DOUBLE INFECTION OF HETEROSEROTYPES OF DENGUE VIRUSES IN FIELD POPULATIONS OF *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE) AND SEROLOGICAL FEATURES OF DENGUE VIRUSES FOUND IN PATIENTS IN SOUTHERN THAILAND

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Abstract. In order to understand more about the epidemiology of DHF, a study of the type of dengue viruses and vectors under natural conditions was carried out. Mosquito vectors in the field and the serum of DHF patients in southern Thailand were examined. The two mosquito species are abundant and DHF incidence remains high in this region. Dengue viruses were examined in field-caught mosquitoes by RT-PCR technique. The mosquitoes were caught in 4 provinces: Krabi, Phuket, Phang-Nga and Surat Thani during the late dry season until the early rainy season in 2005. Three dengue serotypes (DEN-2, DEN-3, DEN-4) were detected in *Ae. aegypti* males and females, and 2 (DEN-2, DEN-3) were detected in *Ae. albopictus* females. Double infection with 2 serotypes of dengue viruses (DEN-2 and DEN-3) were detected in *Ae. aegypti* males and females and *Ae. albopictus* females. DEN-2 and DEN-1 were the most prevalent serotypes found in the serum of the patients in this area, followed by DEN-4 and DEN-3. The prevalence of the predominant dengue serotype varied from province to province. Detection of viruses in adult male mosquitoes reveals the role of transovarial transmission of dengue viruses in field populations of DHF vectors and elucidates circulation of dengue viruses in vectors in the natural environment of endemic areas. The incidence of multiple serotypes of dengue virus in *Ae. aegypti* and *Ae. albopictus* in the same area points toward a high risk for an epidemic of DHF. These findings provide greater understanding of the relationship among mosquito vectors, virus transmission and DHF epidemiology in endemic areas.

INTRODUCTION

Dengue fever (DF) and its more severe form, dengue hemorrhagic fever (DHF), are important mosquito-borne diseases, caused by four serotypes of dengue virus and are transmitted by *Aedes* mosquitoes: *Aedes aegypti* (L.) and *Ae. albopictus* Skuse (Service, 1993). The disease has worldwide distribution in some 100 countries, but is more prevalent in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific (WHO, 2002). It is estimated that 2,500 million people are at risk for DF/DHF, and about 50 million cases of DF/DHF infection are reported annually (WHO, 2002). In Thailand, five decades after the first report of the disease in the late 1950s, DHF has spread across the country and has become a major vector-borne disease, with increasing incidence, especially since the late 1980s. Major efforts using a variety of approaches have been directed toward prevention and control of DF/DHF. *Ae. aegypti* is the major vector of DF/DHF in almost all countries, including Thailand, whereas *Ae. albopictus*, *Ae. scutellaris* Colless and *Ae. polynesiensis* Marks are vectors in some areas

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(Japan, islands of the Pacific) (Kettle, 1995). There is currently no effective vaccine available to prevent this disease (Service, 1993). A tetravalent vaccine for DHF is being investigated but it may take years before an effective vaccine can be widely used in the main disease areas (Gratz and Knudsen, 1997). Therefore, prevention and control of the disease should be conducted through understanding of DHF epidemiology and vector control strategies. To understand more about the epidemiology of the disease, a study of the association between viruses and vectors under natural conditions was carried out evaluating viruses in field populations of mosquito vectors and in the serum of DHF patients in southern Thailand, where the two mosquito species are abundant and DHF incidence remains high.

MATERIALS AND METHODS

Study areas

Four southern provinces of Thailand (Krabi, Phuket, Phang-Nga and Surat Thani) were selected for the study sites (Fig 1). These provinces have been recognized as areas with a high incidence of DHF and the places having two species of DHF vectors: *Ae. aegypti* and *Ae. albopictus*.

Mosquito collection

Mosquito collection using human bait (WHO, 1997) was carried out at the study sites from the late dry season (March) to the early rainy season (May) 2005. The chosen dwellings for mosquito collection were those villages which had experienced recent DHF cases. Five volunteers captured mosquitoes indoors for 20 minutes in each dwelling. The collectors usually situated themselves in dark areas of the room where most biting activity occurs. The collectors bared their legs between knee and ankle and collected all landing and biting mosquitoes individually in vials, which were capped. Using a similar procedure, the collecting was also conducted outdoors (approximately 10 m away from dwellings) to catch *Ae. albopictus* in the same environment. The collecting was carried out from 09 00 to 17 00 hours. The collected mosquitoes were visually identified, as there were only two species (*Ae. aegypti* and *Ae. albopictus*) present. These live mosquitoes were inactivated by putting them in a refrigerator, and separated by species, sexes and localities and then kept individually in cryogenic vials to store in liquid nitrogen for subsequent dengue viral detection.

Viral detection in mosquitoes

The procedure for dengue virus detection in mosquitoes followed the methods described by Tuksinvaracharn *et al* (2004). Viral RNA was extracted from individual mosquitoes. The wings and legs were removed then the mosquitoes were ground in a lysis solution (provided with the kit), centrifuged, then the supernatant was processed for RNA extraction using RNeasy mini kit (QIAGEN, Germany).

The six oligonucleotide primers within the core and pre-membrane protein gene (C-prM) of dengue viruses used in this study were designed by Lanciotti *et al* (1992). Two consensus primers (D1 and D2) were designed to be homologous to the genomic RNA for all four dengue serotypes, whereas the type-specific nucleotide primers (TS1, TS2, TS3 and TS4) were designed to anneal specifically to each of their respective genomes. These primers were positioned such that a differently sized product was generated from each type.

The procedure of semi-nested RT-PCR performed in this study was modified from that of Lanciotti *et al* (1992). The first step was performed using Superscript III one-step RT-PCR with Platinum III® *Tag* (Invitrogen, USA) which followed the manufacturer's protocol. The second step was to identify type-specific DNA products. The RT-PCR products from the second step were examined by agarose gel electrophoresis and visualized by ethidium bromide staining.

Mosquitoes inoculated with dengue viruses were used as positive controls. These viruses were DEN-1 Hawaii, DEN-2 strain TR 1751, DEN-3 strain H87, and DEN-4 strain H241. The method for mosquito inoculation was modified from Pervin *et al* (2003). Briefly, 3-5 day old female *Ae. aegypti* reared in the laboratory were collected and inactivated on ice for 10 minutes. Each mosquito was then inoculated intra-tho-

racically with 0.3 µl of $2.5x10^3$ pfu/ml of dengue virus antigen diluted with PBS diluents (PBS pH 7.4, 0.5% gelatin and 5% fetal calf serum). After inoculation, the mosquitoes were kept in a double door insectary at 28°C with 80% humidity and supplied with 10% sucrose solution for 7-10 days. Uninfected laboratory-reared *Ae. aegypti* mosquitoes were used as negative controls. The sensitivity of the RT-PCR technique used in the present study was approximately 25 viral particles/µl.

Collection of blood specimens

Blood specimens were taken from DHF patients admitted to hospitals in the study areas. The blood samples were drawn into tubes with EDTA anticoagulant, and centrifuged to obtain plasma. The plasma specimens were kept in liquid nitrogen tanks and then transported to the Arbovirus Laboratory, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand for determination of serotypes against dengue viruses.

Viral determination in blood samples

Reverse transcriptase - polymerase chain reaction (RT-PCR) was carried out to identify dengue virus serotypes as described in previous reports (Yenchitsomanus *et al*, 1996; Chanama *et al*, 2004). Viral RNA was extracted from 100 µl of patient plasma with a QIAamp viral RNA mini kit (Qiagen Gmbh, Hilden, Germany). Then, RT-PCR was processed using the one-step RT-PCR kit (Qiagen) and dengue-specific oligonucleotide primers. Positive and negative controls were always included in each batch of tests. Finally, the second PCR products were electrophoresed through agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

Incidence of DHF in Thailand

The data of DHF incidence in Thailand and those of provinces in the study areas between 2000 and 2005 was obtained from the Department of Disease Control, Ministry of Public Health, Thailand. The annual incidence of dengue per 100,000 populations was ranked as percentiles: 71 was 50th, 134 was 75th, and 175 was 90th (Office of the Permanent Secretary for Public Health, 1999). The epidemics with annual dengue incidence ranging between the 75th and 90th percentile were classified as moderately severe epidemics, whereas those greater than the 90th percentile as severe epidemics.

RESULTS

A total of 469 *Ae. aegypti* mosquitoes (145 males and 324 females) were collected from the study sites and subsequently identified individually for dengue virus by RT-PCR assays. The distinct patterns of some products from the RT-PCR assays for dengue viruses are illustrated in Fig 2. Out of those, 75 *Ae. aegypti* (22 males and 53 females) were positive for dengue viruses (Table 1). The relative infection rate varied from place to place, ranging from 5.3% to 25.9%. The three serotypes of dengue viruses: DEN-2, DEN-3 and DEN-4 were found in *Ae. aegypti* individual males and females, whereas no DEN-1 was detected in any mosquito (Table 1). It is interesting to note that the three serotypes (DEN-2, DEN-3 and DEN-4) were detected in both male and female *Ae. aegypti* mosquitoes collected from Krabi but only DEN-4 virus was found in one male. *Ae. aegypti* mosquitoes caught from Phuket and Phang-Nga were positive for two serotypes: DEN-2 and DEN-3, whereas of those collected from Surat Thani, only DEN-3 was found in two *Ae. aegypti* females. Double infections with two serotypes of dengue viruses (DEN-2 and DEN-3) were also detected in *Ae. aegypti* males and females captured from Krabi and Phuket, while this double infection was found in only one *Ae. aegypti* male collected from Phang-Nga.

Since outdoor mosquito collection for *Ae. albopictus* was carried out in the dry period when natural developmental sites were scarce, the number of mosquitoes collected was extremely low. As shown in Table 2, only 58 *Ae. albopictus* females were caught: Krabi (28), Phuket (5) and Phang-Nga (25), whereas no *Ae. albopictus* mosquitoes were collected from Surat Thani in the present study. The relative infection rates were 21.4% in Krabi, 60% in Phuket and 48% in Phang-Nga. Of the field-caught mosquitoes, a total of 21 were positive for DEN-2 (10), DEN-3 (7) and double serotypes: DEN-2 and DEN-3 (4),

Fig 1–Map of Thailand showing the location of the four provinces in this study.

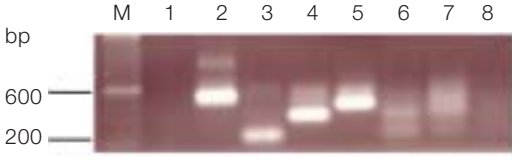

Fig 2–The distinct patterns of products from RT-PCR assays for dengue viruses. Lanes: M = molecular weight marker; 1 = negative control; 2 = positive control (DEN-1); 3 = positive control (DEN-2) ; 4 = positive control (DEN-3); 5 = positive control (DEN-4); 6 = sample 1 positive for DEN-2 and DEN-3; 7 = sample 2 positive for dengue 3; 8 = sample 3 negative.

as shown in Table 2. The mosquitoes collected from Krabi were infected with only DEN-2 serotype, whereas double infections with two serotypes (DEN-2 and DEN-3) were detected in individual mosquitoes collected in Phuket (1) and Phang-Nga (3).

A total of 124 blood specimens were collected from some DHF patients admitted to hospitals in the study areas (Table 3). These included 77 samples from Krabi, 31 from Surat Thani, 10 from Phuket and 6 from Phang-Nga. Overall, DEN-2 (36.3%) and DEN-1 (34.7%) were the most prevalent serotypes found in this study, followed by DEN-4 (20.1%) and DEN-3 (8.9%). Only a single serotype of dengue virus was detected in each patient. As shown in Table 3, all four serotypes of dengue viruses were detected in blood samples of DHF patients from Krabi, but only DEN-2 serotype (100%) was found in those obtained in Phuket. In Krabi, the prevalence of DEN-1 (32.5%) was equal to DEN-4 (32.5%) whereas DEN-2 (26%) and DEN-3 (9%) were less frequent. Three dengue serotypes DEN-1 (54.8%), DEN-2 (32.3%) and DEN-3 (12.9) were found in samples collected from Surat Thani, and only DEN-2 (83.3%) and DEN-1 (16.7%) were detected in samples from Phang-Nga.

The annual incidence and deaths due to DHF in Thailand (whole country), Krabi, Phuket, Phang-Nga and Surat Thani from 2000 to 2005 are presented in Table 4. During the year 2000, the incidence of DHF in Thailand, including all the four provinces was relatively low (58/100,000). Two severe epidemics of DHF occurred in Thailand, with 139,355 reported cases and 245 deaths in 2001, and 114,800 reported cases and 176 deaths in 2002. As a result, the DHF incidence rates were 224/100,000 in 2001, and 185/100,000 in 2002, respectively. Thereafter, the incidences and deaths due to DHF in Thailand and those four provinces dramatically declined in 2003 and 2004, but increased again in 2005.

DISCUSSION

A number of studies have been reported the isolation and detection of dengue virus in field populations of *Ae. aegypti* in Thailand (Watts *et al*, 1985; Rojanasuphot *et al*, 1988; Thavara *et al*, 1996; Tuksinvaracharn *et al*, 2004). In 1978 and 1979, Watts *et al* (1985) failed to isolate any

Table 1
Prevalence of relative infection of dengue virus in *Ae. aegypti* mosquitoes collected from four southern provinces in Thailand.

Province	Total tested mosquitoes[a]	No. of mosquitoes positive for virus	Relative infection rate (%)	Frequency of dengue serotypes found				
				DEN-1	DEN-2	DEN-3	DEN-4	DEN-2&3
Krabi	82 (M)	9	11	0	4	2	1	2
	149 (F)	18	12.1	0	6	9	0	3
	231 (M+F)	27	11.7	0	10	11	1	5
Phuket	61 (M)	10	16.4	0	2	6	0	2
	114 (F)	29	25.4	0	3	17	0	9
	175 (M+F)	39	22.3	0	5	23	0	11
Phang-Nga	17 (M)	3	17.7	0	2	0	0	1
	10 (F)	4	40	0	0	4	0	0
	27 (M+F)	7	25.9	0	2	4	0	1
Surat Thani	2 (M)	0	0	0	0	0	0	0
	36 (F)	2	5.6	0	0	2	0	0
	38 (M+F)	2	5.3	0	0	2	0	0
Total	145 (M)	22	15.2	0	8	8	1	5
	324 (F)	53	16.4	0	9	32	0	12
	469 (M+F)	75	16	0	17	40	1	17

[a]M = Male mosquitoes, F = Female mosquitoes

Table 2
Prevalence of relative infection of dengue virus in *Ae. albopictus* mosquitoes collected from three southern provinces in Thailand.

Province	Total tested mosquitoes[a]	No. of mosquitoes positive for virus	Relative infection rate (%)	Frequency of dengue serotypes found				
				DEN-1	DEN-2	DEN-3	DEN-4	DEN-2&3
Krabi	28 (F)	6	21.4	0	6	0	0	0
Phuket	5 (F)	3	60	0	2	0	0	1
Phang-Nga	25 (F)	12	48	0	2	7	0	3
Total	58 (F)	21	36.2	0	10	7	0	4

[a]F = Female mosquitoes

Table 3
Prevalence of dengue infection detected in blood specimens collected from some DHF patients admitted to hospitals in the study provinces in 2005.

Provinces	Dengue serotypes, no. and (%) positive				Total
	DEN-1	DEN-2	DEN-3	DEN-4	
Krabi	25 (32.5)	20 (26)	7 (9)	25 (32.5)	77
Phuket	0	10 (100)	0	0	10
Phang-Nga	1 (16.7)	5 (83.3)	0	0	6
Surat Thani	17 (54.8)	10 (32.3)	4 (12.9)	0	31
Total	43 (34.7)	45 (36.3)	11 (8.9)	25 (20.1)	124

Percentage in each parenthesis is derived from comparison with the total number of each line in the last column.

Table 4
Annual incidence of DHF in Thailand (whole country) and four provinces in the study areas from 2000 to 2005.

Place	Population (Year 2004)	Total DHF reported cases/deaths and incidence (per 100,000)					
		2000	2001	2002	2003	2004	2005
Thailand	63,079,765	18,617/32 (30)	139,355/245 (224)	114,800/176 (185)	63,657/75 (101)	39,135/48 (62)	44,765/82 (72)
Krabi	377,954	152/0 (40)	1,209/7 (320)	1,853/10 (490)	378/0 (100)	260/0 (69)	714/0 (184)
Phuket	270,438	39/0 (14)	170/0 (63)	503/1 (186)	205/0 (76)	115/0 (43)	163/2 (57)
Phang-Nga	239,401	99/1 (41)	366/0 (153)	984/0 (411)	236/0 (99)	108/0 (45)	234/0 (98)
Surat Thani	920,283	530/2 (58)	2,351/0 (256)	4,963/15 (539)	843/1 (92)	725/1 (79)	1,802/4 (192)

Source: Department of Disease Control, Ministry of Public Health, Thailand.

dengue virus from several thousand specimens of *Ae. aegypti* larvae, pupae and adult males collected from houses in Bangkok in which one or more persons had recent dengue virus infection. However, DEN-2 virus was eventually isolated from dengue patients and individual *Ae. aegypti* females collected in dwellings of DHF patients (Watts *et al*, 1985). Following that, Rojanasuphot *et al* (1988) isolated DEN-1 virus from 5 pools out of 365 pools (1.4%) of *Ae. aegypti* mosquitoes collected from study sites in Rayong between 1983 and 1984. On Ko Samui (an island in the gulf of Thailand in the province of Surat Thani) during a high epidemic of DHF (497/100,000), 5 out of 6 pools (83.3%) of *Ae. aegypti* females were positive for dengue viruses, however serotypes of the viruses were not identified (Thavara *et al*, 1996). Recently, Tuksinvaracharn *et al* (2004) found an infection of DEN-3 in pooled *Ae. aegypti* mosquitoes collected from communities in Bangkok during the dry season.

Our study constitutes the first report of double infection with two different serotypes of dengue viruses found in field-caught individual *Ae. aegypti* males and females and *Ae. albopictus* females. This phenomenon could have occurred due to multiple feedings of mosquitoes on two different dengue-infected persons or a single blood meal taken from a person with a double infection with two different serotypes of dengue viruses. This event is possible in highly endemic areas where two or more serotypes of dengue viruses are circulating simultaneously. It is thus possible for a human to get a double infection with two different serotypes of dengue viruses from a single bite of the *Ae. aegypti* female infected with two serotypes of dengue viruses. Gubler *et al* (1985) reported a case of natural concurrent primary infection with two serotypes of dengue viruses, DEN-1 and DEN-4, during a 1982 outbreak in Puerto Rico. This patient presented with only mild symptoms of dengue infection without hemorrhagic manifestations, however, this illness was uncommon and not similar to many other single-serotype dengue infections found in the same area at that time. It is generally thought that concurrent infection in a person with two different serotypes of dengue viruses may cause severe disease. This hypothesis may be true for patients who get secondary dengue virus infection, as found in earlier studies (Halstead *et al*, 1970; Vaughn *et al*, 2000; Nisalak *et al*, 2003). Disease severity in DHF correlates with secondary dengue virus infection, high viremic titers and is associated with rapid virus clearance (Vaughn *et al*, 2000).

The presence of dengue single or double infection in field-caught individual *Ae. aegypti*

males in the present study provides clear evidence for transovarial transmission occurring in the natural environment in the study areas. Based on experimental results obtained from laboratory infections, Rosen (1987) suggested that *Ae. albopictus* male mosquitoes naturally infected with dengue virus may acquire infection vertically with no sexual transmission of dengue virus from female to male mosquitoes. This phenomenon was found with *Ae. aegypti* in our findings. Transovarial transmission of dengue viruses in mosquito vectors takes place naturally and may be able to maintain the viruses in the environment during dry periods when the population of mosquito vectors is scarce (Rosen *et al*, 1983).

Detection of dengue virus in *Ae. albopictus* mosquitoes collected from Ko Samui, Surat Thani has been reported previously (Thavara *et al*, 1996). In contrast to previous studies, Watts *et al* (1985) failed to isolate dengue virus from *Ae. albopictus* mosquitoes collected in the field in Saraburi between 1978 and 1979. Our present study, thus, adds to previous reports of dengue virus infection found in field-caught male *Ae. albopictus* mosquitoes in southern Thailand. The success of dengue virus detection in field-caught male *Ae. albopictus* mosquitoes in the present study and that of Thavara *et al* (1996) may be due to the sensitivity of the detection method used, as well as other relevant factors, such as the mosquito collection procedure, specimen preparation, period of investigation and study sites. It is interesting to note that a relatively high infection rate of dengue viruses (21-60 %) was found in *Ae. albopictus* mosquitoes even though its populations were extremely low. We suspect that the incidence of infection of dengue viruses in *Ae. albopictus* mosquitoes in the rainy season is high. *Ae. albopictus* mosquitoes are usually abundant in the rainy season (Thavara *et al*, 2001a) and widespread in many provinces in southern Thailand, where the natural habitats of the mosquitoes, such as fruit orchards and rubber and palm plantations are prominent (Thavara *et al*, 2001b). We suggest expanding control methods for *Ae. albopictus* mosquitoes in southern Thailand.

Until recently, only one serotype of dengue virus has been detected or recovered from a single patient (Rojanasuphot *et al*, 1988; Nisalak *et al*, 2003; Anantapreecha *et al*, 2005). However, a single case of natural infection with two serotypes of dengue virus, DEN-1 and DEN-4, was reported by Gubler *et al* (1985). As to the occurrence of the serotypes of dengue virus found in this study, the findings are similar to a previous study (Rojanasuphot *et al*, 1988) which found that DEN-2 (45%) was the predominant serotype among 51 subjects isolated for dengue viruses in DHF patients admitted to the Rayong Provincial Hospital between 1980 and 1984, followed by DEN-1 (31.4%), DEN-3 (11.8%) and DEN-4 (11.8%). Anantapreecha *et al* (2005), in 2,715 confirmed specimens of dengue patients collected from six hospitals scattered throughout Thailand from 1999 to 2002, found that 45% were infected with DEN-1, followed by DEN-2 (32%), DEN-3 (19%) and DEN-4 (5%). The prevalences of the dengue serotypes obtained from these studies are different due to various factors, such as the sensitivity of the technique used for viral detection, sample collection, period of investigation and study site. It is obvious that all four dengue serotypes circulate continuously in Thailand with fluctuations in the dominant serotype from place to place and year to year. Nisalak *et al* (2003) pointed out that each serotype of dengue virus constitutes a distinct influence on disease severity and nature of the dengue epidemic. They found that DEN-1, DEN-2 and DEN-3 were associated with moderately severe dengue epidemic years (annual incidence rate 134 - 175 per 100,000), but DEN-3 was associated with severe dengue years (annual incidence rate >175 per 100,000). DEN-4 may need pre-existing heterotypic dengue antibodies for replication or to create clinical manifestations (Nisalak *et al*, 2003). In Thailand, dengue disease incidence has fluctuated over time, increasing from 9/100,000 in 1958 to 72/100,000 in 2005, with the highest incidence of 325/100,000 in 1987. Moderately severe epidemics occurred in 1984, 1985, 1989, 1990 and 1997, whereas severe epidemics took place in 1987 and 1998 (Nisalak *et al*, 2003). The years 2001 and 2002 also had severe epidemics. The Ministry of Public Health, Thailand, has set a DHF threshold for each province of 50/100,000 or lower. This goal has been difficult to achieve in

many provinces of Thailand. Since an effective dengue vaccine is not yet available. Control strategies have included integrated vector surveillance and control for *Ae. aegypti* and *Ae. albopictus*, monitoring of dengue virus in mosquito vectors and in dengue patients, and the use of geographic information systems (GIS) and remote sensing (RS) to analyze high risk areas and predict dengue epidemics.

Our findings, to our knowledge, are the first report of double infection with two different serotypes of dengue viruses, DEN-2 and DEN-3, in field-caught *Ae. aegypti* females and males, *Ae. albopictus* females and dengue virus infection found in field-caught *Ae. aegypti* males in Thailand. This study reveals the role of transovarial transmission of dengue viruses in field populations of DHF vectors and elucidates circulation of dengue viruses in vectors in the natural environment in endemic areas. The finding of the ability of DHF vectors to transmit dengue virus transovarially is valuable to the health officers and the public in the development of more effective strategies to control DHF and vectors. The incidence of multiple serotypes of dengue viruses found in field populations of *Ae. aegypti* and *Ae. albopictus* in the same area raises the high possibility of a DHF epidemic. These findings provide greater understanding into the relationship of mosquito vectors, virus transmission and DHF epidemiology in endemic areas.

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