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# Knockdown resistance (*kdr*) in dengue vectors, *Aedes aegypti* and *Aedes albopictus*: A post-flood risk assessment

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**ABSTRACT.** Even though dengue remains a major threat in Malaysia, information on insecticide resistance of vectors, along with the underlying genetic basis of such resistance, and the impact of a natural flooding disaster remains sparse. Kelantan was one of the states of Malaysia severely affected by monsoon flooding in December 2014. We examined the resistance profile of *Aedes* mosquitoes in after the big flood, comparing the susceptibility of *Ae. aegypti* and *Ae. albopictus* from flooded and unflooded areas. We also sought to validate a simple molecular assay for detecting knockdown resistance (*kdr*) mutations in the voltage-gated sodium channel (VGSC) gene of the mosquitoes. Mosquito immatures were collected by using ovitraps in Kampung Baru, Pasir Pekan, Tumpat (flooded area), and Bandar Baru Kubang Kerian, Kubang Kerian (unflooded area) five months after the flood disaster. The samples were reared to adult mosquitoes and bio-assayed following World Health Organization (WHO) protocol against deltamethrin (0.05%) and pirimiphos-methyl (0.25%) to evaluate their susceptibility. The DNA molecular assays focused on amino acid substitution in domain II (S989, I1011, L1014, and V1016) and domain III (F1534C) in segment 6 of the VGSC gene. *Aedes aegypti* and *Ae. albopictus* from both locations were found to be susceptible to pirimiphos-methyl

 

(mortality >98%). A high level of resistance to deltamethrin was detected in *Ae. Aegypti*; the unflooded area mosquitoes had significantly lower mortality (17%) than the flooded area (74%). Investigation of *kdr* mutation showed F1534C substitution in the VGSC gene of *Ae. aegypti* from the flooded and unflooded areas, with an insignificant difference in frequency of 83 and 75% ($P > 0.05$). This mutation was not detected in *Ae. albopictus*.

**Key words:** *Aedes aegypti*; *Aedes albopictus*; Flooding; *kdr* mutations; Kelantan; Malaysia; Pyrethroid resistance; Voltage-gated sodium channel (VGSC)

## INTRODUCTION

Dengue fever has been a never-ending public health problem, especially in tropical and subtropical regions across the world. Mosquito-borne flaviviral infection caused by dengue virus (DENV) is now endemic in more than 100 countries, with an estimation of 400 million cases yearly (Bhatt et al., 2013). In 2016, Malaysia reported 100,028 cases, a similar burden compared to the previous year (WHO, 2017). The health risks and threats inflicted by *Aedes* mosquitoes are too much to forbear and likely to aggravate due to emerging insecticide resistance. It is a great misfortune to mankind due to the close association with mosquitoes in terms of time and space. Mosquitoes tend to change their breeding habitats rapidly as a result of vector control activities; therefore, they are capable of adapting to various human habitats for successful reproduction.

Hitherto, no specific treatments are available to cure dengue infection. Therefore, efforts to combat the disease are mainly targeted on vector control and source reduction by the community itself or by the health authorities. Since *Aedes* mosquitoes are container breeders, the first line of control against these mosquitoes is by breeding site reduction. In the case where the container is too large to be removed or cleared, larvicides are often used. In Malaysia, temephos (Abate 1% Sand Granules) is widely used as a larvicide for *Aedes* control by health personnel (Chen at al., 2005). When a dengue outbreak is reported, vector controls, targeting the adult mosquitoes are applied. To date, the most efficient method to suppress the mosquito vectors in a short period of time is by using chemical insecticides, particularly organophosphates and pyrethroids (WHO, 2011). In Malaysia, when a dengue outbreak is reported, the norm is an inspection for vector breeding grounds followed by fogging or ultra-low volume (ULV) by the health authorities afterward. Malathion and fenitrothion (organophosphates) and pyrethroids are the main insecticides used in fogging. Although the insecticide-based strategy has been sometimes successful (da-Cunha et al., 2005; Montella et al., 2007), the monolithic reliance on chemical insecticides has led to adverse effects. Their widespread misuses have caused the development of insecticide resistance in mosquitoes (Das et al., 2007), with the main vector, *Ae. aegypti* is being ranked eighth in the list of arthropod species with the highest case of insecticide resistance worldwide (Whalon et al., 2008). Furthermore, the initiation of vector control activities after the onset of an outbreak might be too late for the efficient management of the disease. Thus, proper surveillance on the vector and the insecticides are crucial for efficient epidemic-preparedness.

 

Kelantan, being the third-ranked state for dengue cases in 2014 in Malaysia, poses a high risk for an increase in dengue incidences due to the massive flood that hit the region in December 2014. Kelantan faced a devastating period of dengue epidemic in the year 2014 with a total of 14,311 cases and 18 deaths. However, after the massive flood struck the region, the number of cases decreased by fivefold to 2,850 cases in the year 2015 with a total of seven deaths. The risk for the emergence of insecticide resistance during a vector-borne disease outbreak, or following a natural disaster is sometimes omitted (PAHO, 1982). It is important to note that dengue and flooding are indirectly associated. Most of the existing mosquito breeding grounds have been washed away by the floodwater, causing a temporary reduction in the mosquito population and subsequent reduction in reported dengue cases. In such a situation, a drastic reduction in the mosquito population may contribute to the selection effect in which mosquitoes with stronger survivability are selected to re-emerge under extreme environmental conditions. Furthermore, over-dependence on 'emergency' vector control after a natural disaster might worsen the situation. Inappropriate and extensive spraying of the insecticides may lead to the emergence of insecticide resistance among the mosquitoes.

In Malaysia, pyrethroids such as deltamethrin and permethrin replaced the use of malathion from early 1998 until today (Low et al., 2013). However, previous studies have proved the existence of multiple resistance to insecticides (malathion, permethrin, deltamethrin, carbamates, DDT) in mosquitoes from dengue-prone areas in Kuala Lumpur, Selangor, Johor, Penang, Kelantan, and Perak (Rong et al., 2012; Ishak et al., 2015). The use of pyrethroids in the control of *Aedes* has increased significantly in the last two decades owing to its relatively rapid mode of action and less hazardousness compared to other classes of insecticides (Bisset et al., 2009; Linss et al., 2014). It is very much expected to develop new *kdr* mutations when pyrethroid remains as the primary control in vector control programs (Amelia et al., 2018). Therefore, the detection of pyrethroid resistance in *Aedes* vector mosquitoes plays a critical role in resistance management efforts.

So far, the resistance profile of *Aedes* mosquitoes in Kelantan has not been investigated, especially after the big flood hit the region in December 2014. The flood chronology started due to a torrential raining period between 14-19$^{th}$ December that caused rivers to exceed the danger level. The flood persisted for more than a week with a continuous intense rain period until the end of December 2014 (Yahaya et al., 2015), which swamped the whole state except a few areas in Kubang Kerian. An appropriate study of susceptibility or resistance status in mosquito vectors is important to detect the problem at an initial stage so that proper vector control can be implemented in areas with high risk. Furthermore, it is also important in providing baseline data for systematic planning and insecticide selection before the commencement of control operational activities, especially after a natural disaster. The aims of this study are (1) to compare the susceptibility status of *Ae. aegypti* and *Ae. albopictus* from the flooded and unflooded region in Kelantan against the main insecticides used in this region, deltamethrin, and pirimiphos-methyl using bioassays and (2) to detect *kdr* mutations in voltage-gated sodium channel (VGSC) gene followed by genotype-phenotype association. The study focused on amino acid substitution in domain II (S989P, I1011M/V, L1014F, and V1016G/I) and domain III (F1534C) in segment 6 of the VGSC gene for the pyrethroid.

## MATERIAL AND METHODS

## Study Area

Kelantan is located in the eastern coastal region of Peninsular Malaysia, with the latitude of 06°10’ N and longitude of 102°20’ E. Kelantan was chosen as a pilot area in this study because it is a floodplain zone that suffered devastating flooding at the end of the year 2014. Kelantan has a tropical climate with an annual temperature of ~23-34°C and an annual rainfall of ~2700 mm (Che Ros et al., 2016). The focus of research and data collection was divided into two areas: (1) Kampung Baru, Pasir Pekan in Tumpat district (flooded area), N 06°06.616', E 102°12.985' and (2) Bandar Baru Kubang Kerian, Kubang Kerian in Kota Bharu district (unflooded area), N 06°05.972’, E 102°16.502’. The flooded area is a suburban residential area which is located a kilometer away from the Kelantan River. The close distance of this area to the river made it prone to the monsoon flood that happened in 2014. The unflooded area in an urban settlement located a few minutes away from a public university and hospital but with a lesser cover of vegetation. The location of this area far from the Kelantan River left the area unaffected from the flooding during the monsoon season.

These stations were randomly selected based on ex-dengue hotspots of human dengue cases and human population density, in which routinely fogging activities occurred, as provided by the local state government. Tumpat and Kota Bharu districts have a population density of 900/km$^2$ and 1,200/ km$^2$, respectively. Both areas suffered a dengue outbreak before the flood incidence in December 2014, specifically with 73 cases in Kampung Baru and 41 cases in Bandar Baru Kubang Kerian (data provided by Kelantan Health Department, MOH). A hotspot is defined as a condition indicating some form of clustering in a spatial distribution (Osei & Duker, 2008). Thus dengue hotspot means areas with more than four confirmed dengue cases in two weeks. Both areas suffered dengue outbreak before the flood stroke the region in December 2014, ranking the whole state in the third place for reported dengue cases in Malaysia. Data collected from the unflooded area served as control as well as a comparison to the flooded area.

## Mosquito sampling

Field sampling for *Ae. aegypti* and *Ae .albopictus* mosquito egg, and the larval collection was conducted by using the ovitraps method. The mosquito samples were collected five months after the flood disaster until enough samples were captured for the bioassay test. Black ovitraps (height 10.4 cm, diameter 7.0 cm) were used in the study. A hardboard paddle (3 cm × 14.7 cm × 0.3 cm) was placed vertically in each ovitrap with the rough surface facing upwards to facilitate oviposition. Dechlorinated tap water was added to each container to a level of 6.0 cm, usingf 200 mL of dechlorinated tap water. We placed approximately 80 ovitraps in the flooded area and 120 ovitraps in the unflooded area. More ovitraps were placed in the unflooded area because the previous study on the mosquito population abundance in these areas revealed lesser mosquito abundance in this area. The ovitraps were placed randomly in the field considering the potential breeding sites that are shaded and not directly exposed to the sunlight; close to mosquito resting sites such as bushes and alongside dark corners and walls with minimal human and animal contact. The ovitraps were collected after 4-5 days. The collected samples were segregated according to the locations. Laboratory strains were obtained from Vector Control Research Unit

 

(VCRU), Universiti Sains Malaysia (USM), 11800 USM, Penang, Malaysia, to be used as susceptible strains.

## Mosquito rearing

Collected eggs and larvae were brought back to the Medical Entomology Laboratory, Vector Control Research Unit, Universiti Sains Malaysia, Penang. The samples were pooled and reared in enamel trays containing dechlorinated tap water. The larvae were fed with a mixture of dog biscuit, beef liver, yeast, and milk powder with a ratio of 2:1:1:1 by weight prepared as a fine powder. The mosquitoes ($F_0$ generation) were reared to adulthood in an adult mosquito cage (30 × 30 × 30 cm) and segregated according to species and sex. The adult mosquitoes were fed with 10% sucrose solution. The culture was maintained at (28±2)°C, 70-85% relative humidity, with a photoperiod of 14-h light and 10-h dark. The $F_0$ generation female mosquitoes were used for the adult bioassay experiment.

## Insecticide susceptibility tests- Adult bioassay

Adult bioassays were carried out on 3-5 days old $F_0$ generation of female *Ae. aegypti* and *Ae. albopictus* mosquitoes separately according to WHO (2016) procedures for insecticide resistance monitoring. Two types of commonly used insecticide by the local health authorities for vector control in the study region: deltamethrin (Type II pyrethroid) and pirimiphos-methyl (organophosphate) were used in this study. The insecticide-impregnated papers followed the recommended insecticide dosages by WHO (2016) for *Anopheline* mosquitoes instead of *Aedes*, 0.05% for deltamethrin, and 0.25% for pirimiphos-methyl. The rationale for using *Anopheline* diagnostic dosage is because of a high level of resistance, especially in *Ae. aegypti* has been observed in Malaysia. Thus the current diagnostic dosage for *Aedes* might be too low for testing this species. Several other studies in Malaysia also used diagnostic dosages for *Anopheline* (Wan Norafikah et al., 2013; Ishak et al., 2015) when evaluating insecticide resistance in *Aedes* mosquitoes in Malaysia. The insecticide-impregnated papers were obtained from VCRU, USM, a WHO collaborating center. The bioassays were carried out with a minimum of four replicates with two controls, each by using approximately 25 active female mosquitoes with a total of 150 individuals, conducted simultaneously under the same conditions. The negative control experiment was conducted by using paper impregnated with carrier oil only; olive oil for deltamethrin control and silicone oil for organophosphate control.

For the bioassay, 25 female mosquitoes were aspirated out from a mosquito cage and transferred into green-doted holding tubes. The slide units were closed, and the holding tubes were set in an upright position for up to an hour. Towards the end of this time, any moribund or dead mosquitoes were removed and replaced with healthy mosquitoes. The mosquitoes were transferred from the holding tube (green-dotted tube) into a red-dotted exposure tube lined with insecticide-impregnated papers by slowly blowing by mouth to avoid injury to the mosquitoes. The separating slides were then detached from the holding tube. For the control set, yellow-dotted tubes were prepared in the same way. The exposure tube was left upright with mesh-screen end uppermost for a period of an hour. At the end of 1-h exposure, the number of knocked-down mosquitoes was recorded. At the end of the exposure period, the mosquitoes were transferred back into the holding tube and provided

with a 10% sucrose solution soaked in cotton wool placed in the mesh-screen end. The mosquitoes were maintained in the holding tube for 24 h provided that the tubes are kept in a shady, sheltered place with a temperature of 27± 2ºC and 75% ± 10% relative humidity. The mortality data were taken after 24 h, and the mosquitoes were classified as alive and dead. Mosquitoes were considered dead if they were motionless, even when they were mechanically stimulated (Chen et al., 2016). This is reasonable because motionless mosquitoes after bioassay exposure rarely recovered and survived. The survived and dead mosquitoes from the bioassay were stored in microcentrifuge tubes individually to avoid DNA cross-contamination and kept in -20ºC for subsequent molecular works.

## Genomic DNA extraction

DNA extraction on the whole body of individual mosquitoes was done by using a standard Geneaid GENEzol$^{tm}$ reagent protocol with slight modifications on the volume of the reagents used to extract the DNA from an individual mosquito. In brief, the whole body of the mosquito was placed in a microcentrifuge tube and homogenized with 100 µL of GENEzol$^{tm}$ reagent. The homogenate was incubated for 5 min at room temperature followed by adding 20 µL of chloroform to the sample. The tube was then centrifuged at 16,000 x g for 15 min at 4°C to separate the phases. For DNA precipitation, the upper aqueous phase was transferred to a new microcentrifuge tube followed by the addition of 30 µL of absolute ethanol. The sample was incubated for 5 min at room temperature before centrifuging at 2000 x g for 5 min at 4°C and the supernatant was discarded. In the DNA wash step, 100 µL of sodium citrate in ethanol solution was added to the sample. The sample was incubated at room temperature for 30 min. The tube was inverted occasionally during the incubation. This step was then followed by centrifuging the tube at 2000 x g for 5 min at 4°C and the supernatant was removed. The wash step was repeated once. About 150 µL of 70% ethanol was added to the sample and incubated for 15 min at room temperature. The tube was inverted occasionally during the incubation period. The sample was then centrifuged at 2000 x g for 5 min at 4°C and the supernatant was removed. The DNA pellet was air-dried for 10 min at room temperature. The DNA pellet was re-suspended by adding 30 µL of Tris-EDTA (TE) buffer. The DNA sample was incubated at 60°C for 15 min to dissolve the DNA pellet. The bottom of the tube was tapped occasionally every three minutes to promote DNA rehydration. The sample was then centrifuged at 16,000 x g for 10 min to remove insoluble particles. The supernatant containing the DNA was transferred to a new microcentrifuge tube and stored at -20°C or immediately proceeded with PCR.

## PCR amplification of VGSC gene

The details on the primer sets and the relative species, sequence and product size to detect five types of *kdr* mutations; S989P, I1011M/V, L1014F, V1016G/I and F1534C are listed in Table 1 which adapted from Kasai et al. (2011) and Kawada et al. (2014). Since all the mosquitoes from the bioassay test were susceptible to pirimiphos-methyl, the amplification of the *ace-1* gene associated with resistance to organophosphates was not conducted in this study. Knockdown resistance genotyping was performed on both live and dead mosquitoes from the WHO bioassay, with a maximum of three mosquitoes chosen for sequencing. Each PCR reaction was carried out in a 25 µL reaction volume, containing 20.475

µL sterilized distilled water, 1.3 µL 10X PCR Buffer, 1.2 µL $MgCl_2$, 0.5 µL dNTP, 0.2 µL each of forward and reverse primer (100 pmol), 0.125 Taq Polymerase (Lucigen) and 1 µL of genomic DNA. The PCR conditions consisted of a denaturation step at 95ºC for 5 min, followed by 35 cycles of 94ºC for the 30s, 57ºC for 30s, 72ºC for 1 min, and a final elongation step at 72ºC for 10 min. The amplified PCR products were size separated by gel electrophoresis on a 0.8% agarose gel in TAE buffer at 60V for 30 minutes. Bands were visualized by nucleic acid (GelRed) stain to confirm the product band size.

**Table 1.** List of primers used to amplify the domain II and domain III of segment six of the voltage-gated sodium channel gene in *Ae. aegpti* and *Ae. Albopictus*.

| | Species | Primer | Sequence (5'-3') | Region | Product size (Base pair, bp) |
|---|---|---|---|---|---|
| PCR | *Ae. aegypti* | AaSCF1<br>AaSCR4 | AGACAATGTGGATCGCTTCC<br>GGACGCAATCTGGCTTGTTA | S989P,<br>I1011M/V, | 480 |
| | *Ae. albopictus* | aegSCF20<br>aegSCR21 | GACAATGTGGATCGCTTCCC<br>GCAATCTGGCTTGTTAACTTG | L1014F,<br>V1016G/I | 380 |
| | *Ae. aegypti, Ae. albopictus* | AaSCF7<br>AaSCR7 | GAGAACTCGCCGATGAACTT<br>GACGACGAAATCGAACAGGT | F1534C | 740 |
| Sequencing | *Ae. aegypti* | AaSCF3<br>AaSCR6 | GTGGAACTTCACCGACTTCA<br>CGACTTGATCCAGTTGGAGA | S989P,<br>I1011M/V, | |
| | *Ae. albopictus* | AaSCF3<br>aegSCR22 | GTGGAACTTCACCGACTTCA<br>TTCACGAACTTGAGCGCGTTG | L1014F,<br>V1016G/I | |
| | *Ae. aegypti,*<br>*Ae. albopictus* | AaSCR8 | GAGAACTCGCCGATGAACTT<br>TAGCTTTCAGCGGCTTCTTC | F1534C | |

## DNA sequencing

The PCR products with bright and clear bands were purified and sequenced commercially (MyTACG Bioscience Enterprise, Taiwan) for further analysis. Single sequence data was checked and aligned using the Mega software version 7.0 (http://www.megasoftware.net/). Samples with both forward and reverse sequences were assembled, and the respective consensus sequences were generated in Bioedit Sequence Alignment Editor version 7.0.5. (http://www.mbio.ncsu.edu//BioEdit/bioedit.html). The sequences were aligned using ClustaW alignment program in Mega. The sequences were deposited in GenBank to obtain the accession numbers. Finally, for each sequenced mosquito sample, the genotypes were compared against the phenotype which previously obtained from the WHO bioassay.

## Data analysis

The susceptibility status of the mosquito samples from the adult bioassay test was interpreted according to the latest WHO (2016) recommendations: (1) susceptible (mortality >98%), (2) suspected resistance (mortality 90-97%) which requires additional molecular tests to identify the underlying resistance mechanisms and (3) confirmed resistance to the insecticide tested (mortality <90%).

The number of knocked-down mosquitoes was recorded in percentage. The percentage mortality of the test samples was calculated using the following formula:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100 \qquad \text{(Eq. 1)}$$

Abbott's formula (Abbot, 1925) will be used to re-calculate percentage mortality if the control population shows mortality between 5% and 20%. However, Abbott's formula was not used throughout this study because all of the control mortality was below 5%.

An independent *T-test* was used to compare the individual effects of area (flooded and unflooded area), species (*Ae. aegypti* and *Ae. albopictus*), and insecticides (deltamethrin and pirimiphos-methyl) on the susceptibility level in SPSS version 22. The allele frequencies for *kdr* mutations were calculated for each mosquito strains. The association between resistance phenotypes (WHO bioassay) and the genotypes (*kdr* frequencies) of the resistance mutation for each location was assessed by estimating the odds ratio (OR) and the statistical significance was calculated using Fisher's exact probability test (for sample sizes less than 5) in Vassar Stats statistical computation Web site (vassarstats.net/odds2x2.html), as shown below. The significance level was set at 5%.

$$\text{Odds of alive or dead mosquitoes} = \frac{No.of\ mutant\ \ mosquitoes}{No.of\ non-mutant\ \ mosquitoes} \quad \text{(Eq. 2)}$$

$$\text{Odds Ratio (OR)} = \frac{Odds\ of\ alive\ mosquitoes}{Odds\ of\ dead\ mosquitoes} \quad \text{(Eq. 3)}$$

# RESULTS

## Knockdown effects

The mean number of knocked-down mosquitoes after 1-hour of exposure to the insecticides are reported in Figure 1. Deltamethrin showed almost a 100% knockdown effect on *Ae. albopictus*, compared to *Ae. aegypti* which only showed 29% and 33% in the flooded and unflooded area, respectively. The number of knocked-down mosquitoes by pirimiphos-methyl was relatively low compared to deltamethrin in both mosquito species tested. Insignificant differences were noticed between the flooded and unflooded area when comparing the number of knocked-down mosquitoes by respective species and insecticide used. (Independent *T-test*, P > 0.05).

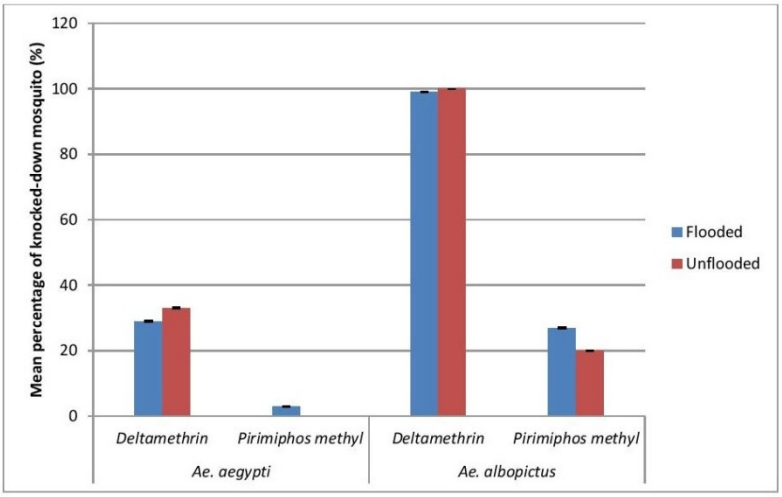


**Figure 1.** Mean percentage of knocked-down mosquitoes from the flooded and unflooded areas after 1 h of exposure to insecticides.

## Adult susceptibility

WHO bioassays indicated that all the *Ae. aegypti* population from both flooded and unflooded areas found to be resistant to deltamethrin, with mortality of 74 and 17%, respectively (Figure 2). However, *Ae. aegypti* from the flooded and unflooded areas were fully susceptible to pirimiphos-methyl with mortality of 98% and 100%. Independent *T-test* showed that the *Ae. aegypti* population from the flooded area had significantly higher mortality than from the unflooded area when tested with deltamethrin ($P < 0.001$), indicating a higher number of resistant mosquitoes to deltamethrin in the latter.

On the other hand, *Ae. albopictus* from the flooded area was found to be susceptible to deltamethrin with 100% mortality, meanwhile in the unflooded area, it showed resistance with 83% mortality (Figure 2). The Independent *T-test* showed significant differences in *Ae. albopictus* mortalities between these two areas ($P < 0.05$). All *Ae. albopictus* population from both areas was susceptible to pirimiphos-methyl with mortality of >98% (Figure 2). The VCRU laboratory susceptible strains as control strain showed 100% mortality against all insecticides tested. All the mosquitoes in the negative control were alive after 24 h in response to bioassay. This indicates no other variables were involved in inducing the mortality in the test mosquitoes other than the insecticides that were being tested (WHO, 2016).

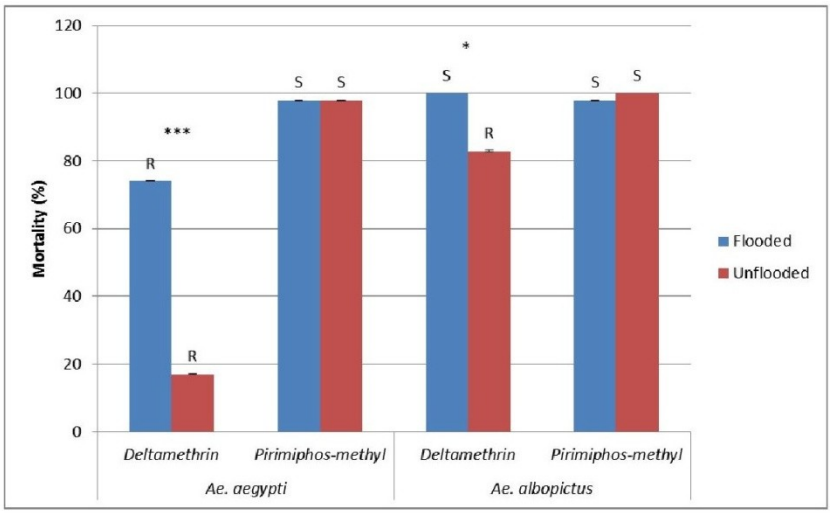


**Figure 2.** Susceptibility status of *Ae. aegypti* and *Ae. albopictus* after 24 h in response to adult bioassay test upon exposure to deltamethrin and pirimiphos-methyl.Significant values denoted by * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). (S: Susceptible, SR: Suspected resistance, R: Resistant)

## Amplification of domain II and domain III genes

The PCR successfully amplified the domain II and domain III genes. The genes amplified for domain III produced a PCR product of approximately 740 bp in both *Ae. aegypti* and *Ae. albopictus*. Whereas, the genes amplified for domain II were different for *Ae. aegypti* and *Ae. albopictus,* with approximately 640 and 480 bp, respectively (Figure 3).

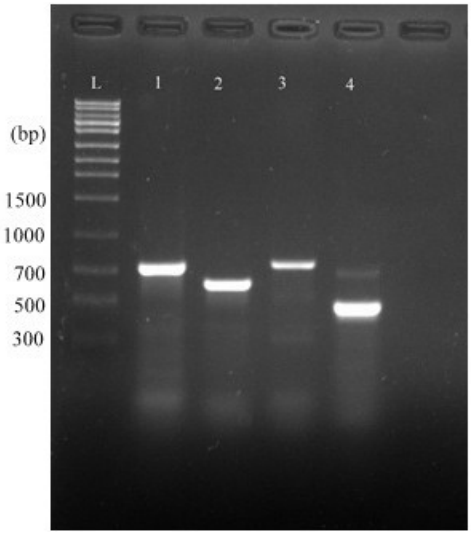


**Figure 3.** Gel electrophoresis picture showing bright bands after *kdr* gene amplification. Lane 1: Domain III, *Ae. aegypti*, Lane 2: Domain II, *Ae. aegypti*, Lane 3: Domain III, *Ae. albopictus*, Lane 4: Domain II, *Ae. albopictus*. L: Ladder 1kb

## Detection of *kdr* mutations in VGSC gene

DNA sequencing from the field-caught $F_0$ mosquitoes from the flooded and unflooded area revealed detection of F1534C mutation: TTC (non-mutant) to TGC (mutant) in the segment 6 of the VGSC gene (Figure 4). Sequencing was not performed for alive *Ae. albopictus* from the flooded area and alive *Ae. aegypti* from the unflooded area since all the mosquitoes were susceptible (100% mortality) to deltamethrin and pirimiphos-methyl, respectively. The substitution of phenylalanine to cysteine (GenBank Accession Nos F1534: MK387305, MK387306) was detected at high frequency in *Ae. aegypti* population from the flooded and unflooded area. All the F1534C mutations in *Ae. aegypti* showed heterozygous resistance T/G genotype (Figure 5). However, mutations spanning the S989, I1011, L1014, and V1016 positions were not detected in this study. A total of 24 *Ae. aegypti* were genotyped from both locations, and 19 of the mosquitoes exhibited F1534C mutation (Table S1). Neither mutations in domain II nor domain III were detected from *Ae. albopictus* population from both study area, despite resistance to deltamethrin in the WHO bioassay (83%) in the mosquitoes from the unflooded area.

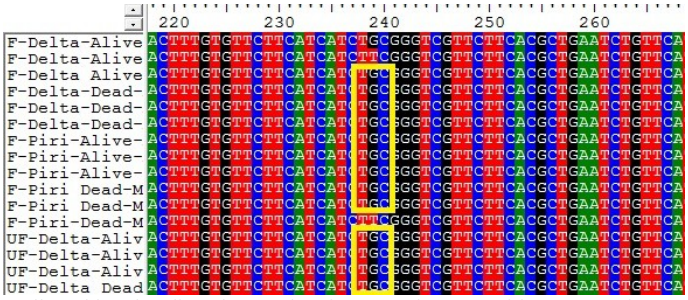


**Figure 4.** Sequences aligned in BioEdit software showing mutation at position 1534. The nucleotide sequence at 239 is showing the mutation (TTC to TGC).

 

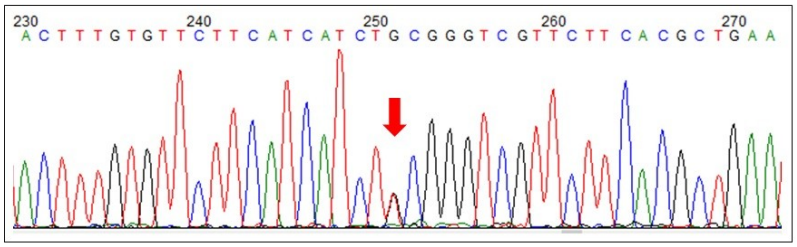


**Figure 5.** Chromatograph of a sequence showing mutation at position 1534 in *Ae. aegypti.* The peak of the mutation marked by the red arrow.

## Distribution of *kdr* allele frequency in *Ae. aegypti* population

The distribution of F1534C mutation in *Ae. aegypti* and *Ae. albopictus* in the flooded and unflooded area are displayed in Figure 6. The distribution of F1534C mutation in *Aedes aegypti* did not vary significantly between the flooded and unflooded areas, with 83% and 75% of mutation from the whole tested population, respectively (Figure 6) ($P > 0.05$). The distribution of F1534C mutation in *Ae. albopictus* from both areas was absent, hence, the population was classified as 100% susceptible (Figure 6).

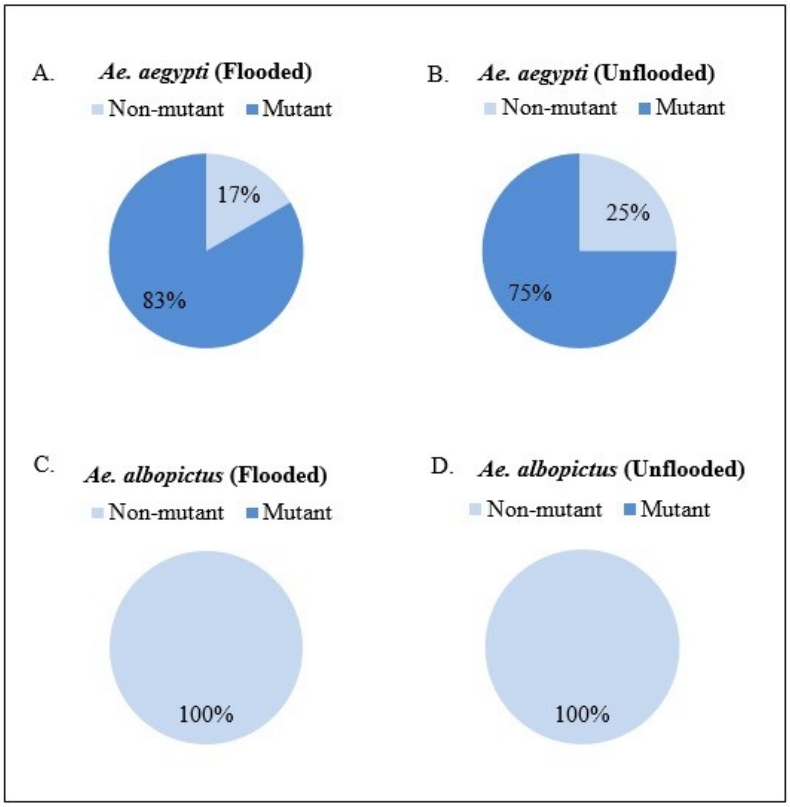


**Figure 6.** Distribution of F1534C mutation in *Ae. aegypti* and *Ae. albopictus* from the flooded and unflooded area.

## F1534C genotype-phenotype correlation

The phenotypes of thealive and dead mosquitoes were correlated with F1534C mutation (Table 2). The odds ratio were calculated. However, the presence of F1534C allele (genotype) was not significantly associated with the live and dead mosquitoes (phenotypes), indicated by low OR values and P > 0.05. The odds ratio was not calculated for *Ae. albopictus* due to the absence of *kdr* mutation. But given the small number of mosquitoes genotyped for *kdr* mutations, this analysis has a limitation.

**Table 2.** Association of F1534C allele counts of the VGSC genes within field-collected mosquitoes with insecticide resistance phenotypes.

| Area | Species | Insecticide | Phenotype | N | Haplotype F1534 (TTC) | C1534 (TGC) | OR | P-value |
|---|---|---|---|---|---|---|---|---|
| Flooded | *Ae. aegypti* | Deltamethrin | Alive (R) | 3 | 4 | 2 | 0.50 | 0.50 |
| | | | Dead (S) | 3 | 3 | 3 | | |
| | | Pirimiphos-methyl | Alive (R) | 3 | 4 | 2 | 0.50 | 0.50 |
| | | | Dead (S) | 3 | 3 | 3 | | |
| | *Ae. albopictus* | Deltamethrin | Alive (R) | 1 | 2 | 0 | - | - |
| | | | Dead (S) | 3 | 6 | 0 | | |
| | | Pirimiphos-methyl | Alive (R) | 3 | 6 | 0 | - | - |
| | | | Dead (S) | 3 | 6 | 0 | | |
| Unflooded | *Ae. aegypti* | Deltamethrin | Alive (R) | 3 | 3 | 3 | 2.00 | 0.50 |
| | | | Dead (S) | 3 | 4 | 2 | | |
| | | Pirimiphos-methyl | Alive (R) | 3 | 4 | 2 | 0.50 | 0.55 |
| | | | Dead (S) | 3 | 4 | 2 | | |
| | *Ae. albopictus* | Deltamethrin | Alive (R) | 3 | 6 | 0 | - | - |
| | | | Dead (S) | 3 | 6 | 0 | | |
| | | Pirimiphos-methyl | Alive (R) | 1 | 2 | 0 | - | - |
| | | | Dead (S) | 3 | 6 | 0 | | |

OR: Odds ratio, P < 0.05, R: Resistant, S: Susceptible, N: Number of mosquitoes.

## DISCUSSION

In this study, the adult bioassays showed a high level of pyrethroid resistance to the 0.05% deltamethrin WHO diagnostic dose in the *Ae. aegypti* population from the flooded and unflooded areas in Kelantan as shown in Figure 2. Prolonged usage of pyrethroid could be one of the reasons since both areas were dengue-prone areas for many years with frequent spraying by the local health authorities. Apart from the aforementioned factor, the resistance to pyrethroids is also attributable to variations in the species habitat. *Aedes aegypti* lives close to human habitats, increasing their risk to be exposed to aerosol spray from household insecticides and also fogging by the Ministry of Health, leading to higher risk for developing resistance to insecticides. On the other hand, *Ae. albopictus* has a wide variety of breeding habitats ranging from man-made to natural containers; thus, the risk of developing resistance is low in *Ae. albopictus* compared to *Ae. aegypti*. However, *Ae. albopictus* from the unflooded area was resistant to deltamethrin in this study. This shows the possibilities of invasion of *Ae. albopictus* to *Ae. aegypti* breeding sites thus increasing their chances to be exposed to the insecticides.

*Aedes aegypti* from the unflooded area showed a relatively higher resistance level compared to those from the flooded area. In the flooded area, resistance was observed with

 

only 74% mortality in the bio-assayed mosquitoes. Flooding has washed away the existing breeding grounds of mosquitoes, thus causing a temporary reduction in the mosquito population. A drastic reduction in the mosquito population might have led to a selection effect whereby mosquitoes with strong survival features to withstand insecticides are selected to survive under selection pressure. However, there is not enough scientific evidence to support the effect of flooding and emergency vector control on the selection effect and the emergence of insecticide resistance, especially in this study. There are a few related pieces of literature available to support flooding and vector control, for example, flooding in Iowa State in 1993 increased the risk for arboviral disease transmission and at the same time, the emergence of insecticide resistance came in the limelight due to emergency vector control activities (CDC, 1993). Similarly, a post-tsunami disease surveillance system revealed increased dengue cases and high resistance to DDT and permethrin in *Ae. aegypti* population in Thailand in 2003 (Paeporn et al., 2005).

In this study, *Ae. aegypti* was highly resistant to deltamethrin, even though higher *Anopheles* diagnostic doses were used. A study by Hamid et al. (2017) in Bali, Indonesia also used anopheline mosquito's diagnostic doses in evaluating *Ae. aegypti* susceptibility to 0.75% permethrin (pyrethroid). Another related work by Iwani (2019) also reported resistance in *Ae. aegypti,* when tested with a three-fold higher dosage of permethrin 0.75% compared to WHO recommended discriminating dose for *Aedes*, permethrin 0.25%. Thus suggesting that the current recommended discriminating diagnostic doses by WHO for *Aedes* mosquitoes could be too low for *Aedes* mosquitoes in Malaysia and need to be re-evaluated for pyrethroid insecticides.

In general, the *Aedes* mosquitoes in this study are still susceptible to organophosphates and resistant to pyrethroids. A previous study in Malaysia also reported a similar finding with resistance to pyrethroids and susceptibility to organophosphates in *Ae. aegypti* larvae (Leong et al., 2018). Evidence on insecticide resistance among *Ae. aegypti* in Malaysia was first reported in 2001, whereby the field strains from Kuala Lumpur were highly resistant to permethrin (Rohani et al., 2001). In another study by Ishak et al. (2015), pyrethroids resistance in *Ae. aegypti* populations were reported in Kota Bharu, Kelantan with 10% and 82% mortality to permethrin (0.75%) and deltamethrin (0.05%), respectively. In this study, the *Ae. aegypti* and *Ae. albopictus* from both study areas were susceptible to pirimiphos-methyl, the organophosphates, showing that this insecticide is one of the suitable insecticides to replace the highly resistant pyrethroids or to be used in insecticide rotation in the vector control program.

Parallel to our study, Hasan et al., 2015 reported full susceptibility of *Aedes* mosquitoes to 0.25% pirimiphos-methyl in Penang. Susceptibility to other organophosphates such as temephos, and malathion also been reported in Malaysia (Rong et al., 2012; Ishak et al., 2017). In Malaysia, temephos is extensively used routine larvicide, and malathion is an adulticide used in fogging activity (Ong, 2016). However, in most cases, resistance to organophosphates, especially temephos and malathion is widespread in the *Aedes* population across Malaysia (Shafie et al., 2012; Elia-Amira et al., 2018). Since there has been evidence on resistance to organophosphates in other countries as well, such as Brazil (Lima et al., 2003), Mexico (Deming et al., 2016) and Thailand (Jirakanjarankit et al., 2007), continuous resistance evaluation and monitoring are still important in vector management.

The mosquitoes used in the bioassay were adult mosquitoes reared from field-collected eggs and larvae using ovitraps. This method allowed us to obtain non-bloodfed and age-standardized samples (3-5 days old). A large number of ovitraps were placed in each study site to ensure broad representation sample of the mosquito population. Also, the ovitraps were placed in different locations, and the samples were pooled to avoid sibling population, which would yield a similar level of resistance during the bioassay. These methods allowed in minimizing the confounding effects on the age and physiological factors on the knockdown and the resistance status. The results showed *Ae. albopictus* exposed to pyrethroid knocked down rapidly within an hour of the exposure followed by high mortality after 24-h, indicating its high susceptibility to deltamethrin compared to of *Ae. aegypti*. This enabled the suggestion that highly susceptible mosquitoes have a high knockdown effect and vice versa. However, this is only applicable to pyrethroid insecticides, also well known as 'knockdown' agent that quickly paralyzes insects. Both species tested in this study were susceptible to pirimiphos-methyl despite a low number of knockdown readings because organophosphates do not show rapid knockdown mechanisms, unlike pyrethroids.

The genotyping of the *kdr* mutations in domain II (S989, I1011, L1014, and V1016) and domain III (F1534C) in the segment 6 of the VGSC gene successfully detected only the presence of F1534C mutation in *Ae. aegypti*. The distribution of F1534C mutant genes in *Ae. aegypti* did not vary much between the flooded and unflooded areas, although there was a significant phenotypic difference between the two areas. This was further supported by the absence of the genotype-phenotype correlation in this study. Several *kdr* mutations in the VGSC genes of pyrethroid-resistant *Ae. aegypti* mosquitoes have been detected worldwide, for example in Thailand (Rajatileka et al., 2008; Yanola et al., 2011), China (Li et al., 2015), Singapore (Kasai et al, 2011), Mexico (Deming et al., 2016), India (Kushwah et al., 2015) and the most common of these such as 1011, 1016, 1534 have been found to associate with the resistant phenotype (Du et al., 2016). The absence of a significant correlation between the resistant phenotype and mutation genotype in this study suggests the possibility of the existence of other resistance mechanisms, such as metabolic pathways related to the overproduction of P450 detoxifying enzymes (Strode et al., 2008). Further investigation of these additional mechanisms may contribute to a better explanation of the observed resistance patterns. Besides, the absence of the genotype-phenotype correlation might have due to the low number of mosquito samples sequenced for detection of the *kdr* mutation. Increasing the sample size for sequencing might have better revealed the genotype-phenotype correlation.

Also, there seems to be an absence of mutations in domain II of the VGSC gene. Of note, the first report on *kdr* mutations was by Ishak et al. (2015) in which F1534C and V1016G mutations were detected in *Ae. aegypti* populations across Malaysia. The F1534C mutation was detected at a high frequency and was found to be closely associated with pyrethroid resistance, whereas the V1016G mutation was detected at a lower frequency and did not associate with resistance in *Ae. aegypti* mosquitoes. Therefore, the point mutations in domain II might be minor in Malaysia, but not negligible as compared to the domain III at F1534C.

None of the *kdr* mutations were detected in *Ae. albopictus* despite bright bands in the PCR amplifications. The presence of pyrethroid resistance and absence of *kdr* mutation in *Ae. albopictus* has also been reported in Malaysia (Ishak et al., 2015) and India (Kushwah

et al., 2015). However, few studies conducted in China had found *kdr* mutation associated with pyrethroid resistance in *Ae. albopictus* (Chen et al., 2016; Gao et al., 2018). Similarly, another study conducted in Singapore reported F1534C mutation in *Ae. albopictus* which was suspected to confer *kdr* resistance in *Ae. albopictus* (Kasai et al., 2011). In Florida, USA, a F1534L (phenylalanine to leucine) mutation was found in *Ae. albopictus* (Marcombe et al., 2014). In *Ae. aegypti*, the resistance to pyrethroid could be due to a *kdr* mutation in the VGSC or metabolic resistance (P450 monooxygenases) (Smith et al., 2016). Thus, there could be chances for the involvement of metabolic pathway conferring resistance in *Ae. albopictus* population, since no *kdr* mutation was detected (Auteri et al., 2018).

## CONCLUSIONS

Pyrethroid resistance was found in this study on *Ae. aegypti* mosquitoes in both the flooded and unflooded areas, which are dengue outbreak hubs. High pyrethroid resistance was detected in *Ae. aegypti* from Kelantan, underscoring the need for re-evaluation of vector control management in these areas. *Aedes aegypti* mosquitoes in both flooded and unflooded areas developed the *kdr* mutation at F1534C allele of the VGSC genes for resistance to the pyrethroid. The overall results provide baseline data for systematic planning and insecticide selection before the commencement of controlling activities, in the light of a flooding disaster. Pirimiphos-methyl is suggested to be used as a substitute or on a rotational basis in vector control programs in this area to minimize over-dependence on pyrethroids and eventually to reduce insecticide resistance problems. Therefore, regular insecticidal resistance surveillance and monitoring of *Aedes* mosquitoes following flooding will aid in setting better goals and allow proper evaluation of mosquito control programs during the high endemic period after a natural disaster.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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