 (95% CI 0.67–5.05). As the 95% CI is entirely above 0, the null hypothesis of no difference (H0: Wt − *w*Mel = 0) can be rejected at a significance level of $P<0.05$. Consequently, it can be concluded that the VT rate of the Wt group is significantly higher than that of the *w*Mel group.

Thirteen DENV-1 infected Wt F1 individuals were identified from the nine positive pools, of which 10 were females. When assessing the frequency of mothers transmitting the virus to their progeny and determining the proportion of progeny born to DENV-infected mothers [9] based on positive pools, we observed a slight increase in rates compared to those found in field-collected mosquitoes [9]. Our estimates indicated that for every 343 DENV-infected Wt *Ae. aegypti* female mosquitoes that survived and laid eggs, 11 individuals (3.21%) transmitted the virus to their offspring. However, experimentally we found that only 0.13% of progeny born to DENV-infected mothers would be infected with DENV-1(13 out of 10,328 eggs acquiring the infection). This proportion increased to 0.21% when calculated based on 6047 F1 adults. The frequency of mothers transmitting the virus to any of their progeny in Wt *Ae. aegypti* (3.21%) compared to the frequency identified in field-collected *Ae. aegypti* (2.43%) using patient-derived blood meals [9] might be attributed to the direct introduction of virus into systemic tissues via IT inoculation.

The MLE of DENV-1 infection rate observed in *w*Mel-infected *Ae. aegypti* was comparable to that found in *w*Mel-infected *Ae. aegypti* from Brazil [40]. In our study, we optimized the conditions to increase the probability of a VT event in Wt *Ae. aegypti*, by employing IT inoculation of virus and a long extrinsic incubation period. Furthermore, our study was conducted

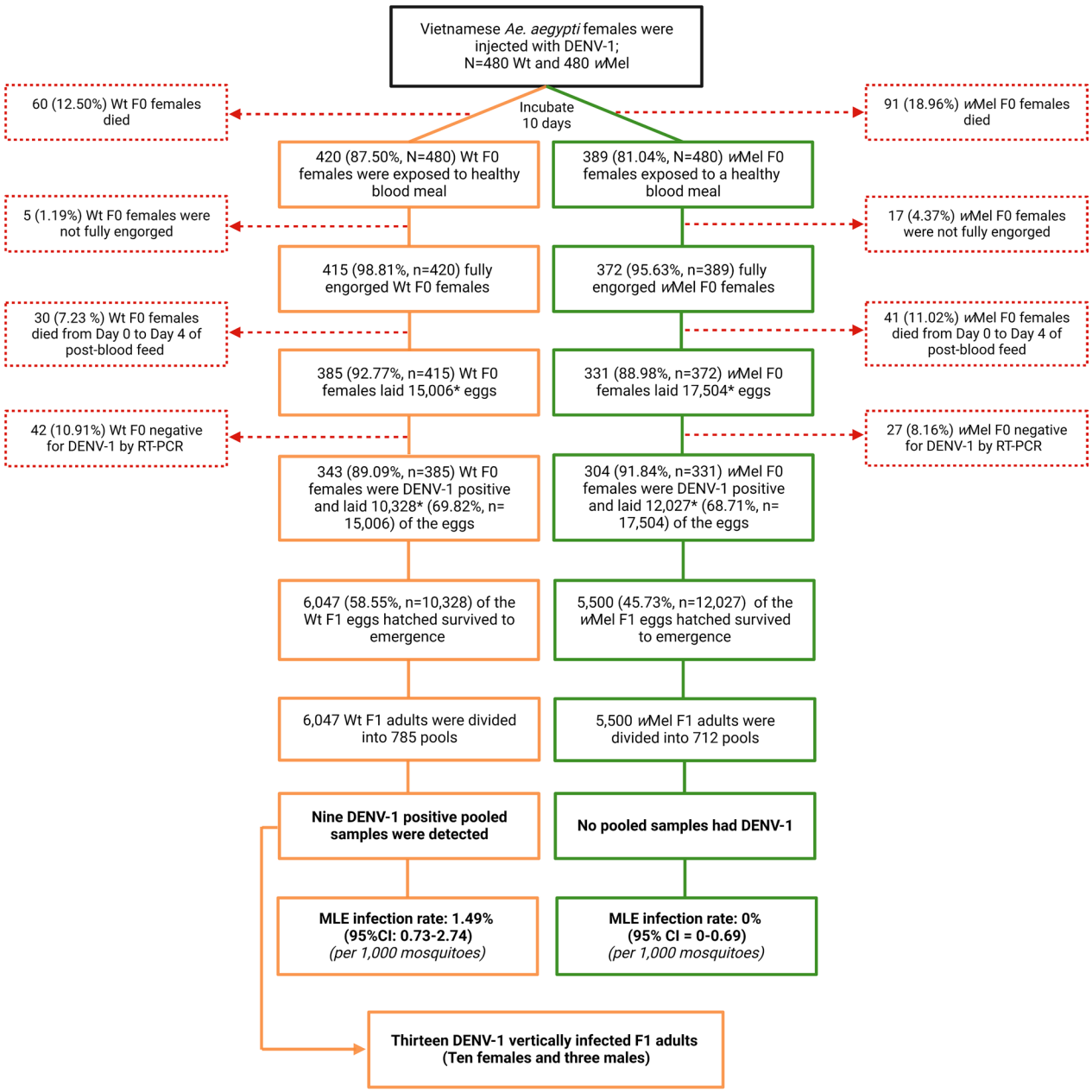

Fig. 1 Flowchart of vertical transmission estimation of DENV-1 in Wt and *w*Mel-infected *Ae. aegypti* with a Vietnamese background. Shown is the fate of mosquitoes as they were processed in order to determine the frequency of vertical transmission of F0 females after being infected by microinjection of DENV-1 (strain FJ432735). The orange boxes represent Wt *Ae. aegypti*, the green boxes represent *w*Mel-*Ae. aegypti* and the red boxes indicate excluded samples. The asterisk (*) indicates the numbers are estimated. DENV-1, Dengue virus serotype 1; MLE, maximum likelihood estimate of infection rate; RT-PCR, reverse transcriptase-PCR; *w*Mel, *Wolbachia* strain *w*Mel-infected *Ae. aegypti*; Wt, wild type

with a large sample size, approximately 11,547 total F1 *Ae. aegypti* (6047 Wt and 5500 *w*Mel-*Ae. aegypti*). Despite these advantages, the VT event was not recorded in *w*Mel-infected *Ae. aegypti*. The absence of DENV infection in *w*Mel-infected F1 mosquitoes and the observed difference in VT rates between mosquito lines provide evidence that *w*Mel is effective in reducing VT. The ability of *w*Mel to decrease DENV replication in *w*Mel-carrying mosquitoes has been used to reduce the incidence of dengue through the deployment of *Wolbachia* mosquitoes in endemic areas [23, 24]. The ability of *w*Mel to reduce the VT of DENV can be attributed to various mechanisms, including resource competition, immune system activation and

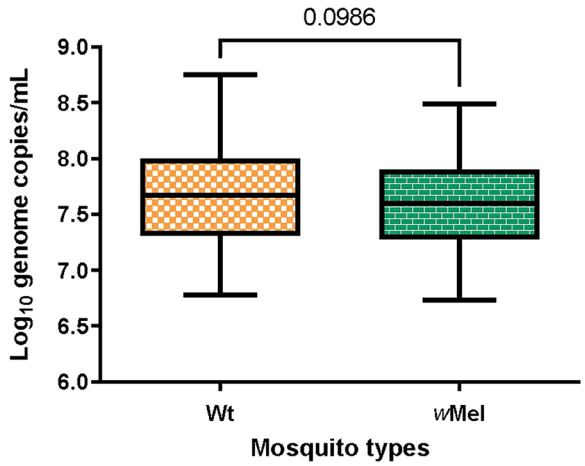

Fig. 2 DENV-1 copy numbers in F0 female *Ae. aegypti* with and without *w*Mel infection. The infectivity of DENV-1 by intrathoracic injection in F0 females represents log10 viral genome copy numbers. Wt mosquitoes are shown in orange and *w*Mel-mosquitoes are shown in green. *w*Mel, *Wolbachia* strain *w*Mel-infected *Ae. aegypti*; Wt, wild type

interference with viral replication in the reproductive tissues of mosquitoes [41–44]. These mechanisms are recognized for their ability to reduce the replication of DENV and could therefore limit its transmission from one generation to the next generation. The capacity of *w*Mel to diminish dengue transmission in both horizontal and vertical modes of transmission is critical in mitigating the incidence of dengue, ultimately decreasing the overall disease burden.

Conclusions

The results of the present study support the belief that VT is a rare phenomenon. *w*Mel infection reduces VT in *w*Mel-carrying *Ae. aegypti* population.

Abbreviations

DENV	Dengue virus
IT	Intrathoracic
MLE	Maximum likelihood estimate (of infection rate)
NPA	Negative percent agreement
RT-PCR	Reverse transcriptase PCR
PPA	Positive percent agreement
VT	Vertical transmission
*w*Mel	*Wolbachia pipientis*, *w*Mel strain
Wt	Wild type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05921-y.

Additional file 1: Table S1. Number of F1 adults distributed in each pooled sample.

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Author contributions

KDTH, DSG, SY and CPS participated in the study design. KDTH, VTT, LTV, DLT, VTTN, GNT, THTX and NVT performed the experiments. KDTH and DSG coordinated data management. KDTH analyzed the data. CPS contributed with funding and reagents. KDTH and CPS wrote the report. All authors read and approved the final report.

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Availability of data and materials

All relevant data generated or analyzed during this study are included in this published article and its additional files.

Declarations

Ethics approval and consent to participate

All work was conducted at the Hospital for Tropical Diseases (HTD), Ho Chi Minh City (HCMC), Vietnam. The enrolment of healthy volunteers to provide non-infectious blood meals to Wt *Ae. aegypti* and *w*Mel-*Ae. aegypti* mosquitoes was approved by the Ethics Committee HTD EC and OxTREC (approval nos. HTD EC CS/NĐ/14/12 and OxTREC 45–14). Informed consent was obtained from all participants by trained study staff.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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