DETECTION OF DENGUE VIRUS FROM FIELD *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* ADULTS AND LARVAE

Rohani Ahmad[1], Asmaliza Ismail[1], Zainah Saat[2] and Lee Han Lim[1]

[1]Division of Medical Entomology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur;
[2]Division of Virology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

Abstract. Mosquito adults and larvae were collected from dengue high risk areas and transported to the laboratory for identification. Identified mosquitos were pooled according to the species, date and locality and stored at -70°C. A total of 1,385 pools of *Aedes albopictus* and 267 pools of *Ae. aegypti* were collected from major towns in 12 states in Peninsular Malaysia. Virus isolation was carried out using cell culture (C6/36 clone) of *Ae. albopictus* and detection of dengue virus by the peroxidase anti-peroxidase staining. All positive isolations were further re-confirmed by the reverse transcriptase-polymerase chain reaction (RT-PCR). Most of the pools were negative with PAP staining and RT-PCR. However, 11 mosquito pools were positive with PAP staining. On the other hand, samples from Terengganu, Pulau Pinang and Johor were positive using both methods.

INTRODUCTION

Dengue virus infection continues to present a serious health problem in many tropical areas of the world including Malaysia. The disease is hyper-endemic in Southeast Asia, where a more severe form, dengue hemorrhagic fever and shock syndrome, is a major public health concern because of the severe and often fatal disease it causes in children (Halsted, 1980). In 1995, 6,520 dengue cases were reported to the Ministry of Health, Malaysia. Of these, 6,150 cases were dengue fever and 370 cases were dengue hemorrhagic fever with 28 deaths (Ministry of Health, Malaysia; unpublished report). In spite of the control and preventive measures, the number of cases reported has been increasing for the last few years.

For many years, members of the subgenus *Stegomyia*, especially *Aedes aegypti (Linn)* and *Ae. albopictus (Skuse)*, have been recognized as the primary vectors of dengue virus (Gubler *et al*, 1979; Boromisa *et al*, 1987; Gubler, 1987). However, since the susceptibility varies among mosquito strains and species (Rosen *et al*, 1985), it is important to evaluate the competence of vectors in each epidemic or endemic area. Methods for identifying areas with dengue activity usually include monitoring of infections in humans and rarely, monitoring of infection rates within mosquito vector populations. Vector surveillance provides estimates of population densities and viral infection rates which are necessary to predict epidemics of dengue and implementing remedial measures. However, the present methods of detecting dengue virus in the vector namely; culture of virus in sucking mice and the use of microinoculation of *Toxorhynchites* adults or larvae and subsequent detection with direct fluorescent antibody staining are laborious and time-consuming. Hence they are not suitable for screening large number of vector mosquitos. Recently, the dengue virion (possessing a single stranded RNA genome of approximately 11 kilobases in length) was sequenced (Rice *et al*, 1985). Based on this information, primers were developed which were specific for detecting RNA of the 4 dengue prototype viruses. These primers are currently being evaluated in detecting dengue viruses in RNA extracted from blood of infected patients. Hence, the potential of this technique for detecting the virus in the vector mosquito should be investigated.

The objective of this study was to isolate dengue virus from field mosquitos using cell culture and subsequent detection using the peroxidase anti-peroxidase staining and reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Field collection of mosquitos

Larval collections: Third and fourth instar of *Ae. albopictus* and *Ae. aegypti* larvae were collected from the field from dengue high risk areas in major

towns in 12 states of Peninsular Malaysia. In the laboratory, larvae were fed on partially cooked cow liver and newly emerged adult mosquitos were maintained on 10% sucrose solution. After emergence, the adults were collected, identified to species and separated by sex. Male and female pools, each containing about 50 mosquitos, were stored separately in cryogenic vials in -70°C freezer for future virus isolation studies.

Adult collection: Male and female mosquitos were captured with 2 × 8 cm vials while landing on human baits or resting inside houses. At times, adults were also collected using a sweep net. All males and females were stored in pools of 20-50 mosquitos at -70°C.

Dengue virus isolation using *Ae. albopictus* clone C6/36 cells (Igarashi, 1978)

The method employed was modified from Maneekarn *et al* (1993). The C6/36 cells were grown in growth media for 2 days until cell monolayers were formed in culture tubes. Pooled mosquitos were homogenized on ice in 1.5 ml MEM medium with 5% fetal calf serum (FCS). A hundred µl each of the homogenate were passed through 0.2 µm filters and inoculated into respective culture tubes. The culture tubes were then vortexed and left to incubate for 2 hours at ambient temperature for adsorption. Maintenance medium with 2% FCS was added and incubated at 28°C for 7 days.

Smear preparation

The culture tubes were vortexed and centrifuged after 7 days incubation period. Cells from the sediments were transfered onto the teflon-coated, 12 well slides where each slide would have a maximum of 7 test smears, 4 positive control and a negetive control. The smear were left to dry at 28°C for 3-4 hours in a laminar flow with the air blower on. The smears were then fixed with cold acetone for 20 minutes. The cultures were stored at -20°C for confirmation by a second or third passage in cell culture if the initial results were to be positive.

Peroxidase-antiperoxidase (PAP) staining

PAP staining procedures were performed as previously described by Igarashi *et al* (1982). The cells that were fixed with cold acetone were reacted with monoclonal antibody against dengue viruses at 1 : 1,000 at room temperature for 40 minutes. The cells were rinsed in PBS and reacted with rabbit anti-mouse IgG at 1 : 1,000 for 40 minutes. The cell were rinsed in PBS again and then reacted with goat-anti rabbit IgG at 1 : 1,000 for 40 minutes. The cells were then rinsed in PBS and reacted with peroxidase-rabbit anti-peroxidase complex at 1 : 1,000 for 40 minutes followed by washing and peroxidase reaction, using 0.2 mg/ml of 3.3 diaminobenzidine (DAB) and 0.2% of H_2O_2 as the substrate. The cells were observed under a normal light compound microscope. The positive samples were reconfirm by a second passage in cell culture.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed as described by Maneekarn *et al* (1993). Briefly, dengue virus-infected culture fluid (10 µl) was treated with an equal volume of a mixture containing 1% Nonidet P-40 and 20 units of RNase inhibitor in phosphate buffer saline (PBS) in a 0.5 ml conical microcentrifuge tube for 5 minutes at room temperature. This was followed by the addition of 90 µl of RT-PCR mixture containing 100pmols of each universal primer (TCAATATGCTGAAACGCGAGAAACCG and TTGCACCAACAGTCAATGTCTTCAGCTTC), 0.2 mM each of deoxynucleoside triphosphate (dATP, dCTP, dGPT, dTTP), 10 mM Tris (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml of bovine serum albumin. 0.1% sodium cholate, 0.1% Triton X-100, 10 units of reverse transcriptase and 2 units of Tth DNA polymerase. The reaction mixture was over layered with 2 drops mineral oil and each tube was placed in a Perkin-Elmer-Cetus thermal cycler which was programmed to incubate at 53°C for 10 minutes (for reverse transcription) followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 1.5 minutes and extension at 72°C for 2 minutes. After amplification, 15 µl of PCR product was electrophoresed on 3% agarose, stained with ethidium bromide, visualized under ultravoilet light and photographed.

RESULTS AND DISCUSSION

A total of 47,804 *Ae. albopictus* and 14, 605 *Ae.*

aegypti larvae were collected from 12 states in Peninsular Malaysia during the study period. The larvae were reared in the laboratory to adults (temperature 28°C, relative humidity 80%) and then were tested for dengue virus. A total of 649 female pools and 351 male pools of *Ae. albopictus* were obtained. On the other hand, a total of 160 female pools and 84 male pools of *Ae. aegypti* were obtained for testing (Table 1).

The total number of adult mosquitos collected were 5,508 and 354 for *Ae. albopictus* and *Ae. aegypti* respectively. The number of mosquito pools at each state and the year of collection are indicated in Table 2. Sixty-five male pools and 330 female pools of *Ae. albopictus* were obtained, but only 1 male pool and 22 female pools of *Ae. aegypti* were obtained.

All the mosquito pools were tested for dengue virus. Positive isolations were further confirmed by the reverse transcriptase polymerase chain reaction (RT-PCR). Most of the pools were negative with PAP and RT-PCR. Table 2 summarizes the results of the virus isolation. Only 11 pools were tested positive with PAP. The positive pools were from 2 larval pools from Kuala Lumpur (1993 sampling), 3 larval pools from Johor (1994 and 1995 sampling), 1 larval pool from Pulau Pinang (1995 sampling) and 2 pools from Perak (1995 sampling). On the other hand, samples from Terengganu, Pulau Pinang and Johor were positive using both methods. All the positive samples came from *Ae. albopictus* except one pool each from Kuala Lumpur and Perak and the virus was isolated from *Ae. aegypti*. However, from our study positive tissue-culture results did not parallel the RT-PCR results. One of the reason could be due to the optimization of PCR condition. Futher work needs to be conducted in this respect. Dengue virus was detected from 9 pools of 1,161 female pools and two pools of 501 male pools.

Our findings thus suggests the possibility of the occurrence of transovarial transmission of dengue virus by *Ae. aegypti* and *Ae. albopictus*. This indicates that the phenomenon is widely distributed in Peninsular Malaysia. It is not known why dengue virus was not detected in other states in Peninsular Malaysia even though dengue cases were reported. Very few pools of *Ae. albopictus* and *Ae. aegypti* were available for testing in some states. The scarcity of this species during the study period was apparently related to the unusually low rainfall in the study area. Thus, the acute shortage of water in nature breeding sites restricted larval collections to artificial containers that were found in the study areas.

Table 1

Different origin of field collected *Ae. albopictus* and *Ae. aegypti* in Peninsular Malaysia.

Origin	Larvae (no. of pools)				Adult (no. of pools)			
	Ae. albopictus		*Ae. aegypti*		*Ae. albopictus*		*Ae. aegypti*	
	female	male	female	male	female	male	female	male
State								
Kuala Lumpur	47	28	20	8	207	19	19	1
Johor	132	108	36	13	36	16	0	2
Kedah	45	14	2	5	9	2	0	0
Kelantan	13	10	18	4	2	0	0	0
Melaka	11	6	9	2	3	2	0	0
N Sembilan	4	0	2	0	3	5	0	0
Pahang	49	39	12	7	11	4	0	0
Perak	144	56	21	14	24	4	3	0
Perlis	4	0	2	1	12	3	0	0
P Pinang	25	14	3	5	2	0	0	0
Selangor	136	60	28	15	2	1	0	0
Terengganu	39	16	7	7	19	9	0	0
Total	649	351	160	84	330	65	22	3

Table 2

Detection of dengue virus in field-collected *Aedes aegypti* and *Ae. albopictus*.

Year	Locality	Species	Origin	Positive pools	PAP	RT-PCR
1993	Kuala Lumpur	*Ae. albopictus* (F)	larval	2	+	-
1993	Kuala Lumpur	*Ae. albopictus* (F)	adult	1	+	-
1993	Kuala Lumpur	*Ae. aegypti* (F)	larval	1	+	-
1994	Johor	*Ae. albopictus* (F)	larval	1	+	+
1994	Terengganu	*Ae. albopictus* (F)	adult	1	+	+
1995	P Pinang	*Ae. albopictus* (M)	larval	1	+	+
1995	Perak	*Ae. albopictus* (F)	larval	1	+	-
1995	Perak	*Ae. aegypti* (F)	larval	1	+	-
1995	Johor	*Ae. albopictus* (F)	larval	1	+	-
1995	Johor	*Ae. albopictus* (M)	larval	1	+	-

Field isolations of dengue virus had recovered type 2 virus from *Ae. aegypti* larvae and adult males in Burma (Khin and Than, 1983) and type 4 virus from larvae of the same species in Trinidad (Hull *et al*, 1984). Dengue type 2 was also isolated in Africa from a pool containing a mixture of *Ae. furcifer* and *Ae. taylori* male mosquitos (in Freier and Rosen, 1988). Studies have shown that transovarian transmission of dengue virus by *Ae. albopictus* varied extensively depending on the strain of virus and geographic strain of mosquito (Rosen *et al*, 1983; Mitchell and Miller, 1990).

Contrary to the results in this study, field investigations conducted in Kelang, Selangor, Malaysia reported that no transovarial transmission of dengue viruses was observed (Ramalingam *et al*, 1986) during their study despite the major dengue fever/ dengue hemorrhagic fever epidemic of 1982. Such transmission was also not observed in the studies done in Thailand (Watts *et al*, 1985).

In addition to the field evidence for transovarial transmission, laboratory studies also suggested that dengue-1 virus was transmitted transovarially by one of 5 strains of *Ae. aegypti* (Rosen *et al*, 1983). Jousset (1981) reported that dengue-2 virus was transovarially transmitted by four different strains of experimentally infected *Ae. aegypti*. Lee *et al*, (1996) similarly reported that transovarial transmission of dengue virus was observed in Malaysian *Ae. aegypti* but not in *Ae. albopictus*. Thus present field data have confirmed the experimental observations of transovarial transmission of dengue virus. Since the rate of the transmission can vary depending on the serotype and strain of virus and on the species or the geographic strain of mosquito; it is important to evaluate the competence of local vectors in each epidemic area. Collectively, these observations suggest that transovarial transmission of flavivirus occurs in nature and that further work is needed to evaluate the role of this phenomenon in their persistence during inter-epidemic periods. Information on the ability of the local vectors to transmit dengue virus transovarially will be useful in assisting the public health personnel and the general public in implementing a more effective campaign against dengue and its vectors; for if transovarial transmission of dengue virus occurs, it is obvious that the control of the immature stages of *Aedes* mosquito and the elimination of breeding sources must be further emphasized and prioritized.

ACKNOWLEDGEMENTS

The authors wish to thank the Director, Institute for Medical Research, Kuala Lumpur for permission to publish. Thanks are also due to Mr Ravindran, Division of Virus Research, IMR for his assistance. This work is supported by a Malaysian Government Research and Development Grant No. IMR-94-25.

REFERENCES

Boromisa RD, Rai KS, Grmstad P. Variation in the vector competence of geographical strains of *Aedes albopictus* for dengue 1 virus. *J Am Mosq Control Assoc*

1987; 3 : 378-86.

Freier JE, Rosen L. Vertical transmission of dengue viruses by *Aedes mediovittatus*. *Am J Trop Med Hyg* 1988; 39 : 218-22.

Gubler DJ, Nalim S, Tan R, Saipan H, Sulianti Saroso J. Variation in susceptibility to oral I infection with dengue viruses among geographical strains of *Aedes aegypti*. *Am J Trop Med Hyg* 1979; 28 : 1045-52.

Gubler DJ. Dengue and dengue hemorrhagic fever in the Americas. *PR Health Sci J* 1987; 6 : 107-11.

Jousset fX. Geographic *Aedes aegypti* strains and dengue-2 virus: susceptibility, ability to transmit to vertebrate and transovarial transmission. *Ann Virol* (Inst Pasteur) 1981; 132E: 357-70.

Halstead SB. Dengue hemorrhagic fever-a public health problem and a field for research. *Bull WHO* 1980; 58 : 170-3.

Hull B, Tikasingh E, Souza MD, Martinez R. Natural transovarial transmission of dengue 4 virus in *Aedes aegypti* in Trinidad. *Am J Trop Med Hyg* 1984; 33 : 1248-50.

Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungungya viruses. *J Gen Virol* 1978; 40 : 531-44.

Igarashi A, Fujita N, Okura Y, *et al*. Isolation of dengue viruses from patients with dengue hemorrhagic fever (DHF) and those with fever of unknown origin (FUO) in Jakarta, Indonesia, in the years of 1981 and 1982. *ICMR Ann* 1982; 2 : 7-17.

Khin MM, Than KA. Transovarial transmission of dengue 2 virus by *Aedes aegypti* in nature. *Am J Trop Med Hyg* 1983; 32 : 590-4.

Lee HL, Mustafakamal I, Rohani A. Does transovarian transmission of dengue virus occur in Malaysian *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). Poster presented at the 32nd Annual Scientific Seminar of the Malaysian Society of Parasitology and Tropical Medicine, Kuala Lumpur, March 29-30, 1996.

Maneekarn N, Morita K, Tanaka M, *et al*. Application of polymerase chain reaction for identification of dengue virus isolated from patient sera. *Microbiol Immunol* 1993; 37 : 41-7.

Mitchell CJ, Miller BR. Vertical transmission of dengue viruses by strains of *Aedes albopictus* recently introduced into Brazil. *J Am Mosq Control Assoc* 1990: 251-3.

Ramalingam S, Shekhar KC, Pang T, *et al*. Does transovarial transmission of dengue viruses occur in Malaysia. *Trop Biomed* 1986; 3 : 87-8.

Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* 1995; 229 : 726-33.

Rosen LD, Shroyer A, Tesh RB, Freier JE, Lien JC. Transovarial transmission of dengue viruses by mosquitoes: *Aedes albopictus* and *Aedes aegypti*. *Am J Trop Med Hyg* 1983; 32 : 1108-19.

Rosen L, Roseboom LE, Gubler DL, 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.

Watts DM, Harrison BA, Pantuwatana S, Klein TA, Burke DS. Failure to detect natural transovarial transmission of dengue viruses by *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol* 1985; 22 : 261-5.