

PERSISTENCY OF TRANSOVARIAL DENGUE VIRUS IN *Aedes aegypti* (LINN.)

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Abstract. A study was conducted to examine the persistency of transovarial dengue virus type 2 (DEN-2) in a Selangor strain of *Aedes aegypti* mosquitoes. Two hundred 4-5 day old female mosquitoes were fed with blood containing dengue virus. The infected mosquitoes were reared to the 7th generation; each generation was screened for the virus using immunological staining methods. The virus was detectable until the 5th generation but absent in the 6th and the 7th generations. Therefore, dengue virus type 2 can be transmitted transovarially in *Aedes aegypti* mosquitoes until the fifth generation under laboratory conditions.

INTRODUCTION

Dengue fever is a cause of serious health problems in many tropical and subtropical areas of the world. The disease is hyperendemic in Southeast Asia, where the more severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are major public health concerns because of its severe and often fatal disease in children. One hundred million cases of dengue fever occur annually, and half a million cases of DHF occur worldwide. Ninety percent of DHF victims are children less than 15 years of age (Malange *et al*, 2004).

Dengue virus is transmitted to humans by the bite of an infected *Aedes* mosquito. Viremic humans in the acute phase of their infection constitute the principal, and perhaps only source of infection for this mosquito. Once infected, a mosquito remains infected for life, transmitting the virus to susceptible individuals while probing and feeding (WHO, 1998). These facts can account for the persistence of

dengue viruses in large urban populations with high birth rates and abundant *Aedes* mosquito populations (Joshi *et al*, 2002).

Transovarial transmission of dengue virus in *Aedes aegypti* mosquitoes, the principal vector, may play a significant role in the maintenance of dengue virus in nature (Lee *et al*, 1997). Transovarial transmission may provide a mechanism to allow the virus to survive dry or cold seasons or the temporary absence of non-immune vertebrate hosts (Rosen *et al*, 1983). Maurya *et al* (2001) found that transovarially infected mosquitoes can transmit the virus orally. Dengue virus has been detected in immature *Aedes aegypti* and *Aedes albopictus* mosquitoes collected in the field (Rohani *et al*, unpublished) and dengue virus has also been isolated from male *Aedes aegypti* mosquitoes (Rohani *et al*, 1997), thus giving evidence that transovarial transmission in these species may be an important cause of maintenance of the virus in nature in Malaysia.

The cyclic nature of dengue epidemics and how the virus is maintained during interepidemic periods in areas where outbreaks have occurred previously raises questions, which have led to studies to evaluate the

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importance of transovarial transmission. Therefore, the present study was conducted to investigate the role of *Aedes aegypti* in the maintenance of DEN-2 virus through transovarial transmission over several generations.

MATERIALS AND METHODS

Mosquitoes

The mosquitoes employed for the experiment were from a laboratory colony maintained for more than 30 years at the Institute for Medical Research, Kuala Lumpur. The mosquitoes were maintained at 70-80% relative humidity and at 24-25°C.

Virus

Dengue type 2 (DEN-2) virus was isolated in 2004 from a dengue fever patients's sera from Damansara Specialist Center, Petaling Jaya, by inoculation into *Aedes albopictus* mosquitoes C6/36 cell line. It was identified by reverse transcription polymerase chain reaction (RT-PCR). The DEN-2 stocks were maintained in *Ae. albopictus* clone C6/36 culture cells incubated at 28°C in Eagle's Minimum Essential Medium (EMEM) supplemented with 2% heat-activated fetal calf serum and 0.2 mM non-essential amino acids. The percentage of infected cells was monitored during incubation. When 100% of the cells were infected the supernatant fluid was collected and filtered with a 0.22 mm filter unit (Nunc) and the virus was concentrated by the Integrated Speed Vac system (Savant ISS 100SC) at 14,000 rpm for 3 hours.

Artificial membrane feeding and transmission procedure

An artificial membrane feeding technique employed was modified from Graves (1980). Briefly, 5-7-day old female *Aedes aegypti* mosquitoes were deprived of sucrose solution 12 hours prior to an infectious meal, then allowed to feed for 30 minutes through a chicken skin membrane covering the base of an arti-

cial feeding apparatus. The feeding mixture of infected samples consisted of 1 ml human blood mixed with 200 µl of dengue virus C6/36 cell culture fluid and was maintained at 37°C during the feeding period. Uninfected samples were obtained by feeding the mosquitoes with a suspension containing 1.0 ml human blood and 200 µl of clean (uninfected) C6/36 cell culture fluid. Fully engorged mosquitoes (100-150) were selected and reared in a cage supplied with 10% sucrose solution, supplemented with 1% vitamin B complex for maintenance feeding. The cage was kept in an isolated room. Precautions were taken to avoid possible escape of infected mosquitoes, including double screening the room, use of double doors, and installation of a screened exhaust fan. After three days, moistened filter paper was provided to the mosquitoes for oviposition. Approximately 1,000 eggs were collected and allowed to hatch in a white plastic tray containing tap water. The first and second instar larvae were fed on liver powder while the third and fourth instar larvae were fed with partially cooked liver. Approximately 250 fourth instar larvae were reared to adults to maintain the next progeny while others were pooled and kept at -80°C for virus isolation to determine the possibility of transovarial transmission. The progeny of the *Aedes aegypti* mosquitoes infected with dengue virus were reared to adulthood. Female mosquitoes of the first generation were allowed to feed directly on mice. For each generation, blood-fed females were confined and the eggs obtained were pooled. The same procedure was repeated until the seventh generation.

Virus isolation

Fourth instar larvae of each batch were pooled in sterile Eppendorf tubes with not more than 10 individuals per pool. They were then homogenized in 1.5 ml of EMEM enriched with Fetal Bovine Serum (FBS). The homogenate was then spun at 3,000 rpm for 15 minutes at 4°C. The supernatant was in-

oculated into C6/36 culture cells in a culture tube. After two hours incubation, the inoculum was removed and replaced by 2 ml maintenance medium (2% FBS EMEM). For the positive control, 50 µl dengue 2 infected culture fluid was inoculated into the cell lines, and for the negative control no inoculum was added. These were then incubated at ambient temperature for seven days.

Smear preparation

After the incubation period, the culture tubes were vortexed and centrifuged at 1,200 rpm for 5 minutes. Cells from the sediment were harvested using a Pasteur pipette and smeared onto 12 well teflon coated slides. The smears were left to air-dry overnight at ambient temperature and then fixed with chilled acetone for twenty minutes. After smear preparation, the cultures were kept at -20°C for confirmation by a second or third passage in a cell culture if the initial results were positive.

Peroxidase-anti peroxidase (PAP) staining

The fixed smears were first reacted with anti-dengue human serum at 1:5,000 dilution inside a moist chamber, which was placed at ambient temperature for 40 minutes. The slides were rinsed three times with phosphate-buffered saline (PBS), after which they were reacted with anti-human rabbit serum at 1:1,000 dilution for 40 minutes. The slides were rinsed again with PBS and then flooded with anti-rabbit goat serum at a 1:5,000 dilution for 40 minutes. The slides were then rinsed again with PBS and peroxidase anti-peroxidase conjugated rabbit serum at a 1:1,000 dilution was applied for another 40 minutes. The slides were then rinsed again with PBS followed by application of 0.2 mg/ml 3,3 diaminobenzidine (DAB) in PBS with 0.01% hydrogen peroxide as a substrate.

Microscopic examination of smears was done at 200 x magnification using a normal compound light microscope. Smears positive for virus showed the presence of distinctive

brown cells, and negative smears had clear or translucent cells.

Minimum infection rate

The minimum infection rate (MIR) was used to compare virus infection rates in laboratory infected *Ae. aegypti* mosquitoes. The MIR was calculated as the number of positive pools ÷ total number tested x 1,000 (Chow *et al.*, 1998).

RESULTS

DEN-2 virus was detectable until the 5th generation of the mosquitoes but absent from the 6th and the 7th generations (Table 1). The MIR for each successive generation decreased. Only about 50% of larvae successfully hatched from eggs.

DISCUSSION

The reason for the low hatch rate is unknown. Fewer positive pools occurred with each generation beginning with the first generation (F1). This showed the "dilution" effect with each successive larval generation. Shroyer (1990) reported the DEN-1 infection rate in *Ae. albopictus* increased with each succeeding generation.

Table 1
Minimum infection rate (MIR) of *Aedes aegypti* fourth instar larvae over successive generations.

Generation (adult)	No. of larvae tested	No. of pools tested	No. of positive pools	MIR
F1	200	10	9	45
F2	200	10	9	45
F3	200	10	7	35
F4	200	10	7	35
F5	200	10	6	30
F6	200	10	0	0
F7	200	10	0	0

In Malaysia, transovarial transmission of dengue virus has been reported in urban and suburban residential areas (Lee and Rohani, 2005). The results in the present study, show that dengue virus is passed on through transovarian transmission to successive generations. Under natural conditions, these mosquitoes may serve as a natural reservoir for the virus.

Information regarding the ability of vectors to transmit dengue virus transovarially is useful in assisting the public health personnel and the general public in implementing a more effective campaign against dengue infection and its vectors. Control of immature stages of *Aedes* mosquitoes and elimination of breeding sources must be emphasized and prioritized. Control measures directed at *Aedes* mosquitoes may not only reduce the risk of dengue transmission during epidemics but also lessen the chances of dengue viruses becoming established in new endemic foci.

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