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Eco-virological survey of *Aedes* mosquito larvae in selected dengue outbreak areas in Malaysia

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ABSTRACT

Background & objectivesi: Transovarial transmission of dengue virus in the *Aedes* vectors is now a well-documented phenomenon reported from many parts of the endemic areas in the world, which played an important role in initiating and maintaining the outbreak in human populations. This study investigated the factors affecting breeding habitats and the relationship with transovarial dengue virus in larvae of *Aedes aegypti* and *Ae. albopictus.*

Methods: Larval surveillance was conducted in dengue outbreak areas in Malaysia from 2008 until 2009. Sampling was carried out based on habitat type, water condition (substrate type), canopy coverage, temperature and pH at breeding habitats. RT-PCR was performed to detect presence of transovarial dengue virus in larvae collected in the study areas.

Results: A total of 789 breeding habitats were identified during this study and the majority of these breeding sites were plastic containers (57.46%). *Aedes albopictus* dominated most of the water condition surveyed, while *Ae. aegypti* indicated preference toward habitats with clear water. *Aedes aegypti* was selective in selecting ovipositional sites compared to *Ae. albopictus* where shaded areas were shown to be the most preferred. From a total of 363 mosquito larvae pools, 23 (6.3%) pools were positive for dengue virus where 18 of them were from *Ae. albopictus* and five were from *Ae. aegypti* mosquito larvae pools.

Interpretation & conclusion: This study indicated the presence of transovarial transmission of dengue virus in immature *Ae. aegypti* and *Ae. albopictus* in the field. This study also showed that combination of water conditions, canopy coverage, temperature and pH of breeding habitats were the factors affecting the larval population. The study suggested that larval survey programme could serve as a tool not only to monitor the local dengue vector distribution but also to provide objective information for taking appropriate action by the community against dengue vectors.

Key words *Aedes aegypti*; *Aedes albopictus*; dengue; larval surveillance; virus infection rate

INTRODUCTION

Dengue is a disease caused by four antigenically distinct single-stranded RNA viruses, denoted as dengue type 1, 2, 3 and 4 of the genus *Flavivirus*, family Flaviviridae[1–2]. The infection can be manifested in a range of symptoms from relatively mild flu-like syndrome with rash, commonly known as dengue fever (DF), to severe and potentially fatal disease known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) which is characterized by capillary leakage, thrombocytopenia and hypovolemic shock. In Malaysia, classical dengue fever was first documented in 1901–02, while the first reported outbreak of DHF occurred in 1962. Since then dengue has remained endemic, with one or more of the four dengue serotypes co-circulating in the country and outbreaks of DHF being reported periodically.

During the first quarter of 2012, the State of Selangor recorded the highest dengue cases in Malaysia with 1271 cases reported from January 2012 onwards. This figure was almost half of the number of cases (2854) reported nationwide[3]. At present, neither an effective vaccine nor a specific drug is available for DF/DHF. Management of patient is via intravenous fluid therapy which helps to maintain patient's body fluid. It is for these reasons that control of dengue is currently focusing on controlling the vectors, *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae) that transmit dengue.

Vector surveillance allows timely implementation of emergency mosquito control measures such as space application of chemical insecticides against adult mosquitoes and destruction of their breeding places to contain an outbreak. Unfortunately, when the adult mosquito density is low, direct entomological monitoring is not sensitive anymore as an indicator to serve as an early warning surveillance system for outbreak prevention. It is in this

particular situation that detection of dengue viruses in vector population becomes a crucial element of an early alert system. Transovarial transmission of dengue virus in the *Aedes* vectors is now a well-documented phenomenon reported from many parts of the endemic areas in the world. Dengue vector control programme should emphasise on the importance of larval control since the immature stages may become the reservoir of the virus during the inter-epidemic periods. Transovarial transmission of dengue virus played an important role in initiating and maintaining the outbreak in human populations. Transovarial dengue virus is infectious in the adult stage of the mosquito when the mosquito develops from the immature stages. The virus is probably transmissible to human bitten by the emerged adults[4]. The possible impact of transovarial transmission on dengue outbreak is also unknown. This study is, therefore, initiated to investigate the factors affecting breeding habitats and the relationship with transovarial dengue virus in larvae of *Ae. aegypti* and *Ae. albopictus.*

MATERIAL & METHODS

Larval survey

Aedes larval surveys were carried out in the State of Johor, Kedah, Malacca, Negeri Sembilan, Pahang, Perak, Perlis, Penang, Sabah, Sarawak and Terengganu based on data provided by the State Health Department. Entomological survey was carried out in urban and suburban areas. Container index was worked out as per standard WHO guidelines. Mosquito larvae collected in the survey were identified using standard taxonomic keys[5]. Identified mosquito larvae were segregated according to the species, site, and date. Mosquito larvae were then stored in pools of 15–20 larvae per pool in cryogenic vials at –70ºC for future virus isolation studies. The mosquito larvae pools were assayed for dengue virus detection by RT-PCR. Serotyping of the dengue positive pools were also performed via RT-PCR.

Larval habitat characterization

During the larval survey, environment variables recorded for each habitat were: (i) habitat type, (ii) substrate type—classified as clear, clear with debris, muddy, greenish, brownish, pinkish and reddish (iii) canopy cover—classified as open, shaded or partially shaded, (iv) water conditions—pH, temperature, dissolved oxygen and turbidity. Correlation between the number of larval population for both *Ae. aegypti* and *Ae. albopictus* and the water conditions were analyzed by correlation coefficient (Spearman's rank-order).

Detection of dengue virus using reverse transcriptase polymerase chain reaction (RT-PCR)

Each pool of mosquito larvae was placed in a nuclease-free 1.5 ml micro centrifuge tube. The larvae were then homogenized and RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen). For positive control, an equal volume of cultured cells infected with dengue virus was used and for negative control, uninfected cultured cells were used. Extracted RNA was kept at –20ºC until used.

RT-PCR method of Lanciotti *et al*[6] was employed. Dengue virus universal consensus primers were (TCAATATGCTGAAACGCGCAGAAACCG and TTGCACCAACAGTCAATGTCTTCAGGTTC)[6]. Master mixes were prepared using Titan One Tube RT-PCR Kit (Roche). Each reaction contained 9.25 µl of double distilled water, 2 µl of dNTP mixture, 1.25 µl of dithiotreitol, 0.5 µl of RNAse inhibitor, 0.5 µl of RT-PCR buffer, 0.5 µl of enzyme mixture and 0.5 µl of each dengue primer. A 10 µl of template RNA was added to 15 µl master mix to make up a final volume of 25 µl of reaction mixture.

For dengue virus detection, the reaction was carried out at 51ºC for 30 min to produce cDNA, which was then amplified by the following PCR steps: Initial denaturation at 92ºC for 3 min, 41 cycles of 92ºC for 30 sec, 51ºC for 45 sec and 72ºC for 1 min; followed by 72ºC for 5 min. For every RT-PCR, a positive control and a negative control were included.

PCR products were analysed by performing electrophoresis in 2.0% Nusieve PCR gel (FC Bio, USA) at 100 volts and staining with ethidium bromide. The gel was viewed under ultraviolet illuminator (Ultra Lum Inc, California, USA) and the resulting bands were photographed with a polaroid camera.

Serotyping was performed on dengue positive pools via RT-PCR. Dengue type-specific primers used in this study were commercially synthesized (D1S and D1C; D2S and D2C; D3S and D3C; D4S and D4C)[7]. The PCR results were analysed by 6 gel electrophoresis. The expected sizes were 490 bp (D1); 230 bp (D2); 320 bp (D3) and 398 bp (D4).

Minimum infection rate (MIR)

The MIR was calculated as: (Number of positive pools/Total specimen tested) × 1000. Data analyses were statistically analysed using the SPSS programme for *t*-test and mean comparison.

RESULTS

A total of 789 breeding sites were identified during this study. These sites included man-made habitats such

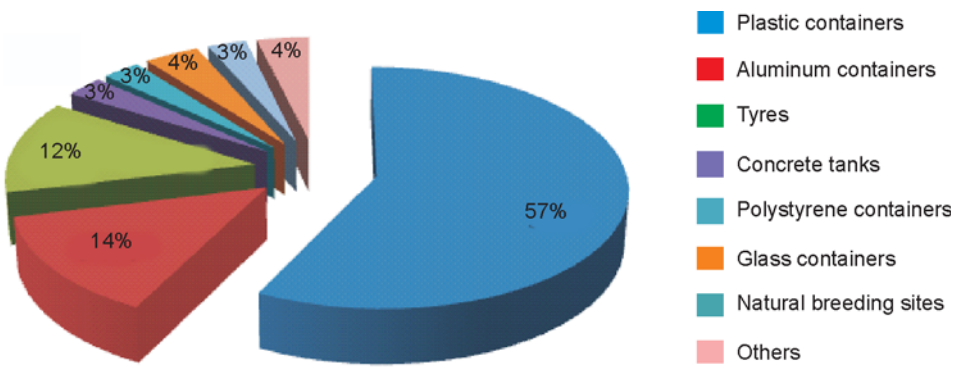

Fig. 1: Distribution of larval population based on habitat type.

as plastic containers, aluminum containers, tyres, concrete tanks, glass containers, and polystyrene containers. Among these sites examined, plastic containers were the most preferred breeding sites (57.46%), followed by aluminum containers (13.76%) and tyres (12.61%) (Fig. 1). The container index (CI) for urban areas (17%) was higher compared to sub-urban areas (9.3%). The dominant mosquito larvae collected during the survey were *Ae. albopictus* (2703) and very small number of *Ae. aegypti* larvae (137).

The distribution of larval population between two mosquito species, *Ae. aegypti* and *Ae. albopictus* in relation to the substrate type of the breeding sites were compared. Figure 2 shows the distribution of larval population of the two species with far higher *Ae. albopictus* larval population than *Ae. aegypti* in all the substrate types, except in the breeding sites with clear substrate types. Here, the larval population of *Ae. aegypti* was seen almost equal to that of *Ae. albopictus.* Significant correlation between substrate type condition and larvae population for both *Ae. albopictus* (r = 0.199, $p = 0.05$) and *Ae. aegypti* (r = 0.133, $p = 0.05$), was observed.

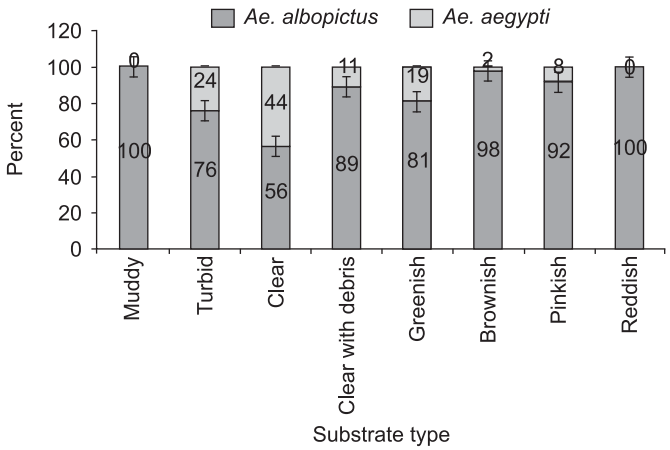

Fig. 2: Distribution of larval population (in %) based on substrate type.

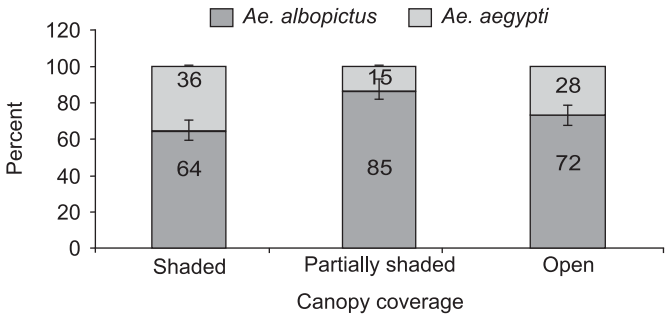

Fig. 3: Distribution of larval population based on canopy coverage of the breeding habitat.

We also examined the effect of canopy coverage on the distribution of larval population of *Ae. aegypti* and *Ae. albopictus.* Three types of canopy were examined which included shaded, partially shaded and open type. Figure 3 shows the distribution of larval population based on canopy coverage at the breeding habitats. Distribution of *Ae. aegypti* larval population was found almost the same for shaded and open canopy and slightly less for partially shaded canopy, whereas in the case of *Ae. albopictus* larval population, it differed from one type of canopy to the other. The larval population was found highest in breeding habitat which was partially shaded, followed by open habitat and the lowest was at fully shaded habitat. The results also clearly demonstrated that for all types of canopy, the larval population of *Ae. albopictus* was higher than *Ae. aegypti.* However, larval population for both the species was not significantly correlated ($p > 0.05$) with canopy coverage.

The effect of temperature of the breeding sites in relation to larval population for both the species was also examined. Our findings showed that breeding activity was highest for both the species in breeding sites having water temperature between 25 and 30°C (Fig. 4). As the temperature increased from 31–35°C, lesser number of larvae was detected. This reduction was seen for both the species with larvae of *Ae. albopictus* reduced by about

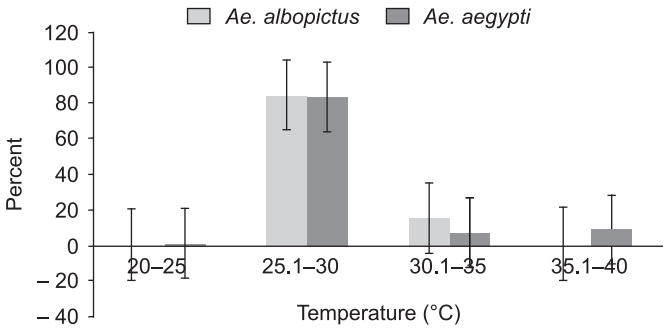

Fig. 4: Number of breeding sites against temperature of the breeding habitat.

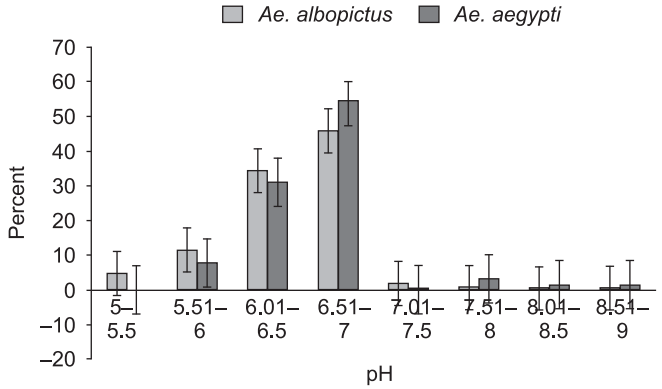

Fig. 5: Number of breeding sites against pH of the breeding habitat.

4.5 times and about 10 times for larvae of *Ae. aegypti.* However, larval population for both the species was not significantly correlated ($p > 0.05$) with temperature.

Regarding the effect of breeding habitat pH in relation to larval population, the findings showed that the breeding activity of both the species was found at breeding sites having pH from 5 to 8.5 (Fig. 5). The activity for both the species increased steadily as pH changed from acidic to neutral. However, increase in the activity of *Ae. albopictus* was greater compared to *Ae. aegypti*. The larval population dropped drastically to almost zero as soon as the pH of the breeding sites changed to alkaline state. However, larval population for both the species was not significantly correlated ($p > 0.05$) with pH.

Other water conditions examined during the survey showed dissolved oxygen of 2–7 mg/l and water turbidity of 0.9–97.8 NTU. However, larval population for both the species was not significantly correlated ($p > 0.05$) with dissolved oxygen. Water turbidity was significantly correlated with larvae population for both *Ae. albopictus* ($r = 0.241$, $p = 0.05$), and *Ae. aegypti* ($r = 0.184$, $p = 0.05$).

The RT-PCR (Fig. 6) performed on 363 pools of mosquito larvae showed 23 pools (6.3%) to be positive with dengue virus and of these positive pools, 18 were of

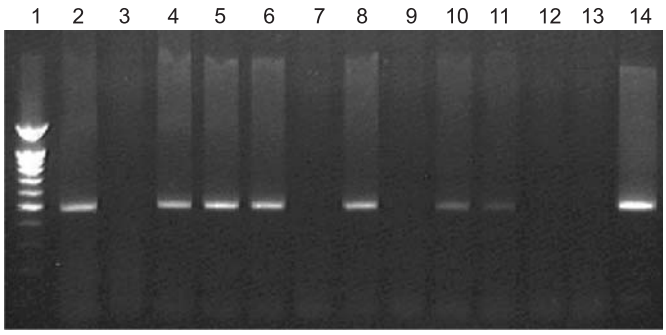

Fig. 6: Detection of transovarial dengue virus by RT-PCR using universal primers—Lane 1: 100 bp ladder; Lane 2: Positive control; Lane 3: Negative control; and Lanes 4 – 14: Mosquito larval samples 510 bp.

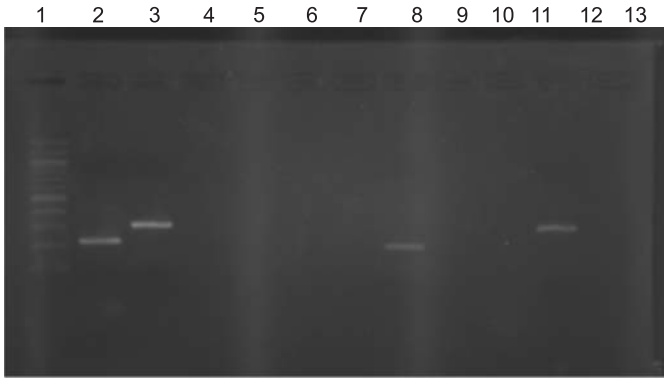

Fig. 7: Detection of transovarial dengue virus by RT-PCR using dengue 2 and 3 primers—Lane 1: 100 bp ladder; Lane 2: Positive control for dengue 2 (230 bp); Lane 3: Positive control for dengue 3 (320 bp); Lanes 4 and 5: Negative control; and Lanes 6–13: Mosquito larval samples.

Ae. albopictus larvae and five were of *Ae. aegypti* larvae. Minimum infection rates; however, were higher in *Ae. aegypti* (36.5) compared to *Ae. albopictus* (6.66). Serotyping via PCR on all positive pools indicated dengue 3 in 15 pools (83.3%) and dengue 2 in the other three pools (16.6%) of the *Ae. albopictus* infected larvae (Fig. 7). Only dengue 3 was detected in infected pools (5-pools) of *Ae. aegypti*. Dengue 1 and dengue 4 were not detected in any of the positive pools.

DISCUSSION

The most dominant mosquito larvae collected in this study were *Ae. albopictus,* with very low number *Ae. aegypti* larvae detected. This finding is in accordance with the study done on the distribution and abundance of *Ae. aegypti* and *Ae. albopictus* in dengue endemic areas in Malaysia which indicated that apparently *Ae. albopictus* was more dominant than *Ae. aegypti*[8]. On the contrary, the study on a nationwide *Aedes* larval survey carried out

in the years 1988–89 in peninsular Malaysia reported that the house index and breteau index for both *Ae. aegypti* and *Ae. albopictus* were similar[9]. The current study could suggest that *Ae. albopictus* is slowly but surely becoming the dominant species in breeding containers, whereas population of *Ae. aegypti* is undergoing the declining process during the last two decades. Nonetheless, with specific treatment and vaccine for dengue still uncertain, targeted environmental and ecosystem management (in relation to mosquito breeding sites) is now becoming an important control/preventive measure in the battle against dengue. It is well-known that the primary dengue vector, *Ae. aegypti* breeds predominantly in artificial water containers and its life-cycle is closely associated with human activities in urban areas where larval habitats are increasing rapidly. *Aedes* mosquitoes breed in water collections in artificial containers such as plastic cups, used tyres, broken bottles, flower pots, *etc*. It has been proven that by periodically draining or removing these artificial containers mosquito breeding grounds were reduced effectively[3].

Our study clearly demonstrated that plastic containers were the major breeding sites for both *Ae. aegypti* and *Ae. albopictus*, followed by discarded aluminum containers and waste tyres. These findings were in accordance with works done by Chen *et al*[10], Thavara *et al*[11], Preechaporn *et al*[12], and Thenmozhi *et al*[13] who detected *Ae. albopictus* larvae breeding in a wide range of artificial containers.

This study also indicated that *Ae. albopictus* was capable of breeding profusely in natural breeding sites having a wide range of water condition (substrate type) from clear to muddy substrate type. *Aedes aegypti* larvae were also detected in almost all the breeding sites, demonstrating that container-breeding *Aedes* have the ability to breed in almost any water condition. *Aedes aegypti* population however was very much lower compared to *Ae. albopictus*, indicating that *Ae. albopictus* larvae were less affected by the substrate type, and therefore, less selective with regards to their breeding site as compared to *Ae. aegypti.* This finding was almost similar to study done by Chen *et al*[10] and Lee[9] in Malaysia which reported that *Aedes* larvae preferred to breed in clear, but not necessarily clean water.

Aedes aegypti larval population was lower compared to *Ae. albopictus* during our survey in relation to different canopy type. *Aedes aegypti* larval population was detected for all canopy types which could indicate that its presence was not very much influenced by light, as opposed to *Ae. albopictus* larvae. The lower in number of larval population compared to larval population of *Ae. albopictus* might suggest that factors other than light is influencing the habitat to be unfavourable for *Aedes aegypti*. *Aedes aegypti* is, therefore, seemed more selective of its oviposition sites compared to *Ae. albopictus* which preferred partially shaded areas for its habitat. Chen *et al*[10], and Wan Norafikah *et al*[14], who conducted outdoor ovitrap surveillance reported that *Ae. albopictus* dominated most of the habitats and no *Ae. aegypti* was detected in the ovitrap. Our findings with respect to canopy type are in general agreement with the finding of Lee[15] who reported that even partially closed containers had larvae breeding in them and only complete coverage of containers prevented *Aedes* breeding.

The fact that increase in temperature caused population reduction in both species, suggested that temperature could be one of the factors that determine suitability of breeding habitats for both the species especially for *Ae. aegypti* since the effect was seen causing greater population reduction in *Ae. aegypti* (11 times compared to 5.5 times for *Ae. albopictus*). Our finding also showed very clearly that the pH of the breeding habitat has influenced the suitability of the habitat for both the species to breed. The most preferred habitat was that having a neutral pH.

Although the number of mosquito larvae detected positive for dengue virus were very small (6.3%), it was evident that dengue virus could be transmitted transovarially. Rohani *et al*[8] suggested the possibility for the transmission to occur from adults to larvae for both the species. Our finding also suggested that transovarial transmission of flavivirus does occur in nature. Lee and Rohani[4] showed that transovarial dengue virus in wild larvae was associated with dengue outbreak. Further, work is, therefore, necessary in order to understand if such phenomenon could have a role to play in facilitating the virus to persist during inter-epidemic periods.

The study also clearly indicated that dengue-infected mosquitoes are capable of breeding in any type of containers especially those with clear water. It is well-recognized that community participation is the key to success in controlling mosquitoes, particularly dengue vectors. The dengue vector surveillance programme serves as a tool not only to monitor the local dengue vector distribution but also to provide objective information for taking appropriate action by the community against dengue vectors.

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