DDT-Pyrethroid Resistance Screening in Association with the *kdr* Allele F1534C in *Aedes albopictus* (Skuse) (Diptera: Culicidae)

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ABSTRACT The emergence of insecticide resistance in *Aedes* against pyrethroid group has become a threat to the vector control program. This study investigates the resistance status and the presence of F1534C *kdr* mutations in *Aedes albopictus* populations of Kota Bharu and Kubang Kerian, Kelantan, Malaysia. The F1 adults of *Ae. albopictus* were assayed using World Health Organization (WHO) susceptibility test with 4% dichloro-diphenyl-trichloroethane (DDT), 0.05% lambda-cyhalothrin, and 0.75% permethrin. For susceptibility analysis, the mortality percentage, 50% cumulative knockdown time (KT50), and resistance ratio (RR) values were calculated. All the mosquito survivors were collected and subjected to the allele-specific polymerase chain reaction (AS-PCR) analysis on the presence of knockdown resistance (*kdr*) mutation F1534C. Results show that *Ae. albopictus* from Kota Bharu was possible resistance to DDT and pyrethroids, while *Ae. albopictus* in Kubang Kerian showed mixed resistant populations which are possible resistance and susceptibility to DDT and pyrethroids, respectively. The *kdr* alleles F/C1534 were detected in both *Ae. albopictus* populations with higher heterozygote resistant alleles (F/C) in Kota Bharu.

KEYWORDS: *Ae. albopictus*, *kdr*, F1534C, DDT, pyrethroids
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Original Article

INTRODUCTION

It has been known that a female *Aedes aegypti* mosquito is the primary vector of dengue fever, while *Aedes albopictus* (Skuse) which has long been considered the secondary vector (McKenzie *et al.,* 2019). In adult mosquito control, various insecticides have been used since 1950 to control the *Aedes* sp. population (Baldacchino *et al.,* 2015). For instance, pyrethroid insecticides have contributed to the massive success of the *Ae. aegypti* control (Bisset *et al.,* 2013). The pyrethroids act by disrupting the insect nervous system, specifically on the voltage-gated sodium channels (VGSC), which trigger to weaken and eventually cause death to the insects (Baldacchino *et al.,* 2015). However, prolonged use of pyrethroids could be the prime factor for the resistance development in *Aedes* sp. (Rocha *et al.,* 2015) and there is evidence that it has compromised the success of control interventions. Resistance could be due to the knockdown resistance *(kdr)* mutation (Sayono *et al.,* 2016) that is associated with the VGSC.

This present study evaluated the presence of *kdr* F1534C alleles by which play a role in the knockdown resistance of *Aedes* sp. against type I pyrethroids (Kushwah *et al.,* 2020) in *Ae. albopictus*. This study, is related with a previous research by Abu Bakar *et al.* (2021) reported on the primary vector, *Ae. aegypti* resistance status against pyrethroid. While *Ae. albopictus* is much more widespread (Kraemer *et al.,* 2019), its presence in the absence of *Ae. aegypti* has raised a concern about the potential vector (Gratz, 2004). In the line of the continuing spread of the speciesand the increase of dengue cases, it is essential to provide the current resistance status of *Ae. albopictus* and therefore the findings can be used to estimate the resistance development, and manage the effective usage of

insecticide in the outbreak area. Thus, the objective of this study was to evaluate the general association of phenotype and genotype resistance of DDT-pyrethroids against *Ae. albopictus* in the affected population.

METHODOLOGY

Mosquitoes

Aedes sp. eggs were collected from two different locations using ovitrap as described by Lee (1992). The locations were situated in Kota Bahru district of Kelantan, Malaysia (i.e., Panji, Kota Bahru (KB): 6° 8' 40.08"N, 102°16' 18.62"E and Universiti Sains Malaysia, Kubang Kerian (KK): 6° 5' 54.64"N, 102° 17' 5.47"E), which were 7.6 km apart. Panji sampling area was situated 5.8 km from the capital city of Kelantan, Kota Bharu. The area was also identified as a dengue hotspot from 2016 – 2018. Frequent fogging activities and larval surveys were conducted actively by the Health Districts Department throughout the three consecutive years. On the other hand, USM is in a gated compound area situated in the Kubang Kerian. The campus area consists of the student's hostels, teaching buildings, offices, cafes, sports complexes, and animal houses.

A soft board paddle (13 cm x 50 mm x 0.2 mm) serves as a medium of the ovitrap for *Aedes* sp. to lay their eggs. On the fifth day, paddles were collected and brought back to the insectarium for the maintenance process. All collected paddles were submerged in the dechlorinated tap water for eggs to hatch. They were given the food every two days until they were successfully developed into pupae and emerged into F1 adult's mosquito. All the female adults of *Ae. albopictus* were identified, separated, and collected for the bioassay testing. The process was repeated throughout the study period. The laboratory mosquitoes used in this study were obtained from the Vector Control Research Unit (VCRU), Universiti Sains Malaysia, Pulau Pinang. The laboratory strain is an established colony of mosquitoes that is maintained continuously under controlled conditions and never exposed to any insecticide for many generations.

Insecticides

Diagnostic dosages of WHO impregnated papers were used in the susceptibility test against adult mosquitoes. The impregnated papers were purchased from the VCRU, Universiti Sains Malaysia, Pulau Pinang. The insecticides were organochlorine (4% DDT), pyrethroid I (0.75% permethrin) and pyrethroid II (0.05% lambda-cyhalothrin) (WHO, 2016).

Bioassay

The susceptibility tests for adults were conducted on filial generation 1 (F1). All adults were supplied with a 10% sucrose solution. Alive mosquitoes from the bioassay susceptibility studies were preserved in the freezer (-80°C) for further studies on the PCR confirmation analysis. The assay conducted followed the WHO (2016) susceptibility testing guideline against *Aedes* mosquitoes. The 4-5 days old sugar-fed adult female mosquitoes were used. Batches of 20 adult mosquitoes were exposed to insecticide-impregnated papers in the test tubes for 60 minutes. All tests were conducted at 26°C ± 2°C. The results were recorded every five minutes for any knockdown observed within one hour of exposure. The mortality was recorded for 24 hours. The bioassays of field mosquitoes were conducted in five (5) replicates per insecticide/per location. The laboratory mosquitoes were used for control and underwent the similar procedure of bioassay testing.

Screening of the kdr F1534C detection

This study was a randomized screening detection of the presence of *kdr* F1534C in the *Ae. albopictus* selected population. The detection of the *kdr* F1534C was conducted by using specimens from preserved alive mosquitoes (stock in -80°C) of the bioassay testing. The reaction was conducted in pools of ten mosquitoes. This was due to various limitations and technical issues that occurred during this phase.

DNA isolation and amplification

Ten mosquitoes per pool from preserved stock were used for each PCR reaction. The DNA of *Ae. albopictus* was isolated by using a commercial DNA extraction kit, Macherey-Nagel NucleoSpin®. The mosquito samples were prepared by removing the wings and legs before grinding their thorax and bodies into small pieces and stored at 4°C. The PCR reaction primers of F1534C, as shown in Table 1 was used to amplify the partial sequence following a standard PCR protocol. The reaction was carried out in a final volume of 25 µl, comprising 10 µl of 2X MyTaq™ Mix, 0.2 µl of 10 µM primer forward (C1534-f), 0.2µl of 10 µM primer reverse (C1534-r), 0.2 µl of 10 µM MyTaq™ primer forward (Ae1534F-r), 0.2 µl of 10 µM primer reverse (Ae1534C-f), 9.62 µl PCR water and 200 ng of 1.5 µl DNA template. The reaction conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of each of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72 °C for 30 seconds, followed by a final extension at 72°C for 2 minutes. Before reaction, a quantification of the extracted genomic DNA was performed by using a biophotometer (Eppendorf, Germany) through the solution at 260 nm. The PCR products were then analyzed in 1.5% agarose gel on gel electrophoresis.

Gel electrophoresis

Gel electrophoresis was used as a quantification and qualification analysis to determine the length and quality of genomic DNA products obtained in DNA extraction and PCR amplification reactions. The amplified products were analyzed with a low molecular weight on 1.5% agarose gel. DNA ladder (DM1100 ExcelBand™ 50 bp DNA Ladder) was used to estimate the band size. 2 µL loading dye was mixed with 8 µL PCR product and the gel was then submerged in 0.5 X TBE buffer and was run for 60 minutes at 90 V for genomic DNA and 60 min at 100 V for PCR products and visualized in a UV transilluminator.

Table 1. Primer used in this study (Saingamsook *et al.*, 2017)

Primer	Primer sequence (5'-3')	Product size bp	Exon
C1534-f	GCGTACCTGTGTCTGTTCCA	368	23
C1534-r	GGCTTCTTCGAGCCCATCTT		
Ae1534F-r	GCGTGAAGAACGACCCGA	232	24

Data analysis

The classification of susceptibility criteria and degree of resistance (resistance ratio, RR) was employed following the WHO (2016) guidelines. Mortality rates were used to classify the susceptibility status of the mosquito's population whereas RR was used to evaluate the development of insecticide resistance among the field mosquito's population. Based on the susceptibility criteria, the mosquitoes were considered susceptible (S) if the corrected mortality >98%, resistant (R) if the mortality rate <90% and possible resistance (PR) if the mortality rate was between 90-97% of the resistance genes was suspected in the tested population and required an additional test for confirmation. When the mortality percentage of the control mosquito was between 5% and 20%, the formula used as (Abbott, 1925):

$$\text{Mortality rate} = \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{\text{control mortality}} \times 100\ \%$$

whereas the degree of resistance was calculated as

$$\text{Resistance Ratio, RR} = \frac{\text{KT50 (field mosquito)}}{\text{KT50 (laboratory mosquito)}}$$

When RR is <5, the field population is considered susceptible (S), when RR is between 5 and 10 mosquitoes are considered to have moderate resistance (MR), and when RR is >10 the mosquitoes are highly resistant (HR). Knockdown time (KT50) calculations were subjected to a Probit analysis using SPSS v24 software (Finney, 1972). In this study, PCR analysis was conducted to confirm the susceptible or resistance genotyping of *Ae. albopictus*.

RESULT AND DISCUSSION

As shown in Table 2, this study indicates that the effective insecticides against laboratory adult *Ae. albopictus* mosquitoes were lambda-cyhalothrin with KT50 of 5.73 minutes and followed by permethrin with KT50 of 6.19 minutes. However, KT50 was not calculated for DDT as there was no knockdown observed during the 60 minutes of exposure. Nevertheless, the mortality rate was 100% for all insecticides were tested against the laboratory mosquitoes within 24 hours post-exposure. From the results obtained, field mosquitoes of *Ae. albopictus* from KB and KK showed variations in susceptibility levels among insecticides tested. Based on the WHO (2016) classification criteria, KB mosquitoes were resistant (R) against permethrin (87.79% ± 2.76) and, respectively, possible resistant (PR) against lambda-cyhalothrin (93.82 % ± 3.01) and DDT (96.91% ± 1.60). However, the calculated KT50 values were not in line with the mortality rates obtained in the insecticides tested. The highest mortality rates of DDT give the longest time of the KT50, 110.49 minutes while both lambda-cyhalothrin and permethrin gave lower KT50 of 28.25 minutes and 28.36 minutes, respectively. In comparison to the field mosquitoes of KK, *Ae. albopictus* was indicated to be susceptible (S) to lambda-cyhalothrin followed by possible resistance (PR) to permethrin and resistant (R) to DDT with the mortality rates of 98.97 ± 1.03%, 90.72 ± 3.01% and 82.83 ± 2.58%, respectively. The KT50 of the field mosquitoes showed an agreement to the mortality rates. Lambda-cyhalothrin gave the fastest knockdown effects of 20.60 minutes in the tested population followed by permethrin (35.51 minutes) and (DDT 88.46 minutes).

However, both *Ae. albopictus* populations gave a higher knockdown time when assayed against DDT. In general, the population of field mosquitoes *Ae. albopictus* in KB consisted of two types of classification, resistant (R) and possible resistance (PR). Whereas, field *Ae. albopictus* mosquitoes in KK were varied with combined populations of resistant (R), possible resistance (PR) and susceptible (S) populations. The variations of resistance or susceptibility among vector mosquito populations observed in this study have also been reported by other researchers. For instance, a study conducted by Rohani *et al.* (2001, 1998) in rural and urban areas of major towns in 12 states of Malaysia revealed that strain of *Ae. aegypti* and *Ae. albopictus* from Kuala Lumpur were highly resistant compared to the Kelantan strain. While another earlier study has found multiple resistance on *Ae. albopictus* from urban strain in Kuala Lumpur to both permethrin and DDT. Another study conducted by Ishak *et al.* (2015) on the *Ae. albopictus* resistance across some major towns in Malaysia reported that there was a mixed resistance pattern observed against DDT with high resistance levels recorded in Kuala Lumpur and Kota Bharu (6 and 14% mortality rates, respectively).

Table 2. Resistance status of field strain *Ae. albopictus* against insecticides

Strains/ Insecticides	KT50 (min)	95% Confidence Intervals	Mean Mortality (24h) (%) ± SE
Laboratory strain			
4% DDT	*	N/A	100.00±0.00 (S)
0.05%Lambda-cyhalothrin	5.73	5.44 - 6.03	100.00±0.00 (S)
0.75% Permethrin	6.19	5.82 - 6.55	100.00±0.00 (S)
KB strain			
4% DDT	110.49	84.28 - 210.68	96.91±1.60 (PR)
0.05% Lambda-cyhalothrin	28.25	25.08 - 30.83	93.82±3.01 (PR)
0.75% Permethrin	28.36	25.60 - 30.96	87.79±2.76 (R)
KK strain			
4% DDT	88.46	72.29 - 133.72	82.83±2.58 (R)
0.05% Lambda-cyhalothrin	20.60	19.06 - 22.04	98.97±1.03 (S)
0.75% Permethrin	35.51	32.64 - 38.56	90.72±3.01 (PR)

Notes: KB - Kota Bharu; R - resistant; MR - moderate resistant;
KK - USM Kubang Kerian; S - susceptible; PR - possible resistant;
*Not calculated due to no knockdown observed in 60 minutes exposure.

Nevertheless, our study also looks at the resistance ratio (RR) of tested insecticides in *Ae. albopictus* populations from KB and KK. The results showed that the RR values of *Ae. albopictus* population from KB was susceptible (S) (RR<5) against lambda-cyhalothrin and permethrin with the values of 4.93 and 4.58. Whereas the KK population gives RR values of 3.60 for lambda-cyhalothrin and 5.74 for permethrin indicating susceptible (S) and moderate resistant (MR), respectively. For DDT insecticide, RR values were not calculated in both *Ae. albopictus* tested populations because no knockdown was observed during the 60 minutes exposure period. This data is used to support the phenotype resistance/susceptible assay of the tested population as part of the mortality rate findings. As reported by Ranson *et al*. (2000), in their previous study, the common target site for DDT and pyrethroid is the VGSC, and have been linked to changes in sensitivity of the target in a range of certain insects. The resistance has occurred in the populations and is being passed to the current generations due to their sharing mechanism in the VGSC.

Based on our studies, susceptibility of DDT and pyrethroids in two *Ae. albopictus* populations varied according to their sampling locations. The susceptibility distribution condition is believed to have associated with their background environment, such as a history of exposure to insecticides as observed in *Ae. albopictus* of KB populations employing possible resistance (PR) and resistant (R). In contrast *Ae. albopictus* populations in KK showed mixed populations of susceptible (S), possible resistance (R) and resistant (R). This can be seen in the bioassay testing and the presence of *kdr* F1534C alleles by which the selection for these alleles likely began with earlier widespread usage of DDT. Thus, we performed an AS-PCR analysis to confirm the association of phenotype and genotype findings. Field specimens of *Ae. albopictus* from KB and KK were successfully amplified on the gel electrophoresis for the F1534C alleles by the AS-PCR (Figures 1 and 2).

Based on Figures 1 and 2, the respective bands obtained were not perfectly captured, with little distortion observed. However, the present results obtained agreed to a certain degree with the bioassay results. Both *Ae. albopictus* populations showed the bands of the F1534C *kdr* with some variations of the heterozygote and homozygote alleles. It is noted, that evaluating the association of phenotype susceptibility with the genotype *kdr* alleles was difficult because of the limited type of samples available and small sample sizes of the exact resistant genes in the tested field mosquito's population due to the constrained conditions as mentioned earlier.

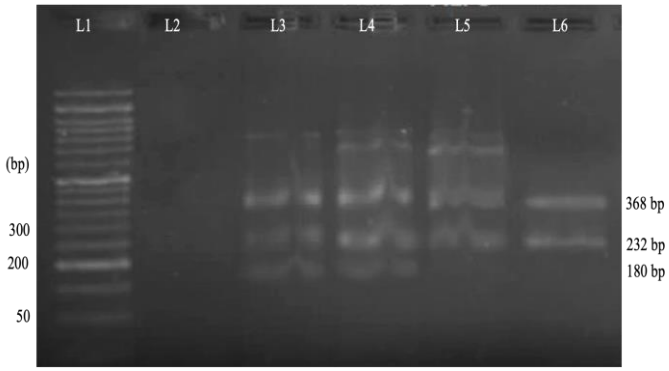

Figure 1. Gel photograph showing AS-PCR assay for genotyping of F1534C alleles in *Ae. albopictus* in KB. Lane 1:50bp DNA ladder, Lane 2: negative control, Lanes 3-4:heterozygote (F/C), Lane 5:homozygote (F/F), Lane 6:positive control

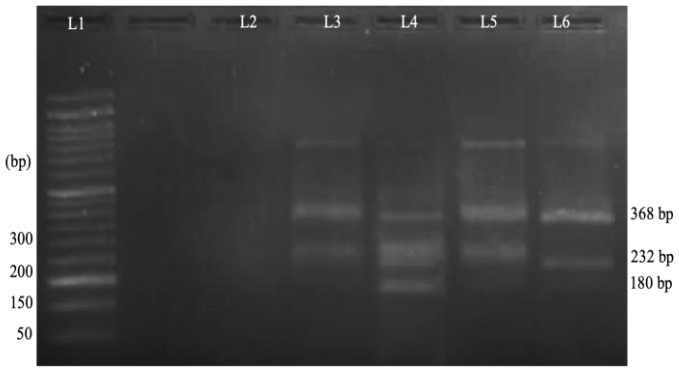

Figure 2. Gel photograph showing AS-PCR assay for genotyping of F1534C alleles *Ae. albopictus* in KK. Lane 1: 50bp DNA ladder, Lane 2: negative control, Lanes 3 and 5: homozygote (F/F), Lane 4: heterozygote (F/C), Lane 6: positive control

Table 3 represents the summary of the allelic frequency of the F1534C *kdr* alleles from Figure 1 and 2 and their distribution in *Ae. albopictus* field mosquitoes (alive/mixed insecticides, stocked in -80°C). In brief, Table 3 shows the presence of F1534C *kdr* alleles in both *Ae. albopictus* populations with a variation of homozygote and heterozygote susceptibility distributions. Of these, two pool samples were heterozygote resistance (F/C1534, 2/3) in KB populations, and vice versa, two pool samples of *Ae. albopictus* in KK were homozygote susceptible (F/F1534, 2/3). The homozygote resistance *kdr* allele (C/C1534, 0/3) was not present in both *Ae. albopictus* populations in KB and KK. Concerning this present study, the susceptible allelic frequency (F/F) of *Ae. albopictus* populations in KB were lower (33.3%) when compared to KK populations (66.7%).

Table 3. Frequency of *kdr* alleles in field mosquito *Ae. albopictus*

Primers	KB	Allelic frequency	KK	Allelic frequency
F/F1534	1/3	33.3%	2/3	66.7%
F/C1534	2/3	66.7%	1/3	33.3%
C/C1534	0/3	0	0/3	0
Total	**3/3**	**100%**	**3/3**	**100%**

Notes: KB-Kota Bharu; KK-USM Kubang Kerian

As described earlier, KB locality has become a dengue hotspot for three consecutive years from 2016 to 2018. As such, occasional fogging was observed throughout the year of sampling collection. The frequent exposure to insecticides has caused notable effects on the populations. Molecular characterization shows that the resistant-associated alleles F1534C were present in both populations studied at high frequencies in KB, mainly on heterozygote resistance (F/C). This suggests that this gene has been subjected to selective pressures in the past and still progressing in these populations. The condition was conferring with the resistance ratio (RR) obtained < 5, which indicates susceptible (S) in which yet to reach fixation in the KB populations. However, the presence of heterozygote resistance (F/C) in both populations would be a significant indicator for the spreading of resistance genes in the populations.

Precautionary should be considered as *Ae. albopictus* has the potential of transmitting the dengue virus in a peri-domestic area. This was reported in a study by Abu Bakar *et al.* (2018), in which the dengue virus was detected in *Ae. albopictus* population samples in the absence of *Ae. aegypti*. In addition, the insecticide resistance in *Ae. albopictus* field mosquito is spreading and continues to increase with many studies evident from a previous researcher (Ishak *et al.*, 2015; Rohani *et al.*, 2001).

These results suggest that *kdr* F1534C allele in both *Ae. albopictus* populations are present in field mosquitoes, however, the effect on physical resistance (phenotype) was different at the population level, whereby this study showed the presence of heterozygote resistance (F/C) allele in *Ae. albopictus* population of the USMKK. This study shows, pyrethroid resistance is widely present in various intensity in *Ae. albopictus* population in both studied areas, Kota Bharu and Kubang Kerian. The distributions of *kdr* alleles in *Ae. albopictus* tested populations were shown moderate of equal susceptible/ resistant levels.

CONCLUSION

Results from the bioassay were generally in agreement with the presence of the heterozygote F/C1534 of the *kdr* mutation allele. This study revealed the presence of *kdr* alleles F1534C in *Ae. albopictus* in Kota Bharu and Kubang Kerian areas against DDT and pyrethroids. Spreading of the mutation genes is possible in both study areas. However, this result was not strongly concrete to determine the definite susceptibility status of *Ae. albopictus* due to the minimal sample size and few technical issues that occurred during the study conducted. The establishment of the ongoing research is important to verify the development resistance condition of field mosquitoes in the affected population

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