RESEARCH NOTE

DENGUE-2 VIRUS CARRYING CAPACITY OF THAI Aedes aegypti STRAINS WITH DIFFERENT SUSCEPTIBILITY TO DELTAMETHRIN

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Abstract. Deltamethrin-resistant Aedes aegypti currently threatens the effectiveness of dengue hemorrhagic fever control operations in Thailand. Although a previous study has suggested that insecticide resistance may increase Ae. aegypti susceptibility to dengue-2 virus infection, our experimental data showed no significant association between laboratory-induced deltamethrin-resistance in a Thai Ae. aegypti isolate and its susceptibility to dengue-2 infection.

Keywords: Aedes aegypti, deltamethrin-resistant, dengue-2 virus, susceptibility

INTRODUCTION

Dengue hemorrhagic fever (DHF) in Thailand was first recognized in 1958 (Wangroongsarb, 1995). Since then, there have been major epidemics of DHF every two to four years, with each succeeding epidemic becoming progressively larger (Nimmannitya, 1987). In 2009, the Thai Ministry of Public Health reported a total of 65,951 cases of DHF, with 83 deaths (Division of Vector-borne Diseases Control, 2009). Vector control is the only current method available to combat outbreaks of DHF in Thailand. Over the past 20 years an intensive space spraying of deltamethrin has been the mainstay control of infected Ae. aegypti during epidemics in Thailand. Unfortunately this strategy may have a limited utility in the future due to the increasing incidence of deltamethrin resistant Ae. aegypti (Srisawat et al, 2010). In some areas of Thailand, such as Chon Buri Province, a recommended lethal dose of deltamethrin kills <50% of Ae. aegypti (Jirakanjanakit et al, 2007). Numerous studies have shown that the susceptibility of Ae. aegypti to dengue virus infection significantly varies among different
mosquito populations (Gubler et al, 1979; Rosen et al, 1985; Sumanochitrapon et al, 1998; Vazeille-Falcoz et al, 1999). In Thailand, it was shown that the ‘type form’ of Ae. aegypti is more susceptible to dengue virus infection than the ‘pale form’ phenotype (Sucharit et al, 1997). However it is not known if newly emerging Thai populations of deltamethrin-resistant Ae. aegypti have a different susceptibility to dengue virus infection. This is an important factor to consider as Gokhale et al (2000) showed that Indian Ae. aegypti surviving insecticide fogging have an increased susceptibility to dengue-2 virus.

This study investigated whether deltamethrin-resistant phenotype of Ae. aegypti linked to changes in the susceptibility to dengue virus infection. We compared dengue-2 virus susceptibility of a deltamethrin-tolerant Thai isolate (Khu Bua Tolerant) to the same isolate selected against sub-lethal doses of deltamethrin (Khu Bua Resistant, S11). The Ae. aegypti New Orleans (WHO susceptible, G77) was used as control.

MATERIALS AND METHODS

Mosquito strains

Three Ae. aegypti strains were used: Khu Bua Tolerant strain (mean deltamethrin KT50 = 40.5 minutes range 37.4-44.4 minutes) kindly provided by Dr Pun gasem Paeporn (Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; Khu Bua Resistant strain [mean deltamethrin KT50 = 117.8 minutes (92.6-149.7 minutes)] selected at Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; and WHO (New Orleans) Susceptible strain [mean deltamethrin KT50 = 10.4 minutes (8.6-12.4 minutes)] was kindly provided by Dr Audrey E Lenhart (Liverpool School of Tropical Medicine, Liverpool, UK), which originated from New Orleans, USA. The care and selection of these strains were as previous described (Srisawat et al, 2010).

This study was approved by the Faculty of Tropical Medicine, Mahidol University, Animal Care and Use Committee (no. 2009/09).

Viral titration by Toxorhynchites splendens inoculation

Dengue-2 virus stock was prepared from a seed stock and stored at -80°C. Infectivity assays, estimated as MID50/ml of bivalent and tetravalent blends of parent strains were compared with the corresponding monovalent dengue strain. Two hundred Toxorhynchites splendens infected with DEN-2 PGMK-3 were tritutated in phosphate-buffered saline (PBS) pH 7.5 containing 50% inactivated fetal calf serum (FCS) and clarified by centrifugation at 11,180g at 4°C for 1 hour. Infectious blood meals were prepared by mixing 1 part of virus suspension with 1 part of washed red blood cells and 1 part of 10% sugar solution.

Female mosquitoes, 3-5 days old, were deprived of food for 1-2 days. An artificial membrane feeder was used for oral feeding for 45 minutes at 37°C (Rutledge et al, 1964; Collin et al, 1964). Fully engorged mosquitoes were kept provided with 10% sugar solution and maintained at 32°C, 70-80% relative humidity for 14 days.

Dengue-2 virus titration was conducted by intrathoracic inoculation of Tx. splendens (0.34 µl/mosquito) at various dilutions (10-27 mosquitoes per dilution) from the highest to the lower dilution using one needle, followed by incubation at 32°C for 14 days. Viral antigen by immunofluorescence (IFA) (Thet-Win, 1982)
on day 14 was performed. *Toxorhynchites* infected with dengue-2 virus obtained from squashed mosquito head showed typical perinuclear fluorescence (data not shown). Calculation of virus titer was as described by Reed and Meunch (1938) and is expressed as MID$_{50}$/ml (Table 1).

**Detection of dengue-2 virus in *Ae. aegypti***

Dengue-2 RNA was extracted from squashed head and thorax of three *Ae. aegypti* using QIAamp® Viral RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions and stored at -70°C until use.

Dengue-2 virus RNA was detected using One Step RT-PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The universal primer sequences were DEN NS5 forward (5’-CAATATGCTGAAACGCGCGAGAAACCG-3’) and DEN NS5 reverse (5’-TTGCACCAACAGTGAATGTCTTCAGGTTC-3’) (Lanciotti et al, 1992). PCR products were analyzed by electrophoresis at 100 V in 1.5% agarose gel for 25 minutes, stained with ethidium bromide and visualized under UV light.

Dengue-2 virus in positive samples of the three mosquito strains were further compared using quantitative RT-PCR (Richard et al, 2003). The first-strand cDNA was synthesized from each RNA sample extracted from the positive mosquito samples in three mosquito strains using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), using forward primer 5’- NNN-NNN-3’ and reverse primer 5’- NNN-NNN-3’, following the manufacturer’s instructions. Quantitative RT-PCR to detect dengue-2 virus cDNA was performed according to QuantiTect SYBR Green PCR (QIAGEN, Hilden, Germany) protocol. Specific primers used were Denv-2 NS5 forward (5’-ACAAGTGCAACACCTGTCCAT-3’) and Denv-2 NS5 reverse (5’-GCGCACCATTGGTCTCT-3’) (Richardson et al, 2006). The reaction mixture of 20 µl consisted of 10 µl of 2x QuantiTect SYBR Green PCR master mix, 1 µl of 0.5 µM Den-F primer, 1 µl of 0.5 µM Den-R Primer, 61 µl of RNase-free

<table>
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<th>Virus dilution</th>
<th>T. splendens no.</th>
<th>IFA test</th>
<th>Total mosquito +ve</th>
<th>Total mosquito -ve</th>
<th>Ratio</th>
<th>%</th>
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</thead>
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<td>5</td>
<td>0</td>
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<td>5</td>
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<td>17/17</td>
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<tr>
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</tr>
<tr>
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<td>3</td>
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<td>5/10</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>$10^7$</td>
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<td>5</td>
<td>0/13</td>
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</table>

Table 1

Dengue-2 virus titer used in *Tx. splendens* inoculation (infectious blood meal).

IFA, immunofluorescence assay
water and 2 µl of DNA template. Thermal cycling conditions (Light Cycler MJ Research Chromo 4; Bio-Rad, Hercules, CA) were as follows: 95ºC for 15 minutes; 40 cycles of 94ºC for 15 seconds; 55ºC for 30 seconds; 72ºC for 30 seconds. Fluorescence was captured at the end of each extension step (72ºC). Data were analyzed using Opticon Monitor 3 program.

RESULTS

*Ae. aegypti* mosquitoes susceptible, tolerant and resistant strains, fully engorged for 14 days after viral infection, were squashed and the head and thorax were determined for dengue-2 virus through amplification of a 648 bp using DEN NS5 forward and reverse primers. The initiated titer of blood meal feeding was 8.92 log MID$_{50}$/ml. The infection rate of the susceptible strain, tolerant strain, and resistant strain was 9% (3/33), 12% (4/32), and 10% (3/31), respectively. Despite a slightly higher infection rate in the local Thai wild type, this does not reach statistical significance (Fisher exact test: $p>0.05$, $\chi^2=0.175$, df=1).

Quantitative RT-PCR was performed on each of the 9 dengue-2 positive mosquitoes, which showed that the WHO Susceptible strain mosquitoes had a lower threshold cycle (C$_T$) (20±2), than that of the Khu Bua Tolerant strain (21±1) and the Khu Bua Resistant strain (24±1). Interestingly there is a significant positive correlation between the degree of deltamethrin-resistance of the mosquito strain (K$_T$) and the relative viral load of the infected mosquitoes ($1/C_T$) (Spearman correlation: $p = 0.023$, $r = 0.7191$) (Fig1).

DISCUSSION

Gubler *et al* (1979) demonstrated that there were significant variations in susceptibility to oral infection with dengue viruses among geographic strains of *Ae. aegypti*, showing that *Ae. aegypti* could be infected with dengue virus when feeding on a virus suspension ranging in infectious blood meal titers from 7.3 to 9.0 log MID$_{50}$/ml, with a range of infection rates of 7.0-90.6%. The amount of virus required to infect 50% of the population was established in each *Ae. aegypti* strain and it was shown that the oral ID$_{50}$ varies inversely with the susceptibility of a particular mosquito strain, and this is an important quantitative factor, which
could affect vector competence. It has been known that dengue viruses could be transmitted by a few mosquito species (Cordellier et al, 1983) other than the classical vector, Ae. aegypti (Freier and Rosen, 1987), with the rate of isolation of dengue viruses being significant for transmission. These observations have raised interest in the range of mosquitoes susceptible to oral infection with dengue viruses (Rosen et al, 1985; Freier and Rosen, 1987; Richard et al, 2003). In addition, the use of mosquitoes in laboratory to isolate dengue virus (Coleman and McLean, 1973; Rosen and Gubler, 1974) has generated a need for information on the range of species susceptible to infection by parental inoculation.

The susceptibility to dengue-2 virus using artificial membrane feeding (AMF) or orally infecting of Aedes aegypti Khu Bua deltamethrin-tolerant strain and Ae. aegypti Khu Bua deltamethrin-resistant strain were compared with Ae. aegypti WHO susceptible strain by RT-PCR. It has been observed that significant numbers of mosquitoes are infected with dengue virus when feeding on humans or animals with circulating virus titers as low as 4.0 log MID₅₀/ml (Gubler, 1978). Our virus titer, by artificial membrane feeding technique, was as high as 8.92 log MID₅₀/ml. Therefore, the infection rate in the three mosquito strains under laboratory conditions may not represent the infection rate in natural population. Field study is needed to further investigate in order to provide precise information regarding susceptibility to oral infection with dengue virus of deltamethrin-resistant and susceptible strains. The current study indicated that insecticide resistance did not play a significant role in altering the vectorial capacity of Ae. aegypti to dengue virus. Susceptible and local strains, however, appeared to be able to support the development of the virus than resistant strain. However, not only mosquito properties affect dengue virus transmission but other factors, such as viremic states, serotype, virus titers and reinfection. The overall information from this study is not sufficient to judge the vector competence the three strains.

Although significant differences in the vectorial capacity of Ae. aegypti populations are known to exist (Donnelly et al, 2009), this capacity has not yet been linked to an insecticide susceptibility phenotype. Data from this study suggests that it is unlikely that deltamethrin-susceptibility affects the vectorial capacity of Thai Ae. aegypti to dengue infection. Despite a slightly higher dengue virus infection rate in the Khu Bua tolerant and resistant mosquitoes, the deltamethrin-sensitive strain supports a slightly higher viral load. Indeed our results support the seminal finding of Gubler et al (1979), which showed a lack of association between dengue infection rate and insecticide resistance. However, as we only examined a limited number of isolates and only one dengue serotype under laboratory conditions, we cannot reject the possibility that deltamethrin-resistant mosquitoes have an altered vectorial capacity. If anything, our limited viral load assay would suggest the deltamethrin-resistant isolates have a slightly reduced capacity to support the dengue-2 virus.

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