Enzymatic Characterization of Insecticide Resistance Mechanisms in Field Populations of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae)

Van Lun Low¹, Chee Dhang Chen¹, Han Lim Lee², Tiong Kai Tan³, Chin Fong Chen¹, Chereng Shii Leong¹, Yvonne Ai Lian Lim³, Phaik Eem Lim¹-⁴, Yusoff Norma-Rashid¹, Mohd Sofian-Azirun¹

¹Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, ²Medical Entomology Unit, WHO Collaborating Centre for Vectors, Institute for Medical Research, Kuala Lumpur, Malaysia, ³Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, ⁴Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur, Malaysia

**Abstract**

**Background:** There has been no comprehensive study on biochemical characterization of insecticide resistance mechanisms in field populations of Malaysian *Culex quinquefasciatus*. To fill this void in the literature, a nationwide investigation was performed to quantify the enzyme activities, thereby attempting to characterize the potential resistance mechanisms in *Cx. quinquefasciatus* in residential areas in Malaysia.

**Methodology/Principal Findings:** *Culex quinquefasciatus* from 14 residential areas across 13 states and one federal territory were subjected to esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase assays. Enzyme assays revealed that α-esterases and β-esterases were elevated in 13 populations and 12 populations, respectively. Nine populations demonstrated elevated levels of mixed function oxidases and glutathione-S-transferase. Acetylcholinesterase was insensitive to propoxur in all 14 populations. Activity of α-esterases associated with malathion resistance was found in the present study. In addition, an association between the activity of α-esterases and β-esterases was also demonstrated.

**Conclusions/Significance:** The present study has characterized the potential biochemical mechanisms in contributing towards insecticide resistance in *Cx. quinquefasciatus* field populations in Malaysia. Identification of mechanisms underlying the insecticide resistance will be beneficial in developing effective mosquito control programs in Malaysia.

**Introduction**

Insecticide resistance mechanisms have been the subject of research interest among researchers from different parts of the world, including Malaysia. It has been proven that increased levels of mixed function oxidases contribute resistance to four major insecticide classes (i.e., organochlorines, carbamates, organophosphates and pyrethroids) [1–3]. It has also been reported that elevated levels of esterases are responsible for the resistance to organophosphates, carbamates and pyrethroids [4–5]. Involvement of glutathione-S-transferase in resistance to organophosphates, organochlorines and pyrethroids has also been noted [6–8]. Furthermore, previous studies have provided evidence on the role of insensitive acetylcholinesterase in resistance to organophosphates and carbamates [9–10]. As far as insecticide resistance mechanisms are concerned in Malaysia, a considerable amount of research indicated that Malaysian mosquitoes have demonstrated variable biochemical mechanisms in resistance to various insecticide classes [11–25].

With regard to *Culex quinquefasciatus* Say, it has been deemed as one of the three ‘world’s resistant mosquitoes’ [26]. The first documented case of insecticide resistance (towards organochlorines) in this mosquito species had been reported in 1952 in California [27]. Subsequently, the widespread development of its biotypes with resistance to 35 insecticide active ingredients has been documented worldwide [28]. In particular, Malaysian *Cx. quinquefasciatus*, the most abundant and annoying mosquito [28–29] has developed resistance towards four major insecticide classes [30–34].

Enzyme assay has been commonly used due to its rapid, simple and sensitive method for the identification of mechanisms underlying the insecticide resistance in mosquito population even at low frequencies [11,35]. However, in Malaysia, the characterization of biochemical mechanisms of *Cx. quinquefasciatus* has been restricted to the districts of Kuala Lumpur [11–14,16,25], Sarawak [18] and laboratory insecticide selected strains [15–16,19,25]. Indeed, there has been a lack of evidence regarding the underlying mechanisms that are involved in insecticide resistance in the field.
Insecticide Resistance in Cx. quinquefasciatus

populations of Cx. quinquefasciatus from other districts in Malaysia. Although previous studies have investigated certain enzymes in insecticide resistance development, there have been no comprehensive studies which concurrently characterize the \( \alpha \)-estersases, \( \beta \)-estersases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase in resistance to four major insecticide classes. It is of great concern that the biochemical mechanisms in Malaysian Cx. quinquefasciatus populations could be underestimated, especially when there is an occurrence of multiple-resistant isolates within the same population.

Recently, multiple resistance to a broad spectrum of insecticides (i.e., DDT, propoxur, malathion and permethrin) ranged from susceptible, low to high resistance has been reported in Malaysian Cx. quinquefasciatus [34]. However, the actual mechanism(s) that cause insecticide resistance in these populations remain uncharacterized. In this context, a nationwide investigation was further conducted to (1) quantify the enzyme activities in field populations of Cx. quinquefasciatus, as part of an ongoing insecticide resistance monitoring from 14 residential areas across 11 states and one federal territory in Peninsular Malaysia and two states in East Malaysia, and thereby attempting to (2) correlate the degree of insecticide resistance with the levels of enzyme activities in this mosquito species. The present study is the first attempt to investigate the potential resistance mechanisms involving \( \alpha \)-estersases, \( \beta \)-estersases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase towards resistance to DDT, propoxur, malathion and permethrin in Cx. quinquefasciatus from all states in Malaysia. With the continued use of insecticides, a better understanding of the prevailing insecticide resistance mechanisms could serve as a justification for changes in control practices and provide baseline data for population monitoring in accordance with appropriate insecticides.

Materials and Methods

Ethical Notes

This research was regulated by the Medical Review & Ethics Committee (MREC), Ministry of Health Malaysia. No specific permits were required for this study which did not involve endangered or protected species. Permission for the study to be conducted on private land/private residences was obtained from owners/residents prior to specimen collection.

Mosquito Strains

Given that there is no specific Culex control program in Malaysia, the study sites were selected on the basis of the incidence of dengue infections and fogging activities, as intense fogging would inadvertently contaminate the breeding ground and exert selective pressure on Cx quinquefasciatus. A standardized larval dipping method developed by Mendoza et al. [36] was conducted. Mosquito larvae were collected from stagnant water in 14 residential areas across 13 states and a federal territory (i.e., Kuala Lumpur) in Malaysia (Figure 1). Details of the studied areas have been described elsewhere [34]. Field-collected larvae were transported to the Laboratory of Zoological and Ecological Research Network, University of Malaya and reared to adulthood for identification, using the taxonomic keys by Rattanarithkul et al. [37]. The identified Cx. quinquefasciatus female mosquitoes were blood-fed for the production of the first generation (F1). The non-blood fed three to five days old female mosquitoes from F1 reared larvae were used for WHO insecticide susceptibility tests (details have been published in previous report [34]) and biochemical assays. For comparison purposes, a laboratory reference strain of Cx. quinquefasciatus from the Institute for Medical Research, Kuala Lumpur, which has been cultured under insecticide free conditions for 117 generations was used. All female mosquitoes which have not been exposed to any chemicals were stored in ~80°C freezer prior to the biochemical tests. In the present study, a total of 1,440 adult Cx. quinquefasciatus with 24 individual mosquitoes representing each of the 60 strains (four enzyme assays in 15 populations, including laboratory reference strain) were used.

Enzyme Assays

Non-specific esterases enzyme assay was carried out according to established protocols [11,38]. A total of 24 individual mosquitoes were homogenized in phosphate buffer solution and were centrifuged at 15,000 rpm for 10 minutes at 4°C. Four replicates of homogenate (50 \( \mu \)l) from each individual mosquito were obtained in this assay. The 50 \( \mu \)l of substrate solution (either \( \alpha \)-napthyl acetate or \( \beta \)-napthyl acetate) was placed in a 96 well plate and left to stand for one minute, followed by the addition of 50 \( \mu \)l of 3 mM indicator solution (fast blue B salt). The reaction was further incubated for 10 minutes and was stopped by the addition of 50 \( \mu \)l of 10% acetic acid. The optical density was measured at 450 nm using absorbance microplate reader (BIO-TEK® ELX800™).

Mixed function oxidases enzyme assay was performed according to the method described by Brogdon et al. [39]. A total of 24 individual mosquitoes were homogenized in sodium acetate buffer solution and four replicates of homogenate (100 \( \mu \)l) from each individual mosquito were obtained in this assay. The optical density was measured at 630 nm after five minutes incubation of individual mosquito homogenate in each well with 200 \( \mu \)l of 2 mM 3,3',5,5'-tetramethylbenzidine (TMBZ) and 25 \( \mu \)l of 3% hydrogen peroxide.

Glutathione-S-transferase enzyme assay was conducted according to previously described protocol [15]. A total of 24 individual mosquitoes were homogenized in potassium phosphate buffer solution and were centrifuged at 14,000 rpm for 10 minutes at 4°C. Four replicates of homogenate (100 \( \mu \)l) from each individual mosquito were placed in a 96 well plate, followed by the addition of 50 \( \mu \)l of 2 mM glutathione and 50 \( \mu \)l of 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction was further incubated for 30 minutes, followed by the measurement of optical density at 400 nm.

With regard to insensitive acetylcholinesterase, enzyme assay was performed according to the method of Brogdon et al. [38], with minor modifications. In this assay, a total of eight replicates of homogenate (50 \( \mu \)l) from each individual mosquito were obtained. Briefly, the first batch of 12 individual mosquitoes which filled in the 96 wells was homogenized in potassium phosphate buffer and was centrifuged at 14,000 rpm for 10 minutes at 4°C. A 50 \( \mu \)l of reaction mixture containing 10% acetone buffer solution of 2.6 mM acetylthiocholine iodide (ACTHI), 0.3 mM of 5, 5-dihiobis (2-nitrobenzoic acid) (DTNB) and 0.1% propoxur inhibitor were added into each well. As for positive control, a 50 \( \mu \)l of reaction mixture without inhibitor was designed. The reaction was incubated at room temperature (28°C) for 30 minutes, followed by the measurement of optical density at 410 nm. This procedure was repeated for the second batch of 12 individual mosquitoes.

Statistical Analysis

Spearman rank-order correlation using SPSS (ver 18) was performed to (1) determine the associations between the survivalability rates in adult bioassays and enzyme activities, (2) investigate the relationships between enzyme activities.
Comparative measure of mean enzyme activities between the study sites was performed by one-way analysis of variance (ANOVA) using SPSS (ver 18). Tukey’s test was used to separate means in significant ANOVAs, \( P < 0.05 \). Independent-samples t-test was performed to indicate significant increase in mean differences.

With respect to insensitive acetylcholinesterase, results were interpreted as a percentage remaining activity in the propoxur inhibited fraction compared to the control (uninhibited) activity [40]. Individual mosquitoes with more than 70% remaining activity are indicative of homozygous resistance (RR), 30–70% remaining activity are indicative of heterozygous (RS) and less than 30% remaining activity are indicative of homozygous susceptible (SS). Because of the light absorbance of propoxur in the microplate, in certain cases, homogenates appear to show higher acetylcholinesterase activity in propoxur-inhibited fraction (>100%) and it is normal in resistant strains [40].

### Results

WHO adult bioassays demonstrated a broad spectrum of susceptibility status against DDT, propoxur, malathion and permethrin across all study sites [34]. Adult mortality recorded 24 h after the initial exposure period of DDT, propoxur, malathion and permethrin ranged from 0.00 to 40.00, 3.34 to 68.89, 0.00 to 100.00 and 36.67 to 100.00%, respectively. Generally, DDT and propoxur resistance were expressed most frequently, as all study sites demonstrated a resistant biotype (less than 80% mortality). With regard to malathion and permethrin, a resistant biotype was detected from 11 out of 14 and 6 out of 14 of the populations, respectively (Table 1).

One-way ANOVA revealed that the mean of all tested enzyme activities in Malaysian *Culex quinquefasciatus* were significantly different across all study sites (\( P < 0.001 \)). In addition, Spearman rank-order correlation indicated a significant correlation between malathion survivability rate in adult bioassays and \( \alpha \)-esterases activity in Malaysian *Culex quinquefasciatus* (\( r = 0.692, P = 0.004 \)) (Figure 2), while no correlation was found with other insecticide survivability rates against enzymes activities. An association between the activity of \( \alpha \)-esterses and \( \beta \)-esterses (\( r = 0.627, P = 0.012 \) was also demonstrated (Figure 3).

In non-specific esterases assay, a significant increase in \( \alpha \)-esterases activity was detected in all populations (except Kelantan). A lack of elevated \( \beta \)-esterses activity was observed in Kelantan and Kedah populations, whereas other populations exhibited a significant increase in \( \beta \)-esterses activity. All populations exhibited higher \( \alpha \)-esterses activity, as compared to \( \beta \)-esterses activity (except Pahang) (Table 2).

As for mixed function oxidases assay, an elevated level of mixed function oxidases activity was found in nine populations (i.e., Kedah, Malacca, Negeri Sembilan, Penang, Perak, Sabah, Selangor, Sarawak and Terengganu) (Table 2).

Of 14 populations, nine populations (i.e., Kedah, Kelantan, Malacca, Pahang, Penang, Perak, Sabah, Sarawak and Terengganu) exhibited a significant increase in glutathione-S-transferase activity (Table 2).

With regard to insensitive acetylcholinesterase assay, all populations revealed a significant increase in acetylcholinesterase activity in the control test (absence of propoxur), except Kuala Lumpur, Perlis and Sarawak. In comparison to the laboratory strain, all populations also revealed a significant increase in acetylcholinesterase activity in the presence of propoxur (Table 2). A quick perusal of the remaining activity data indicated that RS
was detected in all 14 populations. The RS genotype was also the most prevalent, with 246 individuals from a total sample size of 336, followed by SS genotype (55 individuals) and RR genotype (35 individuals). An excess of RR genotype was recorded in *Cx. quinquefasciatus* population from Sarawak (Figure 4).

Summary of insecticide resistance and prevalence of resistance mechanisms in different *Cx. quinquefasciatus* populations was presented in Table 3. Elevated levels of all enzymes activities were demonstrated in four populations (Malacca, Penang, Perak and Terengganu).

**Table 1.** Mortality of Malaysian *Culex quinquefasciatus* adults using a WHOPES treated filter paper assay.

| Strain     | DDT (4.0%) | Propoxur (0.1%) | Malathion (5.0%) | Permethrin (0.25%) |
|------------|------------|-----------------|------------------|-------------------|
| Reference  | 43.34 ± 2.72 | 100.00 ± 0.00 | 100 ± 0.00       | 100.00 ± 0.00     |
| Kelantan   | 24.44 ± 4.44 | 8.34 ± 3.34    | 69.67 ± 3.34     | 43.33 ± 10.00     |
| Terengganu | 25.00 ± 5.00 | 55.00 ± 5.00   | 100.00 ± 0.00    | 95.00 ± 5.00      |
| Pahang     | 13.33 ± 3.33 | 20.00 ± 5.77   | 71.11 ± 2.22     | 71.11 ± 2.22      |
| Perlis     | 4.45 ± 2.22  | 6.67 ± 3.85    | 71.11 ± 5.88     | 71.11 ± 2.22      |
| Kedah      | 35.56 ± 5.88 | 37.78 ± 2.22   | 71.11 ± 5.88     | 71.11 ± 2.22      |
| Penang     | 20.00 ± 0.00 | 6.67 ± 3.85    | 10.00 ± 3.33     | 83.33 ± 10.00     |
| Perak      | 6.67 ± 3.85  | 55.55 ± 2.22   | 0.00 ± 0.00      | 62.22 ± 4.45      |
| Selangor   | 17.78 ± 2.22 | 33.33 ± 3.85   | 11.11 ± 5.88     | 77.78 ± 2.22      |
| Kuala Lumpur | 20.00 ± 0.00 | 10.00 ± 5.77   | 6.67 ± 6.67      | 100.00 ± 0.00     |
| Negeri Sembilan | 11.11 ± 2.22 | 55.55 ± 2.22 | 4.44 ± 4.44 | 82.22 ± 5.88 |
| Malacca    | 22.22 ± 2.22 | 22.22 ± 2.22   | 31.11 ± 4.44     | 100.00 ± 0.00     |
| Johore     | 40.00 ± 3.85 | 62.22 ± 4.45   | 55.55 ± 2.22     | 93.33 ± 0.00      |

Details have been produced in previous study [34].

R = resistant, S = susceptible, M = moderate resistant as determined by WHO [50].

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**Figure 2.** Spearman rank-order correlation between malathion survivability rate and α-esterases activity in Malaysian *Culex quinquefasciatus*.  
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Discussion

Regardless of the associations between the degree of insecticide resistance and the enzyme activities, the enhanced enzyme activities of α-estases, β-esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase in Malaysian Cx. quinquefasciatus confirmed the incidence of insecticide resistance (low to high resistance towards DDT, propoxur, malathion and permethrin), as detected by WHO adult bioassays [34]. An elevated level of esterases [11–13,16], oxidases [17–

Table 2. Mean (± SE) esterases, glutathione-S-transferases and mixed function oxidases and acetylcholinesterase activities in Malaysian Cx. quinquefasciatus populations.

| Strain       | α-EST (μmol/min/mg protein) | β-EST (μmol/min/mg protein) | MFO (Absorbance 630 nm) | GST (μmol/min/mg protein) | AChE (Control without insecticide) | pAChE (ACTH with 0.1% propoxur) |
|--------------|-----------------------------|-----------------------------|--------------------------|---------------------------|-----------------------------------|---------------------------------|
| Reference    | 0.23 ± 0.01                 | 0.21 ± 0.00                 | 0.50 ± 0.02              | 0.13 ± 0.00               | 0.17 ± 0.01                       | 0.05 ± 0.00                     |
| Kelantan     | 0.25 ± 0.01*                | 0.21 ± 0.00*                | 0.53 ± 0.01*             | 0.15 ± 0.00*              | 0.22 ± 0.01bcd                    | *0.09 ± 0.00*                   |
| Terengganu   | *0.29 ± 0.01abc             | *0.24 ± 0.01abc             | *1.08 ± 0.05             | 0.15 ± 0.00*              | 0.32 ± 0.01fg                     | *0.09 ± 0.00*                   |
| Pahang       | *0.27 ± 0.00ab              | *0.32 ± 0.01ef              | 0.42 ± 0.02               | *0.16 ± 0.00*             | *0.24 ± 0.01cde                    | *0.09 ± 0.00*                   |
| Perlis       | *0.32 ± 0.02bc              | *0.26 ± 0.01bcd            | 0.53 ± 0.02               | 0.13 ± 0.01abc            | 0.16 ± 0.01ab                      | *0.09 ± 0.00*                   |
| Kedah        | *0.29 ± 0.01abc             | 0.21 ± 0.01                 | 0.94 ± 0.05ab             | *0.16 ± 0.01*             | 0.35 ± 0.02g                      | *0.10 ± 0.00*                   |
| Penang       | *0.32 ± 0.01bc              | *0.29 ± 0.00bc              | *0.93 ± 0.06              | 0.17 ± 0.00               | 0.28 ± 0.01bc                      | 0.12 ± 0.00                     |
| Perak        | *0.42 ± 0.02ef              | *0.34 ± 0.01f               | 0.57 ± 0.02ef             | 0.15 ± 0.00*              | 0.29 ± 0.02efg                     | *0.09 ± 0.00*                   |
| Selangor     | *0.31 ± 0.01bc              | *0.24 ± 0.01ab              | *0.83 ± 0.02              | 0.12 ± 0.00               | 0.24 ± 0.01cde                     | *0.10 ± 0.00*                   |
| Kuala Lumpur | *0.38 ± 0.01de              | *0.27 ± 0.00cd              | 0.44 ± 0.01               | 0.12 ± 0.00               | 0.16 ± 0.01ab                      | *0.09 ± 0.00*                   |
| Negeri Sembilan | *0.34 ± 0.01cd          | *0.24 ± 0.01abc            | *0.84 ± 0.03              | 0.14 ± 0.00cde            | *0.24 ± 0.02cde                    | *0.10 ± 0.00*                   |
| Malacca      | *0.34 ± 0.01cd              | *0.26 ± 0.01bcd            | 0.96 ± 0.02de             | *0.14 ± 0.00cde           | 0.21 ± 0.01bc                      | *0.10 ± 0.00*                   |
| Johore       | *0.30 ± 0.01abc             | *0.24 ± 0.00abc             | 0.53 ± 0.02ab             | 0.12 ± 0.00ab             | 0.20 ± 0.01bc                      | *0.09 ± 0.00*                   |
| Sarawak      | *0.28 ± 0.01ab              | *0.24 ± 0.00ab              | *0.87 ± 0.04f             | 0.14 ± 0.00cde            | 0.12 ± 0.00a                       | *0.09 ± 0.00*                   |
| Sabah        | *0.47 ± 0.02f               | *0.28 ± 0.00df              | *0.68 ± 0.03ef            | 0.14 ± 0.00cde            | 0.21 ± 0.01bc                      | *0.10 ± 0.00*                   |

One way ANOVA

F = 21.43; df = 13; P = 0.0001
F = 25.99; df = 13; P = 0.0001
F = 46.98; df = 13; P = 0.0001
F = 18.75; df = 13; P = 0.0001
F = 22.25; df = 13; P = 0.0001

*Significant increase in mean differences compared to the laboratory reference strain, P<0.05, t-test.

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in field populations of Malaysian Cx. quinquefasciatus has been previously described. However, the elevated levels of glutathione-S-transferase and acetylcholinesterase in this study indicated contrasting results with previous studies, where there was a lack of elevated level of glutathione-S-transferase and acetylcholinesterase after propoxur inhibition in Malaysian Cx. quinquefas-

![Figure 4. Percentage remaining activity of acetylcholinesterase in individual Malaysian Culex quinquefasciatus after 0.1% propoxur inhibition. *<30% = homozygous susceptible (SS), 30–70% = heterozygous (RS), >70% = homozygous resistance (RR) [40]. doi:10.1371/journal.pone.0079928.g004](image)

| Strain       | DDT | PRO | MAL | PER | α-EST | β-EST | MFO | GST | AChE | pAChE |
|--------------|-----|-----|-----|-----|-------|-------|-----|-----|------|-------|
| Kelantan     | R   | R   | M   | R   | –     | –     | –   | +   | +    | +     |
| Terengganu   | R   | R   | S   | M   | +     | +     | –   | +   | +    | +     |
| Pahang       | R   | R   | S   | R   | +     | +     | –   | –   | –    | +     |
| Perlis       | R   | R   | R   | R   | +     | +     | –   | –   | +    |       |
| Kedah        | R   | R   | R   | R   | +     | –     | +   | +   | +    | +     |
| Penang       | R   | R   | R   | M   | +     | +     | +   | +   | +    | +     |
| Perak        | R   | R   | R   | M   | +     | +     | +   | +   | +    | +     |
| Selangor     | R   | R   | R   | R   | +     | +     | –   | –   | +    | +     |
| Kuala Lumpur | R   | R   | R   | R   | +     | +     | –   | –   | –    | +     |
| Negeri Sembilan | R   | R   | R   | S   | +     | +     | +   | –   | –    | +     |
| Malacca      | R   | R   | R   | M   | +     | +     | +   | +   | +    | +     |
| Johore       | R   | R   | R   | S   | +     | +     | –   | –   | +    | +     |
| Sarawak      | R   | R   | R   | S   | +     | +     | +   | +   | –    | +     |
| Sabah        | R   | R   | R   | M   | +     | +     | –   | +   | +    | +     |

*PRO = propoxur, MAL = malathion, PER = permethrin, α-EST = α-esterases, β-EST = β-esterases, MFO = mixed function oxidases, GST = glutathione-S-transferase, AChE = acetylcholinesterase, pAChE = propoxur-inhibited acetylcholinesterase, R = resistant, M = moderate resistant, S = susceptible, + = presence of mechanism, – = absence of mechanism.

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In conclusion, the results presented here provide the first report on the mechanisms of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase towards resistance to DDT, propoxur, malathion and permethrin in *C. quinquefasciatus* from all states in Malaysia. Evidence of malathion resistance due to elevated α-esterases activity was found. In addition, an association between activity of α-esterases and β-esterases was also demonstrated in the present study. Nevertheless, multiple insecticide resistance involving both metabolic mechanisms and target site alteration in *C. quinquefasciatus* has been reported from many parts of the world [41,47–48]. Furthermore, reduced insecticide penetration (cuticular resistance) in *C. quinquefasciatus* has also been noted [49]. Hence, for future study, investigation of the insecticide resistance involving the factors mentioned above in Malaysian *C. quinquefasciatus* will be beneficial in unraveling the prevailing resistance mechanisms which will therefore contribute to the technical know-how of implementing effective mosquito control programs in Malaysia.

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**Author Contributions**

Conceived and designed the experiments: MSA HLL CDC. Performed the experiments: VLL TKTC CFC CSL. Analyzed the data: VLL. Contributed reagents/materials/analysis tools: YALL YNR PEL. Wrote the paper: VLL.
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