RESEARCH NOTE

NATURAL TRANSOVARIAL DENGUE VIRUS INFECTION RATE IN BOTH SEXES OF DARK AND PALE FORMS OF *AEDES AEGYPTI* FROM AN URBAN AREA OF BANGKOK, THAILAND

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Abstract. Transovarial dengue virus infection status of two forms of adult *Aedes aegypti* (dark or *Ae. aegypti* type form and pale or form *queenslandensis*), reared from field-collected larval and pupal stages, was determined by one-step RT-PCR and dengue viral serotype by nested-PCR. Natural transovarial transmission (TOT) of dengue virus was detected in the two *Ae. aegypti* forms, and in both adult males and females. Male *Ae. aegypti* had a higher rate of TOT dengue virus infection than female. The overall minimum infection rate among the male and female populations was 19.5 and 12.3 per 1,000 mosquitoes, respectively. All four dengue serotypes were detected in mosquito samples, with DEN-4 being the predominant serotype. Thus, both male and female *Ae. aegypti* have influences on the epidemiology of dengue virus transmission.

Keywords: *Ae. aegypti*, dark form, pale form, dengue virus, transovarial infection

INTRODUCTION

Dengue is the most prevalent mosquito-borne viral disease, and is transmitted by *Aedes* mosquitoes. Dengue infection is caused by any of the 4 distinct dengue virus serotypes (DEN1-DEN4) of the genus *Flavivirus*. Manifestations range from mild dengue fever (DF) to a more severe form, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), the latter due to a secondary infection with a different serotype of dengue virus. Dengue is becoming an increasingly important global public health problem due to its rapid geographic spread. In the last 50 years, incidence of dengue has increased 30-fold, with wide geographic distribution in over 100 endemic countries (WHO, 2009).

Aedes aegypti and *Ae. albopictus* are important vectors. However, *Ae. aegypti* is now considered the principal vector

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for the dengue viruses, and has been incriminated in major dengue outbreaks worldwide (Lambrechts *et al*, 2010). In endemic areas, dengue viruses are maintained by the human-*Aedes* mosquito-human cycle (Gubler and Trent, 1994). Laboratory studies have shown that infective female mosquitoes can transmit dengue virus transovarially (vertically) to their offsprings (Lee *et al*, 1997; Joshi and Sharma, 2001; Mourya *et al*, 2001, Joshi *et al*, 2002; Wasinpiyamongkol *et al*, 2003). In addition, several field studies have confirmed natural transovarial dengue virus transmission in infected *Ae. aegypti* larvae, and from adults reared from them or from wild-caught adult male mosquitoes (Khin and Than, 1983; Lee *et al*, 1997; Chung *et al*, 2001; Gunther *et al*, 2007; Angel and Joshi, 2008; Arunachalam *et al*, 2008). This phenomenon has long been suspected as being the inter-epidemic maintenance mechanism for dengue virus (Rodhain and Rosen,1997).

Thailand is highly endemic for dengue, and cases of dengue have been reported at all times of year. However, outbreaks tend to occur during the rainy season due to increased adult survival and longevity, which are related more to temperature and humidity than mosquito density (Thammapalo *et al*, 2005). Bangkok, the largest metropolitan area in the central region of Thailand, was found to be the country's endemic center (Halstead, 2008). In Thailand two distinct morphological forms of *Ae. aegypti* (dark, or *Ae. aegypti* type form; and pale, or form *queenslandensis*), which can be identified by variations in adult abdominal tergal white scale patterns, have been reported (Sheppard *et al*, 1969; Sucharit and Surathin, 1994). Both are domestic in nature (Sucharit and Surathin, 1994), and are susceptible to oral infection with dengue virus type 2 (DEN-2), but are capable of transovarial transmission (TOT) in laboratory experiments (Sucharit *et al*, 1997; Wasinpiyamongkol *et al*, 2003).

Vector status is a dynamic process in the epidemiology of vector-borne disease. Increased knowledge of disease vectors and their importance in dengue infection can enhance dengue surveillance and prevention. In this report, we determined the natural TOT dengue virus infection rates in the two forms of *Ae. aegypti*, reared from field-collected immature stages.

MATERIALS AND METHODS

Study area

The study area was carried out in Bang Khun Thian District, an urbanized residential area of Bangkok, Thailand that has had dengue outbreaks almost every year. Field study was conducted from October 2007 to September 2008. During the study period , the number of dengue cases in the study area had been reported every month ranging from 4 cases in March to 78 cases in September 2008.

Mosquito larval collection

Mosquito larva and pupa were collected monthly for one year from domestic water storage and artificial breeding containers, both in and around houses of reported cases and its surroundings. Mosquitoes were reared continuously at 28°C at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University in Bangkok, Thailand until adult emergence. Prior to adulthood, male and female pupae were separated according to their sizes; males being markedly smaller, were kept in small screen plastic containers and allowed to emerge in separate containers. Any containers with both sex appearances were

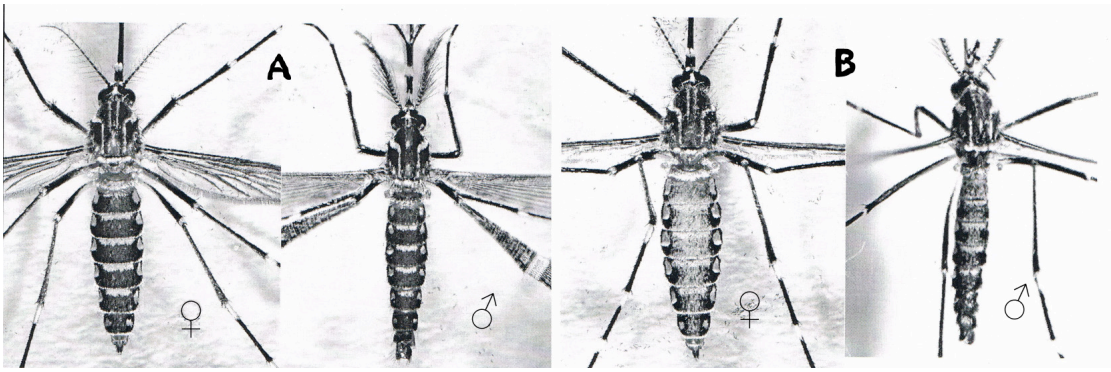

Fig 1–Male and female *Ae. aegypti* dark form (A) and pale form (B).

discarded. Newly emerged adults were anesthetized with cold condition, identified as to species, and the *Aedes aegypti* were classified morphologically into dark and pale forms based on the dorsal scaling on the abdominal tergite (Mattingly 1957, 1958). The dark type has only a white scale on the first abdominal tergite, while the *queenslandensis* form has extended white scaling on the abdominal tergites beyond the first tergite (Fig 1). After identification, mosquitoes were pooled, sorted by form and sex resulting in variable pool sizes (ranging from 8 to 40 mosquitoes/pool), and were stored at -80ºC until assayed for the presence of dengue virus.

Dengue virus detection

Mosquitoes used for dengue virus determination were processed as pooled samples and their dengue virus infection status was determined by one-step RT-PCR and dengue viral serotype by nested PCR, using the method of Lanciotti *et al* (1992), with some modifications. In brief, mosquito pools were ground in 200 µl of cold PBS (Gibco, Gaithersburg, MD) at pH 7.4 in a 1.5 ml microfuge using a sterilized micropestle, and then centrifuged at 18,928*g* at 4ºC for 30 minutes. Viral RNA was extracted from supernatants using a viral RNA mini kit (QIAmp, QIAGEN, Hilden, Germany) according to the manufacturer's protocol. One-step RT-PCR kit (QIAGEN, Hilden, Germany) was used to obtain cDNA as follows: 42ºC for 1 hour, 35 cycles 94ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 2 minutes. Using primer D1 (5´- TCAATATGCTGAAACGCGCGAGAAACCG-3´) and D2 (5´-TTGCACCAACAGTCAATGTCTTCAGGTTC-3´) in order to obtain 511 bp amplicon of C and PrM of DENV. Each dengue virus serotype was identified by nested PCR as follows: The one-step RT-PCR amplicons were diluted 1:50 with water and amplified using primers for DENV 1-4 [D1 and TS1 (5´-CGTCTCAGTGATCCGGGGG-3´), or TS2 (5´-CGCCACAAGGGCCATGAACAG-3´), or TS3 (5´-TAACATCATCATGAGACAGAGC-3´), or TS4 (5´-CTCTGTTGTCTTAAACAAGAGA-3´)]. Thermal cycling consisted of 25 cycles of 94ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 2 minutes, generating amplicons of 482, 119, 290, and 392 bp, respectively. A 5 µl aliquot of each reaction mixture was electrophoresed in 1.5% agarose gel containing ethidium bromide. Positive control, a combination of DEN1, DEN2, DEN3 and DEN4 amplicons and nega-

Table 1
Minimum infection rate of dengue virus in *Ae. aegypti* dark form and pale form.

Ae. aegypti	Female			Male		
	No. of mosquito pools	Total no. of mosquitoes	MIR/1,000	No. of mosquito pools	Total no. of mosquitoes	MIR/1,000
Dark form	160	4,013	12.7	108	3,412	19.1
Pale form	8	201	5.0	2	31	64.5
Total	168	4,214	12.3	110	3,443	19.5

Table 2
Dengue virus serotypes detected two forms of *Ae. aegypti.*

Dengue virus serotype	No. of pools			
	Ae. aegypti (dark form)		*Ae. aegypti* (pale form)	
	Male	Female	Male	Female
DEN 1	4	2	-	-
DEN 2	1	3	-	-
DEN 3	13	3	-	-
DEN 4	34	23	-	-
DEN 1, DEN 3	1	-	-	1
DEN 1, DEN 4	3	6	-	-
DEN 3, DEN 4	8	11	-	-
DEN 1, DEN 3, DEN 4	1	3	2	-
Total number	65	51	2	1

tive control (using H_2O) were included in every experiment. Bands were visualized under an UV transilluminator and recorded using SynGene gel documentation equipment.

Mosquito infection rate

Minimum infection rate (MIR) per 1,000 mosquitoes was calculated as ratio of the total number of positive pools to total number of mosquitoes tested, multiplied by 1,000 , in order to estimate TOT dengue virus infection rates (Chiang and Reeves, 1962).

Statistical analysis

Comparison of TOT dengue virus infection rates of *Ae. aegypti* was determined using chi-square test. Significant level of infection rate is $p \leq 0.05$, using Epi info program version 7.0.

RESULTS

The majority of adult *Aedes aegypti* resulting from field-collected larvae was *Ae. aegypti* type form. The female:male ratio was 1:1 and 8:1 for dark and pale form, respectively.

There were 268 pools containing 7,425 dark form *Ae. aegypti* (ranging from 8 to 40 mosquitoes per pool) and 10 pools containing 232 pale form *Ae. aegypti* (ranging from 11 to 31 mosquitoes per pool). From a total of 278 mosquito pools assayed, 119 pools (43%) consisting of 67 male and 52 female pools were positive for TOT dengue virus, respectively (Table 1). MIR of male and female population was 19.5 and 12.3 per 1,000 mosquitoes, respectively showing that male *Ae. aegypti* had a higher rate of TOT dengue virus infection rate than female ($p \leq 0.05$). However, TOT infection rates of the two *Ae. aegypti* forms could not be analysed statistically due to the small sample size of field-collected pale form.

The numbers of pools positive for the four dengue-virus serotypes present in both forms of *Ae. aegypti* from the study area during the 1-year study are shown in Table 2. All four dengue serotypes were detected in the mosquito samples, with DEN 4 being the predominant serotype.

DISCUSSION

This study showed a difference in population size of the two forms of *Ae. aegypti*, consistent with a previous report that demonstrating the majority of *Ae. aegypti* population in Thailand is *Ae. aegypti* type form (Mc Clelland, 1974; Mogi *et al*, 1989; Sucharit and Surathin, 1994; Wasinpiyamongkol *et al*, 2003). Transovarial and venereal transmission are considered the maintenance modes for vector-borne viruses in nature during unfavorable conditions, and have been studied in several vector species, including *Aedes* vectors of dengue (Rodhain and Rosen, 1997; Mavale *et al*, 2006). In addition, experimental studies showed that TOT-infected male *Aedes* can transfer dengue virus venereally to virgin females during copulation allowing dengue virus to be transmitted vertically to the progeny (Rosen, 1987; Tu *et al*, 1998). However, the actual impact of these two phenomena on the dynamics of dengue has not been elucidated.

Natural TOT dengue viruses were monitored in adult *Ae. aegypti* mosquitoes reared from immature stages. Dengue virus serotype was determined using one step RT-PCR and nested-PCR methods. Furthermore, the RT-PCR amplicons were subsequently sequenced and compared with the DENV2 infected patients (unpublished). There results confirmed the validation of RT-PCR assay in field collected *Ae. aegypti*.

TOT dengue virus infections occurred in both sexes of dark and pale forms of *Ae. aegypti*. The present study revealed that TOT dengue virus infection occurred at a relatively higher rate among the male *Ae. aegypti* population compared with female. Although the dark form population was more prevalent than the pale form, the latter is still important as it can sustain the virus in nature, due to the high rate of TOT dengue virus infection especially in the males. It is possible that transovarially infected male mosquitoes have a significant impact on the natural maintenance of dengue virus by transferring venereally dengue virus to females. In addition, experimental laboratory studies found that pale form *Ae. aegypti* are significantly more susceptible to dengue virus infection (Wasinpiyamongkol *et al*, unpublished data). Thus, the male pale form may play an important role in the epidemiology of dengue virus in places where this pale form predominates. However, morphological variations among *Ae. aegypti*, and their biological significance, remain unclear. Further re-

search into vector status, bionomics, and vector-parasite relationships in relation to temperature and humidity are needed for a better understanding of their roles in the dynamics of transmission and dengue epidemiology.

TOT dengue virus in mosquito larval surveys may provide an alternative way for identifying the history of circulating dengue serotypes and their prevalence in dengue outbreak area. This will provide particularly important information for dengue prevention and control.

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