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EXPRESSION PATTERNS OF DETOXIFICATION ENZYMES UNDERLYING SUSCEPTIBILITY STATUS IN *Culex quinquefasciatus* SAY (DIPTERA: CULICIDAE) FROM DENGUE HOTSPOT AREAS IN PENANG, MALAYSIA

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ABSTRACT

Mosquito control remains a primary strategy for managing vector-borne diseases, relying heavily on various classes of insecticides including organophosphates, organochlorines, carbamates, and pyrethroids. However, widespread and prolonged usage has led to increasing insecticide resistance in mosquito populations. This study investigated resistance levels and detoxification enzyme activities in *Culex quinquefasciatus* larvae collected from two field sites (Minden and Bayan strains) in Pulau Pinang, Malaysia, compared to a susceptible laboratory strain (VCRU strain). Insecticide susceptibility bioassays were carried out using technical-grade malathion (96.0%), temephos (92.6%), deltamethrin (99.7%), and pirimiphos-methyl. Biochemical assays were performed to quantify the activities of acetylcholinesterase (AChE), cytochrome P450 monooxygenases (CYP450), glutathione S-transferases (GST), and esterases. The bioassay results showed varying resistance ratios (RR_{50}) ranging from 3.0- to 10-fold in field strains, with the highest resistance in the Bayan strain exposed to deltamethrin (10-fold), and the lowest in the Minden strain treated with malathion (0.57-fold). These findings confirm resistance to malathion, pirimiphos-methyl, temephos, and deltamethrin in local *Cx. quinquefasciatus* populations, associated with increased detoxification enzyme activity. The study highlights the need for continuous resistance monitoring and supports alternative vector control strategies such as outdoor residual spraying (ORS) to manage insecticide-resistant mosquito populations in urban habitats.

Keywords: *Culex quinquefasciatus;* Detoxification enzymes; Insecticide resistance; Vector control

ABSTRAK

Kawalan nyamuk kekal sebagai strategi utama dalam menguruskan penyakit bawaan vektor, dengan bergantung kepada pelbagai kelas insektisid termasuk organofosfat, organoklorin, karbamat dan piretroid. Walau bagaimanapun, penggunaan yang meluas dan berpanjangan telah membawa kepada peningkatan ketahanan insektisid dalam populasi nyamuk. Kajian ini menilai tahap ketahanan dan aktiviti enzim detoksifikasi dalam larva *Culex quinquefasciatus* yang dikumpul dari dua tapak lapangan (strain Minden dan Bayan) di Pulau Pinang, Malaysia, berbanding dengan strain makmal yang rentan (strain VCRU). Ujian bioassai insektisid dijalankan menggunakan malation gred teknikal (96.0%), temephos (92.6%), deltamethrin (99.7%) dan pirimifos-metil. Ujian biokimia dilaksanakan untuk mengukur aktiviti asetilkolinesterase (AChE), sitokrom P450 monooksigenase (CYP450), glutathione S-transferase (GST) dan esterase. Keputusan bioassai menunjukkan nisbah ketahanan (RR_{50}) yang berbeza antara 3.0 hingga 10 kali ganda dalam strain lapangan, dengan ketahanan tertinggi pada strain Bayan yang terdedah kepada deltamethrin (10 kali ganda), dan paling rendah pada strain Minden yang dirawat dengan malation (0.57 kali ganda). Penemuan ini mengesahkan ketahanan terhadap malation, pirimifos-metil, temephos, dan deltamethrin dalam populasi *Cx. quinquefasciatus* tempatan, yang dikaitkan dengan peningkatan aktiviti enzim detoksifikasi. Kajian ini menekankan keperluan pemantauan ketahanan secara berterusan dan menyokong strategi kawalan vektor alternatif seperti semburan sisa luar (ORS) untuk menguruskan populasi nyamuk tahan insektisid di habitat bandar.

Kata kunci: *Culex quinquefasciatus*; enzim detoksifikasi; ketahanan insektisid; kawalan vektor

INTRODUCTION

Chemical insecticides such as organophosphates, organochlorines, carbamates, and pyrethroids have long been essential tools in controlling mosquito-borne diseases like dengue, malaria, and filariasis. Despite their effectiveness, the widespread and continuous use of these insecticides has resulted in growing resistance among mosquito populations, making control efforts increasingly difficult (Yunta et al. 2019). To ensure ongoing success in vector control programs, it is important not only to monitor resistance levels but also to understand the biological mechanisms responsible for this resistance, especially those involving detoxification enzymes (Fodjo et al. 2018). In Malaysia, recent studies have highlighted increasing resistance trends in *Culex quinquefasciatus* and other mosquito vectors, with biochemical and molecular evidence pointing towards elevated esterase, GST, and cytochrome P450 activities as major contributors (Farouk et al. 2021; Wan Najdah et al. 2020). Reports from Penang and surrounding hotspot areas further revealed altered susceptibility patterns in both *Culex* and *Aedes* species, underscoring the local impact of sustained insecticide exposure (Nazni et al. 2019; Rosilawati et al. 2021). These findings emphasise the need for detailed characterisation of detoxification enzyme expression in Malaysian *Cx. quinquefasciatus*, particularly from Penang, to support locally effective vector control strategies.

One of the main mechanisms by which mosquitoes develop resistance is through metabolic detoxification. Detoxifying enzymes are capable of breaking down insecticides before they can act on their intended targets. These enzymes, often activated by exposure to foreign chemicals or environmental pollutants (xenobiotics), play a critical role during both aquatic and terrestrial stages of the mosquito lifecycle (Ramkumar et al. 2022). Overexpression of detoxification enzymes is typically due to increased activity of certain genes involved in

resistance, and different enzyme types are often associated with resistance to specific classes of insecticides.

Among the most studied detoxification enzymes are cytochrome P450 monooxygenases (P450s). These enzymes have been linked to resistance against pyrethroids, one of the most commonly used classes of insecticides. In *Cx. quinquefasciatus*, the overexpression of P450 genes such as CYP6AA7 and CYPAA10 helps metabolize permethrin and its toxic by-products, reducing their harmful effects (Gong et al. 2017). Further investigations have identified additional P450 genes like CYP6P14, CYP9J33, CYP6BZ2, and CYP9J34, which play a role in converting toxic permethrin derivatives such as 3-phenoxy benzoic alcohol (PBCHO) into less harmful compounds like 3-phenoxy benzoic acid (PBCOOH), enhancing mosquito survival and resistance (Gong et al. 2022). A study in Indonesia found moderate resistance to cypermethrin in *Cx. quinquefasciatus,* which may be linked to the mosquito's minimal involvement in dengue vector control operations compared to *Aedes* species (Subahar et al. 2022). This suggests that even species not directly targeted by control efforts can develop resistance due to environmental exposure.

Another group of detoxification enzymes involved in insecticide resistance is glutathione S-transferases (GSTs). GSTs are stress-inducible enzymes that help detoxify a wide range of organic pollutants and pesticides (Domingues et al. 2010). They also serve as biomarkers indicating chemical exposure in insects. In *Aedes albopictus* larvae treated with permethrin and DDT, overexpression of multiple GST isoforms was observed, especially in the Delta, Theta, and Sigma classes. These findings highlight the significant role GSTs play in insecticide resistance, with some isoforms showing expression levels more than two- to threefold higher in treated populations (Hamzah et al. 2019). Esterases, particularly α- and β-esterases, are also important in resistance to organophosphates and pyrethroids. These enzymes function by breaking down insecticides into less toxic compounds. In one study, SDS-PAGE analysis showed multiple esterase bands in *Cx. quinquefasciatus* exposed to λ-cyhalothrin, suggesting esterase involvement in resistance (Muthusamy & Shivakumar 2015). Other research demonstrated increased esterase activity in *Cx. quinquefasciatus* females treated with malathion, indicating higher resistance to organophosphates than pyrethroids. In contrast, permethrin-treated strains showed lower non-specific esterase activity (Zhang et al. 2021). Similarly, high esterase activity due to gene amplification has been observed in *Cx. pipiens*, suggesting a rising threat of control failure in affected regions (Aboulfadl et al. 2022).

Another mechanism contributing to resistance is target-site insensitivity, particularly involving acetylcholinesterase (AChE). This enzyme breaks down acetylcholine in the mosquito’s nervous system, but mutations in the ace-1 gene can reduce its sensitivity to insecticides such as propoxur and pyrethroids (Low et al. 2013). These mutations are often accompanied by changes in voltage-gated sodium channels (VGSC), which are the primary targets of pyrethroids. Studies from Malaysia and Thailand have confirmed such mutations, including the well-known kdr (knockdown resistance) mutation caused by the L1014F substitution (Chamnanya et al. 2022).

In light of these findings, this study focuses on *Cx. quinquefasciatus* populations collected from dengue hotspot areas in Pulau Pinang, Malaysia. These areas are regularly exposed to insecticides as part of *Aedes* control programs, potentially affecting non-target mosquito species. The study aims to assess the susceptibility status of *Cx. quinquefasciatus* and investigate the expression levels of key detoxification enzymes (GSTs, esterases, cytochrome

P450s, and AChE). Understanding these resistance mechanisms is essential to developing more effective and sustainable mosquito control strategies.

MATERIALS AND METHODS

Mosquito Larval Collection

Susceptible *Cx. quinquefasciatus* larvae were obtained from the Vector Control Research Unit (VCRU), Universiti Sains Malaysia, where the strain has been maintained insecticide-free for over 30 years (Lee et al. 1997). Field strains were collected using the dipper method, with samples obtained at 5°2′12.611″N, 100°1′7.15211″E and designated as the Minden strain, while those collected at 5°3′27.18411″N, 100°2′7.515311″E were designated as the Bayan strain. Field samples were taken from similar habitats of open sewage-polluted water mixed with human and animal waste, decayed vegetation, and debris. The field-collected generation (F_0) larvae were brought to the laboratory for bioassay and biochemical analysis.

Bioassays and Determination of Diagnostic Concentration

The larval bioassay for *Cx. quinquefasciatus* was performed following WHO (2005) guidelines using four insecticides: malathion (96.0%), temephos (92.6%), deltamethrin (99.7%) and pirimiphos-methyl. Ethanol was used to prepare insecticide solutions and controls. Groups of 25 early 4th instar larvae from the susceptible strain were tested in four replicates, each in 250 mL of seasoned water, exposed to various insecticide concentrations for 24 hours. Controls were treated with 10% ethanol under the same conditions (16°C temperature, 12:12 h light-dark cycle). If mortality in controls exceeded 10%, results were discarded. The LC_{50} dose from this test was then used to calculate LC_{50} and resistance ratios (RR_{50}) in field strains.

Protein Quantification

In three replicates, 5 µL of the homogenate supernatant was mixed with 250 µL of Coomassie Reagent. The mixture stood for 30 seconds and then incubated for 10 minutes at room temperature. Bovine Serum Albumin was used as a protein standard, and optical density was measured at 595 nm using a microplate reader, following the method described by Hamzah et al. (2022).

Biochemical Assay

Biochemical tests for detoxification enzymes were performed on both susceptible and field strains (Minden and Bayan) of *Cx. quinquefasciatus* following WHO guidelines (Hemingway & Brogdon 1998). Early 4th instar larvae were homogenized in 200 µL of seasoned water on ice to preserve enzyme activity. From each homogenate, 25 µL was used for the acetylcholinesterase (AChE) assay, while the rest was centrifuged at 14,000 rpm for 30 seconds at 4°C, and the supernatant was used for the other enzyme assays. The AChE assay involved mixing homogenate with Triton phosphate buffer, DTNB solution, and acetylcholine iodide to start the reaction, with one replicate inhibited by propoxur to measure enzyme activity. Optical density was measured at 405 nm over one hour and activity was calculated using Beer's Law. For esterase assays, 20 µL of supernatant was incubated separately with α-naphthyl acetate and β-naphthyl acetate solutions, followed by staining with fast blue, and absorbance was read at 570 nm. Glutathione S-transferase (GST) activity was determined by combining 10 µL supernatant with glutathione and CDNB solutions, incubating for 20 minutes, and measuring absorbance at 340 nm. Cytochrome P450 activity was measured by mixing 2 µL supernatant with potassium phosphate buffer, tetramethylbenzidine-methanol solution, and hydrogen peroxide, incubating for 2 hours, then reading absorbance at 650 nm. Enzyme specific activities

were calculated according to standard methods and expressed as nmoles per minute per mg of protein.

Statistical Analysis

Bioassay results were analysed using log-probit analysis in SPSS software (version 27), and LC_{50} values were used to assess mosquito susceptibility. The resistance ratio (RR_{50}) was calculated by dividing the LC_{50} of field strains (Minden and Bayan) by the LC_{50} of the susceptible strain (VCRU). Resistance levels were classified as follows: RR_{50}<1 (susceptible), 1–10 (low resistance), 11–30 (moderate resistance), 31–100 (high resistance), and >100 (very high resistance). Detoxification enzyme activity data were tested for normality and variance using Kolmogorov-Smirnov and Levene's tests. Enzyme levels were reported as mean±standard deviation. Differences in total and specific enzymatic activities among all strains were compared using one-way ANOVA, with significance set at $P<0.05$ (Khan et al. 2011).

RESULTS

Susceptibility Assay

The susceptibility of the VCRU control strain and two field strains of *Cx. quinquefasciatus* (Minden and Bayan) was tested using WHO (2005) guidelines. Four insecticides (malathion, pirimiphos-methyl, temephos, and deltamethrin) were used to determine the LC_{50} (lethal concentration for 50% mortality) in the control strain. These LC_{50} values were then used as diagnostic doses to assess resistance and calculate resistance ratios (RR_{50}) in the field strains.

Table 1 presents the LC_{50} values for each insecticide. With malathion, LC_{50} values were: 0.019 mg/L, 0.011 mg/L, 0.081 mg/L for VCRU (susceptible strain), Minden and Bayan respectively. With pirimiphos-methyl, LC_{50} values were: 0.006 mg/L, 0.030 mg/L, 0.037 mg/L for VCRU (susceptible strain), Minden and Bayan respectively. With temephos, VCRU (susceptible strain), and Minden had LC_{50} values of 0.001 mg/L, and Bayan had 0.003 mg/L. For deltamethrin, the LC_{50} was 0.0003 mg/L in VCRU, 0.0026 mg/L in Minden, and 0.003 mg/L in Bayan.

Table 1. Susceptibility of *Culex quinquefasciatus* larvae towards various classes of insecticides

Strains	Insecticides	LC_{50} (mg/L)	95% Confidence Limits (mg/L) LCL-UCL	LC90 (mg/L)	95% Confidence Limits (mg/L) LCL-UCL	Resistance Ratio (RR_{50})
VCRU		0.019	0.017–0.020	0.033	0.031–0.037	0.00
Minden	Malathion	0.011	0.092–0.136	0.165	0.139–0.268	0.57
Bayan		0.081	0.060–0.097	0.139	0.113–0.216	4.26
VCRU		0.006	0.005–0.007	0.012	0.009–0.017	0.00
Minden	Pirimiphos methyl	0.030	0.019–0.049	0.108	0.063–0.344	5.00
Bayan		0.037	0.029–0.047	0.086	0.066–0.137	6.16
VCRU		0.001	0.00079–0.019	0.003	0.001–0.002	0.00
Minden	Temephos	0.003	0.005–0.002	0.007	0.005–0.022	3.00
Bayan		0.003	0.003–0.004	0.006	0.005–0.007	3.00
VCRU		0.0003	0.00029–0.00035	0.001	0.0001–0.0017	0.00
Minden	Deltamethrin	0.0026	0.0018–0.0026	0.009	0.0074–0.0013	8.66
Bayan		0.0030	0.0020–0.0030	0.009	0.007–0.012	10.0

Result of the WHO Bioassay LC_{50} and LC_{90} of VCRU (susceptible strain), Minden and Bayan strains. RR_{50} refers to the Resistance Ratio of the field strains divided by the susceptible (control) strain.

These results indicate that both field strains showed varying degrees of resistance to all tested insecticides. The calculated resistance ratios (RR_{50}) ranged from 0.57-fold to 10-fold. The highest resistance was seen in the Bayan strain exposed to deltamethrin (10-fold), while the lowest was in the Minden strain treated with malathion (0.57-fold). Notable resistance levels were also observed in the Minden strain treated with deltamethrin (8.66-fold) and the Bayan strain treated with pirimiphos-methyl (6.16-fold). Moderate resistance was seen in the Minden strain treated with pirimiphos-methyl (5.0-fold) and the Bayan strain treated with malathion (4.26-fold). Both field strains showed a 3-fold resistance to temephos. Overall, the resistance ratios observed in this study were generally low but indicate the presence of insecticide resistance in field populations of *Cx. quinquefasciatus*.

Total Protein Content

The total protein content of the larvae field strains of Minden and Bayan was evaluated with the susceptible *Cx. quinquefasciatus* larvae (control/susceptible strain) and determine the fold change and the elevation after Coomassie brilliant blue binding in the Bradford assays conducted (Table 2). The total protein content of the susceptible *Cx. quinquefasciatus* larvae (control) (1.29±0.13 mg) was lower compared with the Minden and Bayan samples at 1.58±0.19 mg and 1.61±0.15 mg respectively, showing a significant difference elevation at 1.22-fold in Minden and 1.24-fold change in Bayan respectively. Collectively, both the field samples show a significant elevation with the control at $P<0.05$ level of confidence. However, there was no significant difference between the field samples ($P=0.05$).

Table 2. Mean of Total Protein (mg) and Total detoxification enzyme activities (Mean±S.D) of Acetylcholinesterase (AChE), Esterase (α-est and β-est), Glutathione S-Transferases and Cytochrome P450 (CYP450) of different strains of *Culex quinquefasciatus* larvae

Developmental Stages	Total protein (mg)	TPF	AChE (nmol/min)	TAF	α-est (nmol/min)	TAF	β-est (nmol/mi)	TAF	GST (nmol/min)	TAF	CYP450 (nmol/min)	TAF
VCRU	1.29±0.13[a]	1.00	11.35±8.87[b]	1.00	59.31±9.91[a]	1.00	203.28±85.75[a]	1.00	7.04±0.46[a]	1.00	13.01±2.89[b]	1.00
Minden	1.58±0.19[b]	1.22	6.51±4.64[a]	0.57	617.84±175.51[b]	10.41	674.02±228.44[c]	3.31	12.66±1.67[b]	1.79	5.96±2.22[a]	0.45
Bayan	1.61±0.15[b]	1.24	7.46±4.70[a]	0.65	538.88±203.49[b]	9.08	472.20±170.52[b]	2.32	21.51±2.05[c]	3.05	5.15±1.68[a]	0.39

Mean and standard deviation (Mean±SD) of total protein contents and total enzyme activities of *Cx. quinquefasciatus* larvae (Control/susceptible strain) and the field samples of Minden and Bayan were obtained by one-way ANOVA. Superscripts indicate a significant difference with the control. TPF: Total protein fold-change. TAF: Total activity fold-change. Calculations of the fold changes were conducted by the division of each field strain by its control strain.

Total Enzyme Activities

The total enzymatic activities of AChE, α-esterase, β-esterase, GST and CYP450 of the field strains of Minden and Bayan with the VCRU (control/susceptible strain) were higher across all the field strains and different total enzyme activity fold changes (Table 2). High AChE activities were observed across all the field strains. The AChE total enzymatic activities of the control were 11.35±8.87 nmol/min while the result of the Minden was 6.51±4.64 nmol/min and Bayan 7.46±4.70 nmol/min. Both the Minden and Bayan total enzymatic activities were lower than the control enzymatic total activity with a fold change difference of 0.57-fold in Minden and 0.65-fold in Bayan respectively. Overall, there was a significant difference in the AChE total enzyme activity of the susceptible *Cx. quinquefasciatus* larvae (control/susceptible strain) with AChE of all the field strains at ($P<0.05$) confidence interval.

α-esterase, β-esterase and GST results of one-way ANOVA of Minden and Bayan were higher compared with their individual VCRU *Cx. quinquefasciatus* (control/susceptible strain) activities at $P<0.05$ level of significance. Therefore, high elevation of α-esterase of the Minden strain (617.84±175.51 nmol of 1-NA/min) and Bayan strain (538.88±203.49 nmol of 1-NA/min) were significant compared with the VCRU *Cx. quinquefasciatus* (control) (59.31±9.91 nmolof 1-NA/min) at a high fold change of 10.41-fold and 9.08-fold respectively. Furthermore, β-esterase activity was also higher in both the field strains. In the Minden strain, high-level total enzymatic activities of 674.02±228.44 nmol of 2-NA/min were observed and in Bayan high activity (472.20±170.52 nmol of 2-NA/min) was also observed. Both were compared with the VCRU *Cx. quinquefasciatus* of 203.28±85.75 nmol of 2-NA/min, high fold changes of 3.31-fold and 2.32-fold were observed, showing high activity in both the field strains tested. Moreover, GST activity was also higher in the Bayan strain (21.51±2.05 nmol/min) and in the Minden strain (12.66±1.67 nmol/min) when compared with the VCRU *Cx. quinquefasciatus* of 7.04±0.46 nmol/min. observing fold changes of 3.05-fold and 1.09-fold at ($P<0.05$) respectively.

Finally, the mean total enzyme activity of CYP450 of the VCRU *Cx. quinquefasciatus* was 13.01±2.89 nmol/min showing high activity compared with the field strains of Minden of 5.96±2.22 nmol/min and bayan of 5.15±1.68nmol/min. Overall, the result found a low fold-change activity of 0.45-fold in Minden and 0.39-fold in Bayan at $P<0.05$ respectively.

Specific Enzymes Activities

The specific enzymatic activities of *Cx. quinquefasciatus* was also presented in (Table 3). AChE-specific enzymatic activities of *Cx. quinquefasciatus* Minden strain (4.11±2.93 nmol/min/mg) and the Bayan strains of *Cx. quinquefasciatus* (4.62±2.91 nmol/min/mg). A comparison of both of the field strains of AChE-specific enzymatic activities shows there was no significant relationship at $P>0.05$. However, the susceptible VCRU strain of the *Cx. quinquefasciatus* (control) specific activity (8.79±6.87 nmol/minute/mg) was higher than both Minden and Bayan-specific activities. Overall, there was a highly significant difference in specific enzymatic activities between the VCRU strain of the *Cx. quinquefasciatus* and the Minden and Bayan specific activities ($P<0.05$) at the specific enzymatic activities fold changes of 0.46-fold and 0.52-fold respectively.

Table 3. The mean of detoxification enzymes specific activities (Mean±SD) of Acetylcholinesterase (AChE), Esterases (α-est) and (β-est), Glutathione S-Transferases (GST) and Cytochrome P450 (CYP450) of different field strains of *Culex quinquefasciatus* larvae

Cx. quinquefasciatus	**AChE (nmol/min/mg)**	**SAF**	**α-est (nmol of 1-NA/min/mg)**	**SAF**	**β-est (nmol of 2-NA/min/mg)**	**SAF**	**GST (nmol/min/ mg)**	**SAF**	**CYP450 (nmol/min/ µg)**	**SAF**
VCRU	8.79±6.87[b]	1.00	2.29±0.38[a]	1.00	7.87±3.23[a]	1.00	5.45±0.35[a]	1.00	10.07±2.23[b]	1.00
Minden	4.11±2.93[a]	0.46	19.51±5.54[b]	8.51	21.29±7.21[c]	2.70	8.00±1.05[b]	1.46	5.96±2.22[a]	0.59
Bayan	4.62±2.91[a]	0.52	16.69±6.30[b]	7.28	14.62±5.28[b]	1.85	13.33±1.27[c]	2.44	5.15±1.68[a]	0.51

Comparison of Mean and standard deviation (Mean±SD) of one-way ANOVA of the detoxification enzymes specific activities of the *Cx. quinquefasciatus* larvae (Control/susceptible strain) VCRU strain, Minden and Bayan field strains. Superscripts indicate a significant difference with the control at p=0.05. All calculations of the activities were made per individual mosquito and of the fold-change by the division of each field strain by the control strain. SAF: specific activity fold-change.

Furthermore, higher specific enzymatic activities of α-esterase in both Minden and Bayan strains were observed. In Minden, there was high α-esterase activity at 19.51±5.54 nmol of 1-NA/min/mg and in Bayan at 16.69±6.30 nmol of 1-NA/min/mg upon comparison with the control at 2.29±0.38 nmol of 1-NA/min/mg, both the Minden and Bayan show a high increase α-esterase specific enzymes activities fold change at 8.51-fold and 7.28-fold respectively. Also, there was a highly significant difference in the specific enzyme activities of both Minden and Bayan strains compared with the control (*P<0.05*). However, no significant difference was observed between α-esterase of Minden and Bayan observed at *P<0.05*. Moreover, in β-esterase specific enzyme activities of Minden and Bayan strain. High activity was observed in Minden at 21.29±7.21 nmol of 2-NA/min/mg and in Bayan at 14.62±5.28 nmol of 2-NA/min/mg. Comparing these results with the control at 7.87±3.23 nmol of 2-NA/min/mg. The result shows specific enzymatic activity fold changes of 2.70-folds in Minden and 1.85-folds in Bayan respectively. Overall, both the field strains of Minden and Bayan-specific activities were significantly different to the control (*P<0.05*). For GST-specific enzymatic activities of all the field strains, Minden (8.00±1.05 nmol/min/mg) and Bayan (13.33±1.27 nmol/min/mg) upon comparison with the control (5.45±0.35 nmol/min/mg) shows a fold change of 1.46-fold and 2.44-fold respectively. These results were also significantly different at *P<0.05*.

Finally, the specific enzymatic activities of CYP450 in all the field strains of *Cx. quinquefasciatus* in Minden (5.96±2.22 nmol/minute/µg) and Bayan (5.15±1.68 nmol/min/µg) were also observed. These specific enzymatic activities were compared with the *Cx. quinquefasciatus* (control) (10.07±2.23 nmol/min/µg) and the result shows low specific activities in both the Minden and Bayan at the fold change of 0.59-fold and 0.51-fold. But shows the existence of a significantly different relationship between the control CYP450 and both the Minden and Bayan CYP450-specific activities compared with the control (*P<0.05*). However, no significant relationship at *P=0.05* was finally observed between Minden CYP450 and Bayan CYP450-specific enzymatic activities respectively.

DISCUSSION

Susceptibility Status

The susceptibility status and the detoxification enzymes activity assay of the susceptible strain of *Cx. quinqefasciatus* developmental stages were conducted in this study. The 4th instar larvae of the susceptible strain were treated with serial doses of four different insecticides: malathion, primiphos methyl, temephos and deltamethrin for 24 hours following WHO guidelines (2005) and obtain the lethal doses that can kill 50% of the larvae (LC_{50}). These doses were further used as the control dose against the Minden and Bayan field strains in this study. Furthermore, detoxification enzymes biochemical analysis was further conducted on the larvae of the Minden and Bayan field strains. The result of the detoxification enzymes of the susceptible larvae of *Cx. quinqefasciatus* collected from VCRU was used as a control and compared with the results of the detoxification enzymes of the field strains of Minden and Bayan that were previously exposed to the various doses of insecticides during control intervention that have been periodically conducted which triggers the metabolic detoxification enzymes high activities in mosquitoes that functions in the detoxification of chemical insecticides and other xenobiotics into tolerable and more water soluble compounds through the process of conjugation, oxidation reduction and hydrolysis reactions that can easily be removed out of their bodies (Hamzah et al. 2022).

The results of the susceptibility tests conducted among the four different insecticides show higher efficacy in the deltamethrin-treated larvae in both the Minden and Bayan field strains indicating the existence of resistance in both the field strains. However, Bayan strains show the possibility of high resistance. This finding was similar to the recent study in Sabah and Sarawak where a new formulation of deltamethrin K-Othrine was used in the control of Malaria caused by *P. knowlesi* transmitted by the mosquito vector, *An. balabacensis*. This *Anopheles s*pecies was well known for its exophilic behaviour. The result of the study shows the high efficacy of the deltamethrin used in the IRS control intervention (Rohani et al. 2020). Similarly, deltamethrin was also shown to be highly effective in the control of dengue in Johor Bahru, Malaysia where various formulations of K-Othrine deltamethrin were used in outside residual spray (ORS) targeting the outdoor resting *Ae. aegypti* and *Ae. albopictus*. The findings of the study show a high effectiveness of deltamethrin towards the dengue vectors (Ab Hamid et al. 2020). In a recent study, *Cx. quinquefasciatus* was reported to be highly susceptible to temephos and deltamethrin in Jakarta, Indonesia. The result of the study shows 100% mortality of *Cx. quinquefasciatus* larvae (Subahar et al. 2022). Furthermore, the result of this study was also similar to the results of the previous study in India where deltamethrin was used in treating different strains of *Cx. quinquefasciatus* at army cantonment in different study sites. The strains of *Cx. quinqefasciatus* collected at Benganajuli and that of the Rikamari were shown to have developed a considerable deltamethrin resistance (Sarkar et al. 2009).

Total Protein Content Variation

The biochemical detoxification enzymes assay conducted also shows a high activity of α-esterase, β-esterase and GST with a clear indication of the presence of resistance phenotypes in all the study sites (Sarkar et al. 2009). Total proteins in mosquitoes increase after exposure to oxidative stress by insecticides resulting in sequestration or metabolism leading to resistance (Riaz et al. 2009). The amount of total protein contents obtained in the larvae (control) (1.29±0.13 mg) was shown to be less than the total protein amounts in the field strains Minden (1.58±0.19 mg) and Bayan (1.61±0.15 mg**)**. This increase in the total protein in the field strains shows clear high activity as a result of insecticide or xenobiotic exposure in the field caused as a result of the control interventions. The total proteins in the Minden (1.58±0.19 mg) and Bayan

(1.61±0.15 mg**)** strains were higher upon comparison with the susceptible control strain (1.29±0.13 mg**)** at varying fold changes of (1.22-fold) and (1.24-fold). Such an increase was greater than 1-fold due to the nature of the low level of resistance detected. These findings also agreed with the results of the previous study in Selangor, Malaysia on the multiple insecticide resistance in *Cx. vishnui* after treatment with malathion, temephos and permethrin which the result of the study shows a low level of resistance at RR<5 (Leong et al. 2014).

Furthermore, a recent study shows the existence of low resistance in *Cx. quinquefasciatus* after treatment with malathion and permethrin in Nigeria (Shehu et al. 2023). Moreover, a similar study on biochemical enzyme profiling in Thailand shows a higher pyrethroid resistance of up to (10-fold) after *Cx. quinquefasciatus* insecticide treatment (Pusawang et al. 2022). The high protein content activity of the field strain detected in this study was due to the vector control intervention measures taking place in the study area.

Total and Specific Enzymatic Activity

Variable amounts of total detoxification enzyme activities of AChE, α-esterase, β-esterase, GST and CYP450 were obtained across all the field strains. The enzymatic activity variation was due to the differential expression of enzyme isoforms and their densities (Hamzah & Alias, 2016). Previously, studies on other insects also affirm that the amount of enzymes and the detoxification activity varies after prolonged exposure to xenobiotics (Pedersen et al. 2020). Furthermore, specific detoxification enzyme activity signifies the induction of the pure detoxification enzymes by insecticides in conjugation, hydrolysis and oxidation-reduction processes. This activity process was commonly used to study insecticide induction activities in mosquitoes (Hemingway & Brogdon 1998).

Enzymatic Detoxification Activity in *Culex quinquefasciatus* Larvae

AChE is a crucial enzyme that hydrolyses acetylcholine (Ach) at cholinergic synapses, ensuring normal nerve signal transmission. Inhibition of AChE by organophosphates and carbamates leads to the accumulation of Ach, causing hyperexcitation, paralysis, and eventually death in mosquitoes (de Melo et al. 2018). Resistance associated with altered AChE activity has been documented in mosquito species like *Cx. quinquefasciatus*, *Ae. aegypti*, and *Ae. albopictus* (Farouk et al. 2021; Leong et al. 2019).

In this study, the total and specific AChE activities in Minden and Bayan field strains were significantly lower than the susceptible VCRU strains (control). Minden recorded 6.51±4.64 nmol/min and 4.11±2.93 nmol/min/mg (total and specific activity), while Bayan recorded 7.46±4.70 nmol/min and 4.62±2.91 nmol/min/mg, compared to the control (11.35±8.87 nmol/min and 8.79±6.87 nmol/min/mg, respectively). This reduction may impair synaptic function and is consistent with insect movement impairment and mortality (Santos et al. 2023). Environmental factors such as water pH and pollution levels may influence AChE activity. Zayed et al. (2019) observed lower AChE activity in larvae from freshwater compared to sewage environments. A similar downregulation in AChE expression was also reported in honey bees after RNA interference (Kim et al. 2023), supporting the idea that both genetic and environmental factors modulate AChE-mediated resistance.

Esterases detoxify insecticides by sequestering and degrading them. Their role in resistance is often linked to gene amplification and overexpression (Karunaratne et al. 2018). In this study, α-esterase and β-esterase activities were significantly higher in Minden and Bayan strains than in the control. Minden exhibited α-esterase activity of 617.84±175.51 nmol/min (total) and 19.51±5.54 nmol/min/mg (specific), with fold increases of 10.41 and 8.51,

respectively. Bayan strain showed 538.88±203.49 nmol/min (total) and 16.69±6.30 nmol/min/mg (specific), with 9.08 and 7.28-fold increases. Similarly, β-esterase activity in Minden (674.02±228.44 nmol/min for total; 21.29±7.21 nmol/min/mg for specific) and Bayan (472.20±170.52 nmol/min for total; 14.62±5.28 nmol/min/mg for specific) showed elevated values with 3.31/2.70 and 2.32/1.85-fold increases, respectively. These elevated esterase levels likely contribute to resistance against malathion and temephos observed in bioassays. Past research in *An. stephensi* and *Cx. quinquefasciatus* identified esterase gene families like CPIJ018232–CPIJ018233 as upregulated in resistant populations (Strode et al. 2006; Vivekanandhan et al. 2021). Excessive fogging and larviciding in the study areas may have been selected for these traits (Rahim et al. 2016). Additionally, Bharati & Saha (2018) reported a 12-fold overexpression of α-esterase in Indian field strains of *Cx. quinquefasciatus*. Elevated esterase activities have also been found in *Ae. aegypti* and *Ae. albopictus* populations exposed to routine insecticide application (Jangir & Prasad 2022).

GSTs play a key role in detoxification by catalysing the conjugation of toxic compounds with glutathione, making them more water-soluble and easier to excrete (Enayati et al. 2005). They are cytosolic enzymes involved in metabolizing insecticides, oxidative by-products and other xenobiotics (Hamzah et al. 2019). GSTs contribute to resistance against multiple insecticide classes, including organophosphates, carbamates, organochlorines, and pyrethroids (Jin et al. 2023; Tao et al. 2022). This study showed significantly elevated GST activities in both Minden and Bayan strains. Minden had total and specific activities of 12.66±1.67 nmol/min and 8.00±1.05 nmol/min/mg (1.79- fold and 1.46-fold increases), while Bayan recorded 21.51±2.05 nmol/min and 13.33±1.27 nmol/min/mg (3.05- fold and 2.44-fold increases) compared to the control (7.04±0.46 nmol/min/mg). These elevations are consistent with GST's known function in resistance and stress adaptation. In *Apis mellifera macedonica*, both acidic and alkaline GST isoenzymes protected bees against environmental stressors (Papadopoulos et al. 2004). Studies also show that GSTs eliminate oxidative stress by-products (Hassan et al. 2019), reinforcing their importance in insecticide resistance mechanisms (Song et al. 2022).

CYP450 enzymes are central to detoxifying insecticides, metabolizing endogenous compounds, and enabling mosquito adaptation to toxic environments (Liu et al. 2015). Resistance develops through overexpression of CYP450 genes, leading to enhanced metabolism of xenobiotics (Gao et al. 2023). In this study, however, Minden and Bayan strains showed decreased CYP450 activity compared to the control. Minden recorded specific activities of 5.96±2.22 nmol/min/mg (0.59-fold decrease), while Bayan showed 5.15±1.68 nmol/min/mg (0.51-fold decrease) compared to control values of 10.07±2.23 nmol/min/mg. This decline may relate to the developmental stage of larvae or inhibitory effects from environmental factors or plant-derived compounds. For instance, compounds like MTT flavonoids and pentachlorophenol have been shown to suppress CYP450 activity (Kotewong et al. 2014; Lin et al. 2022). Similarly, reduced CYP450 activity has been observed in permethrin-treated larvae of the diamondback moth (Bautista et al. 2009).

CONCLUSION

Bioassay results confirmed resistance in both Minden and Bayan *Cx. quinquefasciatus* strains across four insecticides: malathion, pirimiphos-methyl, temephos, and deltamethrin, with the highest resistance observed for deltamethrin. The resistance mechanisms were supported by elevated enzymatic activities of AChE (though reduced compared to the susceptible strain), esterase (both α and β), and GST, suggesting their significant roles in detoxification.

Conversely, CYP450 activity was reduced, which may reflect life stage-specific expression or inhibition by environmental or chemical factors. These findings highlight the complex interplay of multiple detoxification enzymes in conferring resistance in field strains and underscore the need for integrated vector management approaches to mitigate resistance development in mosquito populations.

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AUTHORS DECLARATIONS

Author Contribution Declaration

Conceptualization, SNH, SAR; Data curation, AB, SNH; Formal analysis, AB, SWA; Investigation, AB; Methodology; SNH, AB; Project administration, SAR, SNH; Resources, SNH; Writing -original draft; SA, SNH; Writing—review and editing, SNH.

Conflicts of Interest

The authors declare no conflict of interest.

Ethics Declarations

This study did not require ethical approval, as it only involved insect organisms.

Data Availability Statements

All data generated or analyzed during this study are included in the article.

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