# RESEARCH NOTE

# DENGUE-2 VIRUS CARRYING CAPACITY OF THAI *AEDES AEGYPTI* STRAINS WITH DIFFERENT SUSCEPTIBILITY TO DELTAMETHRIN

Theerawit Phanpoowong[1,2], Usa Lek-Uthai[2], Supatra Thongrungkiat[3], Narumon Komalamisra[3], Raweewan Srisawat[3], Bruce Russell[4] and Laurent Renia[4]

[1]Infectious Diseases and Epidemiology Program (Parasitology), Faculty of Graduate Studies, Mahidol University, Bangkok; [2]Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University, Bangkok; [3]Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [4]Singapore Immunology Network (SIgN), Agency for Science Technology, and Research (A*STAR), Biopolis, Singapore

**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

Correspondence: Dr Usa Lek-Uthai, Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University, 420/1 Ratchawithi Road, Ratchathewi, Bangkok10400, Thailand.
Tel/Fax: +66 (0) 2644 5130
E-mail: phulu@mahidol.ac.th

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, $S^{11}$). The *Ae. aegypti* New Orleans (WHO susceptible, $G^{77}$) was used as control.

## MATERIALS AND METHODS

### Mosquito strains

Three *Ae. aegypti* strains were used: Khu Bua Tolerant strain (mean deltamethrin $KT_{50}$ = 40.5 minutes range 37.4-44.4 minutes) kindly provided by Dr Pungasem Paeporn (Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; Khu Bua Resistant strain [mean deltamethrin $KT_{50}$ = 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 $KT_{50}$ = 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 -80ºC. Infectivity assays, estimated as $MID_{50}$/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 triturated in phosphate-buffered saline (PBS) pH 7.5 containing 50% inactivated fetal calf serum (FCS) and clarified by centrifugation at 11,180*g* at 4ºC 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 37ºC (Rutledge *et al*, 1964; Collin *et al*, 1964). Fully engorged mosquitoes were kept provided with 10% sugar solution and maintained at 32ºC, 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 32ºC for 14 days. Viral antigen by immunofluorescence (IFA) (Thet-Win, 1982)

Table 1
Dengue-2 virus titer used in *Tx. splendens* inoculation (infectious blood meal).

| Virus dilution | *Tx. splendens* no. | IFA test | | Total mosquito +ve | Total mosquito -ve | Ratio | % |
|---|---|---|---|---|---|---|---|
| | | +ve virus | -ve virus | | | | |
| $10^{0}$ | 27 | 5 | 0 | 27 | 0 | 27/27 | 100 |
| $10^{-1}$ | 22 | 5 | 0 | 22 | 0 | 22/22 | 100 |
| $10^{-2}$ | 17 | 5 | 0 | 17 | 0 | 17/17 | 100 |
| $10^{-3}$ | 13 | 4 | 1 | 12 | 1 | 12/13 | 92 |
| $10^{-4}$ | 11 | 3 | 2 | 8 | 3 | 8/11 | 73 |
| $10^{-5}$ | 10 | 3 | 2 | 5 | 5 | 5/10 | 50 |
| $10^{-6}$ | 10 | 2 | 3 | 2 | 8 | 2/10 | 20 |
| $10^{-7}$ | 13 | 0 | 5 | 0 | 13 | 0/13 | 0 |

IFA, immunofluorescence assay

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 -70ºC 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'-CAATATGCTGAAAC GCGCGAGAAACCG-3') and DEN NS5 reverse (5'-TTGCACCAACAGTGAAT GTCTTCAGGTTC-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' 2- NNN-NNN-3' and reverse primer 5' 2- 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'-ACAAGTC GAACAACCTGGTCCAT-3') and Denv-2 NS5 reverse (5'-GCCGCACCATTGGTCT-TCTC-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

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.

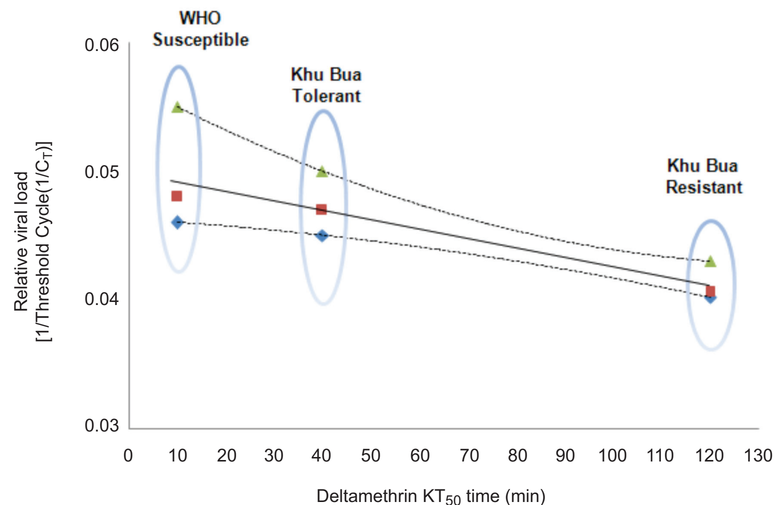


Fig 1–Susceptibility of the three *Ae. aegypti* strains with differing deltamethrin-resistance levels [deltamethrin-susceptible WHO (New Orleans) strain, Khu Bua deltamethrin-tolerant strain, and Khu Bua deltamethrin-resistant strain] to dengue-2 virus infection. (1/$C_T$ ≅ high viral load); KT, the time in minutes taken to kill 50% of the mosquitoes).

## 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 $MID_{50}$/ml (Gubler, 1978). Our virus titer, by artificial membrane feeding technique, was as high as 8.92 log $MID_{50}$/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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## REFERENCES

Coleman JC, McLean DM. Dengue virus transmission by *Aedes aegypti* mosquitoes following intrathoracic inoculation. *Am J Trop Med Hyg* 1973; 22: 124-9.

Collin WE, Harrison AJ, Skinner JC. The use of membrane feeding technique to determine infection and transmission thresholds of Semliki Forest virus in *An. quaderimaculatus* and *An. albimanus*. *Mosq News* 1964; 24: 25-7.

Cordellier R, Bouchite B, Roche JC, Monteny N, Diaco B, Akoliba P. Circulation silvatique du virus dengue 2 en 1980, dans les savannes subsoudaniennes du Cote d'Ivoire. *Cah ORSTOM Ser Entomol Med Parasitol* 1983; 21: 165-79.

Division of Vector-borne Diseases Control, Ministry of Public Health, Thailand. Annual report 2009: Dengue hemorrhagic fever/Dengue fever situation. Nonthaburi: Ministry of Public Health, 2009.

Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, Black WC. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? *Trends Parasitol* 2009; 25: 213-9.

Freier JE, Rosen L. Vertical transmission of dengue viruses by mosquitoes of the *Ae. scutellaris* group. *Am J Trop Med Hyg* 1987; 37: 640-7.

Gokhale MD, Jacob PG, Mourya DT. Dengue virus and insecticide susceptibility status of *Aedes aegypti* mosquitoes from Belagola village, Mandya District, Karnataka State: during and post-epidemic investigations. *J Commun Dis* 2000; 32: 247-53.

Gubler DJ. Facters influencing the distribution and spread of epidemic dengue hemorrhagic fever, Asian. *J Infect Dis* 1978; 2: 128-31.

Jirakanjanakit N, Saengtharatip S, Rongnoparut P, Duchon S, Bellec C, Yoksan S. Trend of temephos resistance in *Aedes* (Stegomyia) mosquitoes in Thailand during 2003-2005. *Environ Entomol* 2007; 36: 506-11.

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992; 30: 545-51.

Nimmannitya S. Dengue hemorrhagic fever in Thailand. *Southeast Asian J Trop Med Public Health* 1987; 18: 291-4.

Reed RJ, Meunch H. A simple method of estimating fifty percent end points. *Am J Trop Med Hyg* 1938; 27: 493-7.

Richard M, Bates E, Davies C, Newman J. Development of a real time quantitative PCR assay for detecting human DNA. *Croatian Med J* 2003; 44: 273-80.

Richardson J, Molina-Cruz A, Salazar MI, Black W. Quantitative analysis of dengue-2 virus RNA during the extrinsic incubation period in individual *Aedes aegypti*. *Am J Trop Med Hyg* 2006; 74: 132-41.

Rosen L, Gubler D. The use of mosquito to detect and propagate dengue viruses. *Am J Trop Med Hyg* 1974; 23: 1153-60.

Rosen L, Roseboon LE, Gubler DJ,Lien JC, Chaniotis BN. Comparative susceptibility of mosquito species and strains to oral and parenteral infection with dengue and Japanese encephalitis viruses. *Am J Trop Med Hyg* 1985; 34: 603-15.

Rutledge LC, Ward RA, Gould DJ. Studied on the feeding response of mosquitoes to nutritive solution in a new membrane feeder. *Mosq News* 1964; 24: 407-19.

Srisawat R, Komalamisra N, Eshita Y, *et al*. Point mutations in domain II of the voltage-gated sodium channel gene in deltamethrin-resistant *Aedes aegypti* (Diptera: Culicidae). *Appl Entomol Zool* 2010; 45: 275-82.

Sucharit S, Jirakanjanakit N, Thongrungkiat S, Komalamisra N, Surathin K. The discriminative infection of dengue virus in *Aedes aegypti* at subspecific level. *Trop Med* 1997; 39: 75-80.

Sumanochitrapon W, Strickman D, Sitiprasasna R, Kittayapong P, Bruce L. Effect of size and geographic origin of *Aedes aegypti* on oral infection with dengue-2 virus. *Am J Trop Med Hyg* 1998; 58: 283-6.

Thet-Win. Detection of dengue virus by immunofluorescence after intracerebral inoculation of mosquitoes. *Lancet* 1982; 319: 53-4.

Vazeille-Falcoz M, Mousson L, Rodhain F, Chungue E, Faillox AB. Variation in oral susceptibility to dengue-2 virus of population of *Ae. aegypti* from the islands of Tahiti and Moorea, French Polynesia. *Am J Trop Med Hyg* 1999; 60: 292-9.

Wangroongsarb Y. Development and approaches for controlling dengue/dengue haemorrhagic fever in Thailand. Strategy for prevention and control of dengue/dengue haemorrhagic fever in South-East Asia Region. Presented at the Regional Consultative Meeting, 1995. New Delhi: WHO SEARO, 1995.