Limited Transmission Potential of Takeda's Tetravalent Dengue Vaccine Candidate by *Aedes albopictus*

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Abstract. Recombinant live-attenuated chimeric tetravalent dengue vaccine viruses, TDV-1, -2, -3, and -4, contain the premembrane and envelope genes of dengue virus serotypes 1–4 in the replicative background of the attenuated dengue virus type-2 (DENV-2) PDK-53 vaccine strain. Previous results have shown that these recombinant vaccine viruses demonstrate limited infection and dissemination in *Aedes aegypti* and are unlikely to be transmitted by the primary mosquito vector of DENVs. In this report, we expand this analysis by assessing vector competence of all four serotypes of the TDV virus in *Aedes albopictus*, the secondary mosquito vector of DENVs. Our results indicate that these vaccine viruses demonstrate incompetence or defective infection and dissemination in these mosquitoes and will likely not be transmissible.

INTRODUCTION

Dengue virus (DENV) infects an estimated 390 million people annually throughout tropical and subtropical regions of the world.¹ Although most cases are clinically inapparent, infection with any of the four serotypes of DENV can lead to a variety of clinical symptoms ranging from mild to severe disease and death. Lasting immunity is considered to be type specific, and infection with a heterologous serotype is associated with a higher likelihood of developing severe disease.^{2,3} Therefore, an ideal DENV vaccine should protect against infection with all four serotypes of DENV.

Takeda's tetravalent dengue vaccine (TDV), previously known as DENVax, is a tetravalent live-attenuated DENV vaccine candidate currently in Phase III human clinical trials. Phase I and II trials have shown promising safety and immunogenicity results.^{4–6} The recombinant TDV contains chimeric DENVs engineered on the replicative genetic backbone of the live-attenuated DENV-2 PDK-53 vaccine virus,^{7–9} which is derived from a wild-type (wt) DENV-2 16681 strain through serial passages in primary dog kidney cells.¹⁰ The PDK-53 strain contains three major attenuating determinants, at nucleotide position 57 of the 5' non-coding region, amino acid 53 of NS1, and amino acid 250 of NS3.^{10–12} A recombinant, clone-derived DENV-2 PDK-53 comprises the DENV-2 component of TDV (TDV-2).^{8,10} The structural genes prM and E from wt DENV-1 16007, DENV-3 16562, or DENV-4 1036 were cloned into the PDK-53 attenuated replicative backbone to create the other three TDV components, TDV-1, -3, and -4, respectively.^{7–9}

It is critical that live-attenuated dengue vaccine viruses will not be transmitted by mosquitoes from vaccinees to the unvaccinated population, and part of the vaccine safety evaluation requires confirmation of low mosquito transmission potential.^{13–18} To be transmissible by mosquitoes, the virus needs to be able to infect the mosquito midgut (infection), escape the midgut infection barrier, replicate in the salivary glands (dissemination), and be released into the mosquito

saliva (transmission).¹⁹ The TDV viruses were previously found to be inefficient at infection, dissemination, and transmission in *Aedes aegypti*, the primary vector of DENV.^{7,20} In this study, we further evaluated the potential for oral infection, dissemination, and transmission of TDV viruses in the DENV secondary vector, *Aedes albopictus*.

MATERIALS AND METHODS

Cells and virus. Vero cells used in the study were amplified from WHO Vero RCB 10-87 (World Health Organization [WHO] Reference Cell Banks). The TDV viruses were prepared in the Centers for Disease Control and Prevention (CDC) laboratory by one time Vero cell passage of Current Good Manufacturing Practice–manufactured premaster DENVax seeds obtained from Inviragen (now Takeda Vaccines, Inc., Cambridge, MA).⁷ The premaster DENVax seeds are the genetically characterized virus stocks used to generate the master vaccine seeds as starting material for manufacture of clinical TDV lots. Thus, the viruses amplified from the premaster seeds were laboratory-surrogate master TDV seeds to be used for characterization in this mosquito infection study. The CDC laboratory strains of wt DENV-1 16007, DENV-2 16681, DENV-3 16562, and DENV-4 1036 used as the prM-E donors for the recombinant TDV viruses were included as parental wt virus controls in the study. All viruses were subjected to one further passage in Vero cells at a multiplicity of infection (MOI) of 0.01–0.001 for 6–14 days at 37°C with 5% CO₂ to generate fresh virus stocks for preparing blood meals used in mosquito oral infection, and were monitored daily for cytopathic effect.

Mosquitoes and infectious blood meals. *Aedes albopictus* mosquitoes were derived from a colony established in 1987 from Lake Charles, Louisiana.²¹ Previous studies have observed significantly reduced infection in mosquitoes exposed to blood meals made with frozen stock of DENV or some other flaviviruses.^{22,23} Therefore, we made blood meals with viruses freshly harvested on the day of oral infection. Back titration of the blood meals and multiple experiments were conducted for each virus in an attempt to achieve sufficient mosquitoes exposed to similar level of virus titer between the wt and TDV for comparisons. Titers from 2 to 5 blood meal preparations were averaged (log-transformed

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mean), weighted according to the number of mosquitoes processed, and tested in each trial (Table 1).

On the day of oral infection, TDV or wt DENV culture supernatants freshly amplified in Vero cells were harvested and mixed with defibrinated calf blood (Colorado Serum Company, Denver, CO) at a ratio of 1:1. An aliquot of each infectious blood meal was retained at -80°C for later back titration of virus doses. Five-to-seven day old mosquitoes were sugar-starved overnight and then offered the infectious blood meal for one hour using a Hemotek membrane feeding system (Discovery Workshops, Lancashire, United Kingdom). Fully engorged females were sorted and incubated as previously described.⁷ After 14 days, all surviving mosquitoes were anesthetized via exposure to triethylamine (Flynap[®], Carolina Biological Supply Company, Burlington, NC) before body, leg, and saliva collection. Saliva was collected by inserting the proboscis of the anesthetized mosquito into a capillary tube containing immersion oil.²⁴ After allowing salivation for at least 30 minutes, the capillary tubes were expelled into tubes containing 300 μL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 5 $\mu\text{g}/\text{mL}$ Fungizone[®] (Thermo Fisher Scientific). Saliva, legs (4–6 per mosquito), and bodies were stored separately at -80°C .

Sample processing and virus titration. Body and leg samples were homogenized and titrated as previously described.^{7,20} Briefly, mosquito body parts were homogenized with copper coated BBs using a mixer mill and then clarified by centrifugation. Virus titers were measured by plaque titration in infected Vero cells with medium/agarose overlay and neutral red staining. Plaque titration results from bodies, legs, and saliva were used to determine the infection, dissemination, and transmission rates, respectively. The limits of detection for these assays were 5 plaque-forming units (pfu)/body or leg sample and 3 pfu/saliva sample.

Statistical analysis. Statistical analysis was conducted with R Stat Package v3.3.2 (<https://www.r-project.org>) and Prism 6 (GraphPad). Logistic regression with Firth's bias correction was used to compare proportion of infected mosquitoes, which was adjusted for individual blood meal titer each mosquito was exposed to. Multiple testing with an overall Type I error of 0.05 was used to compare across viruses and serotypes. Fisher's exact test was used to compare the proportion of dissemination in wt versus TDV groups. Unpaired two-tailed *t* tests were used for statistical

comparison of geometric mean titers (GMT) from wt DENV- and TDV-infected mosquitoes. The *t* test was applied to two versions of the data; one includes only positive samples, and the other includes all samples with the negative samples (<5 pfu) assigned an arbitrary value of 1 pfu for analysis, to more accurately analyze groups with all or mostly negative results. Both analyses resulted in identical *P* values as shown in Figure 2. We also analyzed the GMT data using a "worst-case scenario" method, in which negative samples in wt groups were assigned a value of 1 pfu/mL, and negative samples in TDV groups were assigned a value of 4 pfu/mL. This method would decrease the statistical significance of the findings, but in this worse-case scenario, all comparisons remained significant at $P \leq 0.02$.

RESULTS

The wt strains of DENV-1 and DENV-2 resulted in high *Ae. albopictus* infection rates (84–97%), whereas TDV-1 and TDV-2 infected only 6% of exposed mosquitoes each (Table 1 and Figure 1). Wt DENV-3 infected 47% of mosquitoes, whereas TDV-3 at a 10-fold higher titer than that of the wt DENV-3 blood meal failed to infect any mosquitoes. TDV-4 also did not infect any mosquitoes, but the blood meals containing TDV-4 did not attain titers comparable with those containing wt DENV-4 or to the other three TDV strains in these experiments. Because of the difficulty in obtaining a comparable blood meal titer for TDV-4, an additional experiment was performed in which wt DENV-4 1036 was serially diluted in medium before preparation of blood meals. Similar to wt DENV-1 and DENV-2, wt DENV-4 was highly infectious at titers ranging from 6.70 to 7.46 $\log_{10}$ pfu/mL (99.1%, shown as "DENV-1036 high" in Table 1) to the mosquitoes, and it still infected 40% of mosquitoes at the lower titer range (3.85–5.11 $\log_{10}$ pfu/mL, "DENV-4 1036 low"). By contrast, TDV-4 at 5.09–5.64 $\log_{10}$ pfu/mL did not infect any mosquitoes tested (Table 1). Overall, body infection rates with all four TDV viruses were significantly lower than those with wt DENVs by logistic regression ($P < 0.001$) (Figure 1). Furthermore, once infected, all 4 wt DENVs replicated effectively in mosquitoes (body GMT at 4.57–5.89 $\log_{10}$ pfu), whereas mosquitoes infected by TDV strains attained 10^4 -fold lower GMT ($P < 0.0001$ by two-tailed *t* test) (Table 1 and Figure 2A).

Virus dissemination efficiency (escaping the midgut and spreading to mosquito legs) is reported as the percentage of total exposed mosquitoes containing infectious virus in their

TABLE 1
Infection and dissemination of DENVs and TDVs in *Aedes albopictus*

Virus	Blood meal*	Infection†		Dissemination‡	
	GMT $\pm$ SD	Positive bodies	Body titers	Positive legs	Leg titers
DENV-1 16007	6.61 $\pm$ 0.36	21/25	5.89 $\pm$ 0.40	15/25	2.89 $\pm$ 0.96
TDV-1	6.67 $\pm$ 0.38	2/36	1.30 $\pm$ 0.85	1/36	0.7
DENV-2 16681	6.40 $\pm$ 1.05	35/36	5.36 $\pm$ 0.65	18/36	2.77 $\pm$ 1.08
TDV-2	7.16 $\pm$ 0.84	2/33	1.35 $\pm$ 0.07	0/33	N/A
DENV-3 16562	5.47 $\pm$ 0.29	31/66	5.60 $\pm$ 0.65	12/66	2.00 $\pm$ 1.07
TDV-3	6.96 $\pm$ 0.19	0/66	N/A	0/66	N/A
DENV-4 1036 (high)	6.76 $\pm$ 0.60	109/110	5.31 $\pm$ 0.60	98/110	3.16 $\pm$ 0.99
DENV-4 1036 (low)	4.63 $\pm$ 0.63	10/25	4.57 $\pm$ 1.08	7/25	1.79 $\pm$ 1.30
TDV-4	5.10 $\pm$ 0.13	0/51	N/A	0/66	N/A

DENV = dengue virus; GMT = geometric mean titers; TDV = tetravalent dengue vaccine.

* Virus geometric mean titer $\pm$ SD ($\log_{10}$ pfu/mL) in blood meals, weighted by the number of mosquitoes exposed to each titer.

† Positive virus-infected mosquito bodies among total exposed mosquitoes. Body titers ($\log_{10}$ pfu/body) are calculated as GMT from positive samples only.

‡ Mosquitoes with positive virus-infected legs among total exposed mosquitoes. Leg titers ($\log_{10}$ pfu/body) are calculated as GMT from positive samples only.

FIGURE 1. Infection and dissemination rates of wild-type dengue and tetravalent dengue vaccine (TDV) viruses-infected *Aedes albopictus*. *** $P < 0.001$ by logistic regression comparison of mosquito body infection rates, adjusted for blood meal titer each experiment group received. **** $P < 0.0001$ by Fisher's Exact test used to compare dissemination rates.

legs. All 4 wt DENVs at similar or lower titers to their counterpart TDVs showed dissemination ranging from 18.1% (DENV-3 16562) to 60.0% (DENV-1 16007) (Figure 1). On the contrary, no virus dissemination was detected in mosquitoes exposed to TDV-2, TDV-3, or TDV-4, and only one of the two mosquitoes with TDV-1 in the body displayed a minimal titer in legs (5 pfu/leg sample, which was the limit of detection) (Table 1). Compared with their corresponding parental wt DENVs, all TDV viruses had significantly lower dissemination rates ($P < 0.0001$, Fisher's exact test) (Figure 1). In addition, the $2.89 \pm 0.96 \log_{10}$ pfu/leg sample GMT from all 15 positive legs of mosquitoes infected with wt DENV-1 16007 was significantly higher than the 5 pfu from the single TDV-1 positive sample ($P < 0.0001$) (Table 1 and Figure 2B). Virus was detected in saliva of only two mosquitoes, both of which were infected with wt DENV-4 1036. Because of the undetectable or very low transmission rates of both wt DENVs and TDV viruses, we cannot meaningfully compare transmission efficiencies of TDV viruses to those of their parental DENVs. However, because infection and dissemination rates by TDV viruses were undetectable or much lower than their parental DENVs, it is expected that any transmission potential of TDV viruses would also be substantially lower than that of their cognate wt DENVs.

DISCUSSION

Poor replication in *Ae. albopictus*-derived C6/36 cells in vitro has been determined to be one of the attenuation phenotype markers of the DENV-2 PDK-based vaccine viruses,^{8,11,25} and the TDV master seeds exhibit marked growth reduction in C6/36 cells compared with their parental wt DENVs.⁷ This study describes the first characterization of TDV oral infection and replication in live, biologically relevant *Ae. albopictus* mosquitoes. We showed here that oral infection rates of *Ae. albopictus* with TDV strains (0–6%) were strikingly lower than those that occurred with the wt DENV strains (40–97%). In addition, in those few cases where infection with TDV-1 or TDV-2 occurred, body titers of virus were also substantially lower than those seen with wt DENVs. These data extend our previous

FIGURE 2. (A) Virus titers in bodies and (B) legs of mosquitoes. The dashed line indicates the detection limit. Negative samples were assigned an artificial value at 10^0 for the graph, but are not included for geometric mean titers (GMT) calculation (black solid bar). Standard deviation for each GMT value can be found in Table 1. **** Indicates $P < 0.0001$ by unpaired two-tailed t test. T tests were analyzed in two ways, as described in Materials and Methods (one includes only positive samples, and the other compared all samples including negative samples assigned an arbitrary value of 1 pfu). For pairs that can be compared in both ways, the P values were identical from both methods.

findings that TDV attains significantly lower infection rates and body titer in *Ae. aegypti* (0–3.2% infection and GMT $\leq 0.7 \log_{10}$ pfu) than their wt DENVs (8–44% infection and GMT of 3.1–3.8 $\log_{10}$ pfu).⁷

Similar to the observation in *Ae. aegypti*, dissemination of TDVs in *Ae. albopictus* was mostly undetectable. Among a total of 201 mosquitoes exposed to any TDV, dissemination was found only in one mosquito (infected by TDV-1). On the contrary, wt DENVs disseminated in 18–60% of *Ae. albopictus*, and their leg GMT titers were all significantly higher than that in the single TDV-1 mosquito. The virus-positive saliva samples were obtained only from the wt DENV-4 high dose group, suggesting that higher titers are likely required for even these wt DENV strains to establish detectable infection in salivary glands. Lambrechts et al.²⁶ reported a positive correlation between titers in legs and transmission potential of DENV-1 in *Ae. aegypti*, with no transmission detected in mosquitoes with leg titers below 3 $\log_{10}$ pfu/leg. As the mean leg titers of the wt DENV strains in our study were lower than or near this limit, it is unsurprising that minimal virus was detected in saliva. Although no meaningful comparison can be made between wt DENVs and TDVs from our saliva results, comparative results of infection and dissemination between TDVs and wt DENVs were sufficient to demonstrate marked attenuation of the TDV in *Ae. albopictus*. Therefore, it is reasonable to conclude that any transmission potential (if ever possible) of TDV would also be much lower than their parental wt DENV counterparts.

Multiple studies have shown that *Ae. albopictus* is generally more susceptible to midgut infection with DENV than *Ae. aegypti*, but less susceptible to dissemination and transmission of the virus.^{26–29} One study, which exposed mosquitoes by direct blood feeding on viremic patients, demonstrated a similar minimum 50% infectious dose (MID₅₀) required to infect both *Ae. species*, but *Ae. albopictus* exhibited significantly higher DENV RNA concentrations in abdominal tissues.³⁰ However, the frequency of infectious saliva was lower for *Ae. albopictus* than that for *Ae. aegypti*.³⁰ Our data on the 4 wt DENV strains are in agreement with these studies and showed higher infection susceptibility and viral titers in the abdomens of *Ae. albopictus* than in those of *Ae. aegypti*.⁷ However, our dissemination results with the wt DENVs were also higher in *Ae. albopictus* than in *Ae. aegypti*, which differ from the conclusions previously described. This disagreement in virus dissemination may be due to our use of an *Ae. albopictus* colony that has been maintained in the laboratory for many generations. Variable results regarding the relative susceptibilities of *Ae. albopictus* and *Ae. aegypti* to DENVs are mainly attributable to different DENV isolates and/or different histories of mosquito colonies in the laboratory.²⁸ A meta-analysis of 14 studies found that susceptibility of *Ae. albopictus* to DENV increases with time (> 5 generations) during colonization. The mechanism of the enhanced susceptibility overtime is not well understood, but loss or attenuation of *Wolbachia* in high generation colonized *A. albopictus* was hypothesized as a possible cause.²⁸ *Wolbachia*, an endosymbiotic bacterium associated with viral inhibition in infected insects, naturally infects *Ae. albopictus* but not *Ae. aegypti*. However, the effect of *Wolbachia* is dependent on both mosquito population and virus strain,^{31–34} and the *Wolbachia* infection status of our laboratory *Ae.*

albopictus colony and its original field ancestor is unknown. Therefore, we cannot determine whether loss of *Wolbachia* could be a plausible reason that the *Ae. albopictus* colony in this study resulted in higher dissemination than *Ae. aegypti* by the same wt DENV strains. Nevertheless, we chose to characterize TDV infection and replication in this highly sensitive laboratory colony for the present vaccine safety evaluation.

In phase I and II clinical trials, human volunteers vaccinated with TDV attained titers of infectious virus that were typically lower than 2 $\log_{10}$ infectious focus forming units per mL (iffu/mL, a measure that is typically similar to pfu/mL), with a single TDV-4 vaccinated individual achieving a titer of 3.11 $\log_{10}$ iffu/mL.^{5,6} Although the blood meal TDV titers used in this study were more than 10- to 1,000-fold higher than those observed in vaccinees, the TDV viruses exhibited minimal infection and dissemination in highly sensitive colonized *Ae. albopictus* mosquitoes. In addition, mosquito infection via direct feeding on DENV viremic subjects indicated that the MID₅₀ for *Ae. albopictus* averaged 7.10 $\log_{10}$ genome equivalents (ge)/mL for all four serotypes.³⁰ The average peak viremic RNA titers in TDV human trials were approximately 4 $\log_{10}$ ge/mL (~1,000-fold lower than the previously reported MID₅₀) among viremia-positive samples.^{5,6} Thus, the magnitude of the TDV titers tested here are likely above the upper bounds for the real TDV mosquito infection, dissemination, and transmission potential in a natural setting.

In conclusion, the very low infection rates seen here with TDV strains, in combination with the minimal virus titers in the infected bodies, and absent or minimal dissemination observed, suggest that all four serotypes of TDV viruses are highly attenuated in *Ae. albopictus*. Based on this study and our previous *Ae. aegypti* study, transmission of TDV via the primary or secondary mosquito vectors for DENVs from vaccinees to other populations is very unlikely.

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