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ORIGINAL PAPER

Laboratory development of permethrin resistance and cross-resistance pattern of *Culex quinquefasciatus* to other insecticides

Govindaraju Ramkumar[1] · **Muthugoundar S. Shivakumar**[1]

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Abstract Resistance of mosquitoes to insecticides is a growing concern in India. Since only a few insecticides are used for public health and limited development of new molecules is expected in the next decade, maintaining the efficacy of control programs mostly relies on resistance management strategies. Developing such strategies requires a deep understanding of factors influencing resistance together with characterizing the mechanisms involved. Among factors likely to influence insecticide resistance in mosquitoes, agriculture and urbanization have been implicated but rarely studied in detail. In the present study, we evaluate the permethrin resistance and cross-resistance pattern of several insecticides in *Culex quinquefasciatus* mosquitoes. After 10 generation of selection with permethrin, the LC_{50} value for both larvae and adult *Cx. quinquefasciatus* was increased by 17.3- and 17.1-folds compared with susceptible strain. Detoxification enzyme profiles and native PAGE electrophoresis of esterase isoenzyme further revealed that esterase and CytP450 may be involved in permethrin resistance (PerRes) strain compared with susceptible strain. In addition to cross-resistance, study revealed that high resistance to cypermethrin (RR=6.3, 8.8-folds). This study provided important information for understanding permethrin resistance and facilitating a better strategy for the management of resistance. These studies conclude that a strong foundation for further study of permethrin resistance mechanisms observed in *Cx. quinquefasciatus* mosquitoes.

✉ Muthugoundar S. Shivakumar
skentomol@gmail.com

[1] Molecular Entomology Laboratory, Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India

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Introduction

Mosquitoes are among the most important insect vectors that transmit numerous devastating insect-borne diseases, such as malaria (White et al. 2011), dengue (Gubler et al. 2009), yellow fever (Peng et al. 2011), filariasis (Koelle et al. 2008), West Nile fever (Styer et al. 2010), and chikungunya (Powers et al. 2011), thereby threatening public health. Vector-borne diseases account for about 17 % of the estimated global burden of infectious diseases (WHO 2006). Therefore, considerable efforts have been taken to fight against these diseases, including drug development, vaccine research, and vector control (Molyneux et al. 2011).

Chemical control has been the main effective measure to reduce the population of these disease vectors since the 1950s (Hemingway et al. 2006). Four classes of chemical insecticides are the mainstay of vector control programs, namely, organochlorines, organophosphates, carbamates, and pyrethroids. Pyrethroids account for approximately 25 % of the world insecticide market and are used extensively because they kill insects rapidly and have low mammalian toxicity. As per WHO, insecticide resistance is defined as "The development of an ability in a strain of an organism to tolerate doses of toxicants, which would prove lethal to a majority of individuals in a normal (susceptible) population of the same species." Elucidation of resistance mechanisms becomes crucial to guide the use of permethrin and the development of its substitutes, and should be considered one of the most challenging issues in modern applied entomology (Muthusamy et al. 2013; Hansen et al. 2012; Nazni et al. 2000). However, a major control problem is that these species have developed resistance to all major

insecticides, including pyrethroids (Mebrahtu et al. 1997) which has led to failures in vector-borne disease control, especially in dengue control (Strode et al. 2008).

Resistance mechanisms in mosquitoes have been extensively studied and are known to be predominantly classified into two classes: metabolic resistance (degradation of the active ingredient by detoxification enzymes) and target site resistance (mutations in the target proteins). A number of genes associated with insecticide resistance, including cytochrome P450, esterases, elevated glutathione S-transferase (GST), and sodium channel gene were identified (Nardini et al. 2012; Hu et al. 2011). In earlier studies, many researchers identified multiple resistance mechanisms, such as P450- and GST-mediated detoxification and target site insensitivity, which were involved in pyrethroid resistance in this *Cx. quinquefasciatus* strain (Xu et al. 2008). Many studies have been conducted throughout the world to understand the mechanisms of pyrethroid resistance in insects, especially in mosquitoes. Biochemical and molecular methods have been used to detect resistance mechanisms in these vectors. Using biochemical methods, the increase of enzyme activities of P450-mediated monooxygenases and mixed function oxidases (MFOs) has been reported to play a role in the metabolism of pyrethroids (Muthusamy and Shivakumar 2014). Permethrin is a broad-spectrum pyrethroid insecticide and has been widely used in the vector control program throughout the world. As a consequence, it is believed that permethrin resistance has developed in mosquitoes, especially among filariasis vectors in throughout world. In the present study, an effort was taken to understand the possible resistance mechanisms to permethrin in the filariasis vectors *Cx. quinquefasciatus* larvae and adult mosquitoes.

Materials and methods

Mosquitoes

Two strains of *Cx. quinquefasciatus* were used for this study: a laboratory strain (Sus) and permethrin-selected strain (PerRes). The laboratory strain was used as a reference in this study. It was originally collected in NCDC, Mettupalayam, Tamil Nadu, India. It is free from exposure to any insecticides. The permethrin-selected strain of *Cx. quinquefasciatus* which was also collected in Salem district, Tamil Nadu, India (July 2011) was continuously exposed to permethrin for 1 year (Aug 2011–Dec 2012).

Insecticide

For the WHO larval and adult bioassay, commercial grade of permethrin (25 % EC) was purchased from Coromandel Fertilizers Ltd., Secunderabad, India

WHO larval bioassay

The larval bioassay was performed according to the standard WHO susceptibility or resistance test protocol (WHO 1981).Twenty-five early fourth (4th) instar larvae were introduced into 250 mL of test solution containing permethrin and ethanol in a 300-mL paper cup for 24 h. The concentrations were obtained by diluting commercial grade of permethrin stock solution (25 % EC). For the control, 250 mL distilled water alone were used. Concentrations of permethrin causing 10 to 90 % mortality were used in this bioassay. The experiments were replicated three times per concentration. The mortality of larvae was assessed after 24 h. Larvae were considered dead if they sank to the bottom of the paper cups and failed to move or float after being probed (Kasai et al. 2007; Hardstone et al. 2009).

WHO adult bioassay

The adult mosquito bioassay was performed according to the Standard WHO susceptibility or resistance test protocol (WHO 1981; CDC 2004; Ramkumar et al. 2015). Using a clean glass test tube coated with diagnostic dosage (0.25 %) of permethrin recommended by WHO and allow to fifteen (15) sucrose-fed 3–5 days old adult mosquitoes per replicate. For the control, 250 mL distilled water alone were used. There were three (3) replicates per bioassay. All mosquitoes were exposed to the diagnostic dosage of permethrin and for the respective exposure period. Cumulative mortality counts were recorded every minute during the exposure time. After the exposure period, the mosquitoes were held for a 24-hour recovery period before the mortality was recorded again. Sucrose solution was provided for the mosquitoes. All survivors were collected and kept at −70 °C before being used in the enzyme biochemical assay.

Selection of permethrin-resistance (*PerRes*) strain

Based on the initial bioassay result, 4th instar larvae of *Cx. quinquefasciatus* laboratory colony were subjected to resistance selection with permethrin (PerRes) using the WHO, bioassay method. Unselected control was treated identically, without exposure to any insectides. The concentration of insecticides used for resistance selection in each generation was based on the result of bioassays LC_{50} from the previous generation. Surviving larvae were kept in plastic trays containing tap water and were maintained in the laboratory, and all the experiments were carried out at 27±2 °C and 75–85 % relative humidity under 14:10 light and dark photoperiod cycles. Larvae were fed with dog biscuit and yeast powder in the ratio of 3:1. They were maintained and reared in the mosquito cage.

Preparation of enzymes

Whole body of 4th instar larvae and adult mosquitoes were used for enzyme preparation. The tissue homogenized with 1.5 ml of 0.1 M ice cold sodium phosphate buffer (pH 7.2). After centrifugation at 10,000 rpm for 30 min, the supernatant was recentrifuged at 12,000 rpm for 15 mins. Then the supernatant was collected and used as enzymes for analysis of the activity of esterase, P450, and GST. All enzymes assays were carried out on ice box.

Total protein

Total protein content of the tissue homogenate was determined by Lowry et al. (Lowry et al. 1951) using bovine serum albumin as the standard.

Detoxification enzymes assay

Mixed-function oxidase

The MFO activity was determined using peroxidation of tetramethylbenzidine (TMBZ) assay according to Brogdon (1989) with slight modifications. Two-fifty microliter of 0.05 M potassium phosphate buffer (pH 7.0) were added to 50 μl microfuged supernatant and 500 μl tetramethylbenzidine solution (0.05 % 3,3′,5,5′ tetramethylbenzidine, i.e., TMBZ+ 5 ml methanol+15 ml sodium acetate buffer 0.25 M pH 5.0). Two-hundred microliter of 3 % hydrogen peroxide were added, and the mixture was incubated for 30 min at room temperature. Absorbance was read at 630 nm and values calculated from a standard curve of cytochrome C.

Glutathione S-transferase

Glutathione S-transferase assay were performed according to the method developed by Kao et al. (1989) using 1-chloro 2, 4-dinitrobenzene and reduced glutathione (GSH) as substrate. The total reaction solution contained 2.79 ml of phosphate buffer saline 0.1 M pH −6.5, 10 μl of diluted enzyme supernatant (the stock solution was diluted 10-fold with0.1 M pH −6.5, sodium phosphate buffer), 50 μl of 50 mM CDNB (dissolved in the 0.1 % (*v/v*) ethanol), and 150 μl of reduced glutathione in Tris HCL (0.05 M, pH 7.5). The changes in absorbance were measured continuously for 5 min at 340 nm using the time scan mode of UV-visible spectrophotometer.

Esterase activity

Esterase activity was determined using the method described by Kranthi (2005). The reaction mixture contains 0.1 ml of supernatant (10 μl of enzyme sample with 99 μl of 40 Mm PBS pH 6.8) was added to the 15 ml clean test tube, containing 5 ml of substrate (5 mg of 30 mM α-napthyl acetate/ml of acetone dissolved in 99 ml of 40 mM PBS), tubes were incubated in dark for 20 min at 30 °C. Then 1 ml of staining solution (5 % SDS and 1 % fast blue BB salt dissolved in 16 ml of sodium phosphate buffer pH 6.8) were added to the reaction tube in the ratio of 1:5. The esterase activity was measured continuously at 590 nm for 10 min. The esterase activity was calculated as micromole/min/mg protein by using 1-napthyl as the standard.

Esterase isoenzyme analysis

Non-denaturing PAGE was carried out following the method of Kranthi (2005) on vertical polyacrylamide gel electrophoresis using the 10 % separating gel and 4 % stacking gel with continuous Tris-glycine buffer running system (50 mM, pH 8.3). Ten microliters of each midgut sample containing 20 μg protein was diluted with 8 μl of 1× sample buffer (1.5 M Tris HCl, pH 6.8, 30 % *v/v* glycerol, 0.02 % bromophenol blue) before loading. Electrophoresis was carried out at a constant current of 75 V at 5–8 °C for approximately 1.5 to 2 h. Gel was stained by incubating for 30 min in a 2 % α-napthyl acetate (in 40 mM phosphate buffer pH 6.8), which contain 1 % acetone, and then placing the gels in 1 % (*w/v*) fast blue BB salt for 1 h. Staining reaction was stopped by adding the gel in 5 % acetic acid solution. After staining, the gel was visualized and photographed in gel documentation unit (Alpha Inotech, USA).

Cross-resistance pattern of permethrin to different insecticides

Cross-resistance of the susceptible (Sus) and permethrin-selected strain (PerRes) was determined at 10th generation for both larval and adult *Cx. quinquefasciatus* mosquitoes use to conventional and new chemical insecticides. These applications were also performed in sets of one control and five insecticides with three replications of each. For each insecticide tested, the cross-resistance rate was calculated as the ratio of the LD_{50} value of the PerRes population to the LD_{50} value of the susceptible population (Sus) (Tikar et al. 2009).

Statistical analysis

For bioassay data, the LC_{50} and their confidence interval were estimated by Probit Analysis using the SPSS software (version 16.0). Resistance ratio (RR) were estimated at the LC_{50} level as RR=LC_{50} of collected field (or resistance) strain/LC_{50} of susceptible strain. The synergism ratios (SR) were calculated as follows: SR=LC_{50} value of insecticide alone/LC_{50} value of insecticide after synergist. All the enzyme assays above were with three replications. The data obtained from enzyme

Table 1 Toxicity of three insecticides against 4th instar *Cx. quinquefasciatus* mosquito larvae

Insectides	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	*df*
Neem	375	1±0.5	41.12 (38.7–42.1)	0.62	3
Permethrin	375	1.2±0.8	40.4 (38.2–47.2)	1.3	3
Temephos	375	1.8±0.02	48.2 (46.7–50.2)	1.1	3

LC_{50} lethal concentration for 50 % killing of the exposure larvae, *C.L.* confidence limit (95 %), n^a number of larvae, *S.E.* standard error, *G* generation, χ^2 chi-square, *df* degrees of freedom

assays were subjected to analysis of variance followed by Bonferroni multiple comparison post hoc test using PRISM 5 software (Graph Pad Software Inc, USA).

Results

Insecticides toxicity

The toxicity of three insectides to the 4th instar larvae *Cx. quinquefasciatus* mosquito was tested using WHO, protocol. The result showed that larvae were most sensitive to permethrin compare with other insecticides (Table 1) but adult bioassay result showed tolerant to permethrin (Table 2). Therefore, the subsequent generations were concentrated on comparison of resistance risk evaluation in *Cx. quinquefasciatus* larvae to permethrin

Permethrin resistance in *Cx. quinquefasciatus*

After 10 generation of selection with permethrin, the larvae were resistance ratio increased by 17.3-fold and adult resistance ratio by 17.1 compared with susceptible strain (Table 3 and 4). This result suggests that *Cx. quinquefasciatus* mosquitoes more readily developed resistance to permethrin.

Detoxification enzymes activities

The detoxification enzymes activities of esterase and MFO may contribute to permethrin resistance in *Cx. quinquefasciatus* was sought by studying the specific activity of detoxification enzymes. There were significant differences in MFO activities between the permethrin-selected strain and laboratory strain of *Cx. quinquefasciatus* both larval and adult mosquitoes. Esterase activity was significantly increased ($p< 0.05$) in resistant strain (Figs. 1 and 2). There was no correlation of GSTs activities. This suggested that the resistance was associated with esterase and MFO.

Table 2 Toxicity of three insecticides against *Cx. quinquefasciatus* adult mosquitoes

Insecticides	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	*df*
Neem	300	1.2±0.5	43.81 (41.0–45.3)	0.63	2
Permethrin	300	1.3±0.8	51.57 (49.1–54.2)	0.85	2
Temephos	300	1.8±0.02	36.82 (34.6–39.4)	0.96	2

LC_{50} lethal concentration for 50 % killing of the exposure adults, *C.L.* confidence limit (95 %), n^a number of adult, *S.E.* standard error, χ^2 chi square, *df* degrees of freedom

Table 3 Development of permethrin resistance in 4th instar *Cx. quinquefasciatus* mosquito larvae

Strains	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	*df*	RR*
Susceptible	375	0.5±0.2	5.42 (3.48–7.62)	0.638	2	–
PerRes	375	2±0	93.8 (92.1–96.8)	3.1	2	17.3

LD_{50} lethal concentration for 50 % killing of the exposure larvae, *C.L.* confidence limit (95 %), n^a number of larvae, *S.E.* standard error, χ^2 -chi square, *df* degrees of freedom, *RR** resistance ratio -LC_{50} of the selected generation/LC_{50} of the original generation

Native page

Native page electrophoresis of esterase banding pattern revealed six bands in permethrin-resistant larvae. Similar pattern also revealed in resistant adult mosquito, whereas in susceptible strain, the number of esterase bands reduced into 5. However, the staining intensity was very low in susceptible strain compare to the resistant strains (designated as Est 4 and Est 5) (Fig. 2).

Cross-resistance pattern

The toxicity result of the susceptible and PerRes exposed to 5 different insecticides. The PerRes showed high-level development of cross resistance to cypermethrin RR=6.3, 8.8 folds (Table 5 and 6).

Discussion

While an enormous amount of information on the inheritance and stability of insecticide resistance in mosquito and other

Table 4 Development of permethrin resistance in *Cx. quinquefasciatus* mosquitoes

Strains	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	*df*	RR*
Susceptible	300	0.8±0.01	4.19 (3.6–6.2)	0.86	2	–
PerRes	300	2±0.02	71.8 (69.1–75.8)	2.81	2	17.1

LD_{50} lethal concentration for 50 % killing of the exposure adults, *C.L.* confidence limit (95 %); n^a number of adult, *S.E.* standard error, χ^2 chi-square, *df* degrees of freedom, *RR** resistance ratio -LC_{50} of the selected generation/LC_{50} of the original generation

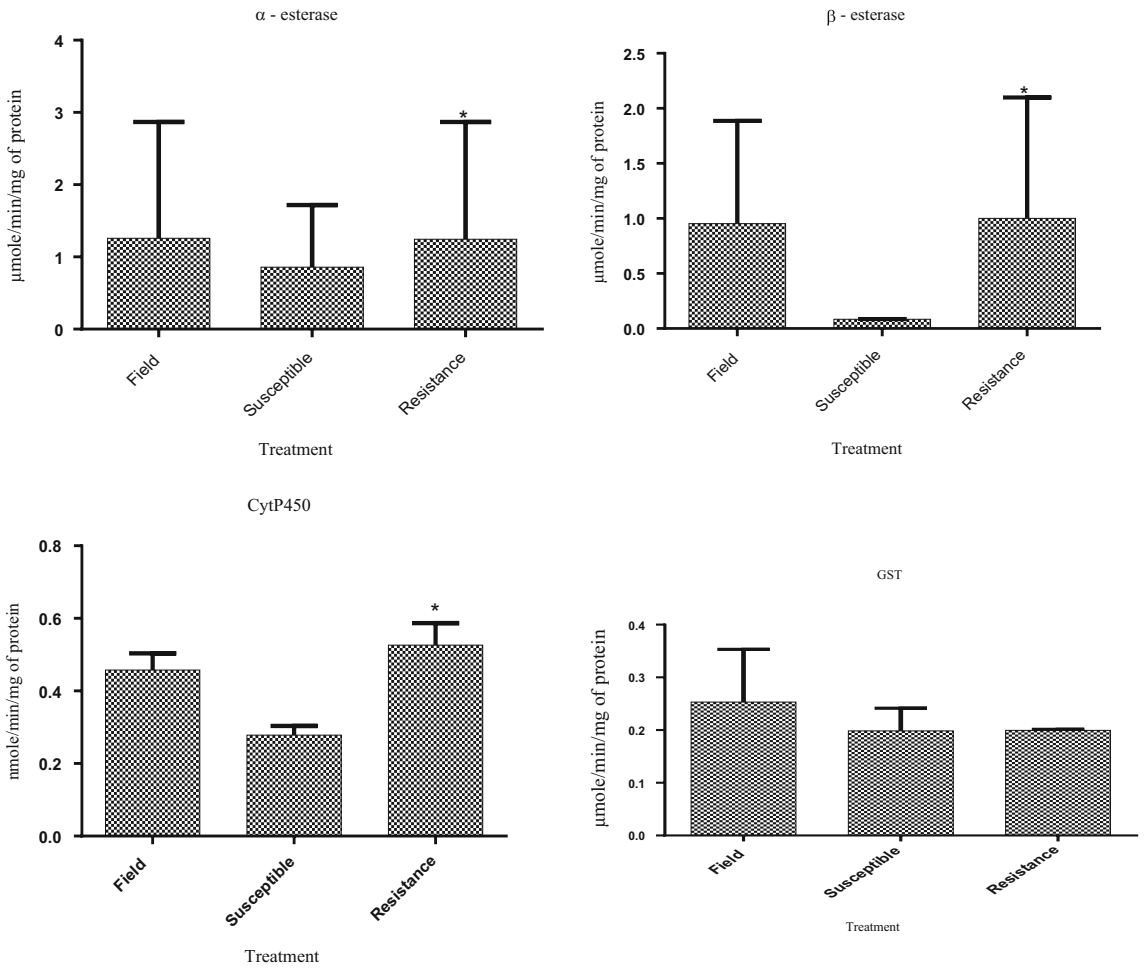

Fig. 1 Detoxification enzyme activities between the field- and permethrin-selected resistance strains compared with susceptible strain larvae of *Cx. quinquefasciatus*. The *asterisk* (*) on the top of the *bars* indicate statistically significant differences ($p < 0.05$; one-way ANOVA with Dunnett's multiple comparison test)

insects is available in the literature, most of these studies are based on general bioassays which demonstrate only the phenotypical aspects of the phenomenon. The present study investigated the resistance mechanism in *Cx. quinquefasciatus* mosquito against permethrin insecticide. Pyrethroid insecticides used for wide range control of mosquito throughout the world. Pyrethroid insecticides are considered as most successful chemical classes of insecticide (Muthusamy et al. 2014). In the present work, the bioassay results confirmed that permethrin insecticide exhibited insecticidal activity in larvae and adult *Cx. quinquefasciatus* mosquito. These results provide evidence to resistance selection of permethrin can occur after few successive generations to develop resistance risk to permethrin (Table 1 and 2). Biochemical-based insecticide resistance mechanism involved by esterase activity against *Culex quinquefasciatus* (Swain et al. 2008, 2009)

In the past several decades, known metabolic resistance mechanisms contributed to insecticide resistance and certain detoxification enzymes, including esterase, MFO, and GST families, have been extensively investigated (Brogdon 1989; Shankarganesh et al. 2012). However, resistance mechanism underlying in *Cx. quinquefasciatus* against pyrethroid is mostly not reported elsewhere. Hemingway et al. (2004) suggested the involvement of cytochrome P450 and esterase in pyrethroid insecticides in mosquitoes. Conversely, Gunning et al. (1991) and Feyereisen (2005) also reported the association between Cytp450 played and development of pyrethroid resistance in insects.

Permethrin resistance in *Cx. quinquefasciatus* mosquito larvae is revealed that α esterases activity had changed in permethrin resistance strain (PerRes). But β esterases activity remained unchanged. It indicates that α esterases, (not β esterases), are responsible for the resistance to permethrin at larval stage. But low α and β esterase activity was observed in adult mosquitoes. This result suggest that permethrin selected strain may be tolerant to permethrin at larval stage, but does not lead to a significant tolerance in adults (Figs. 1 and 2). Pyrethroid resistance owing to increased level of

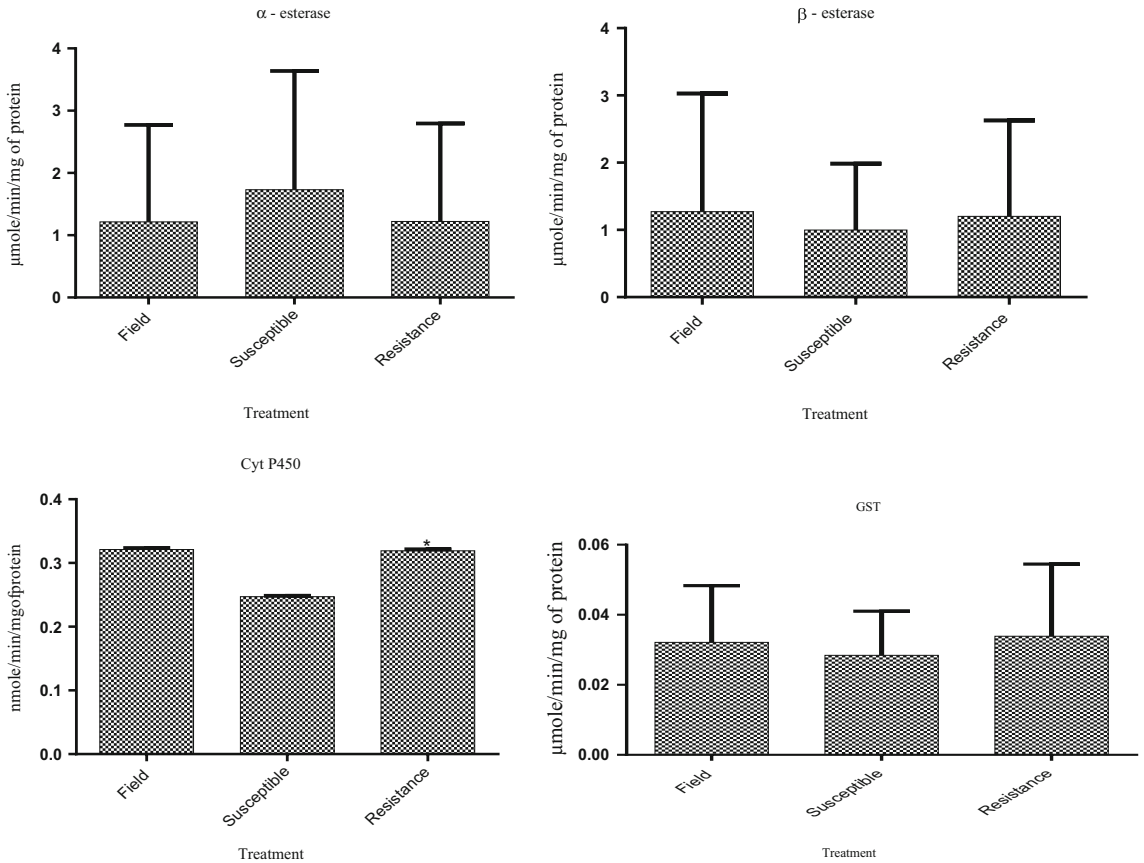

Fig. 2 Detoxification enzyme activities between the field- and permethrin-selected resistance strains compare with susceptible strain adult *Cx. quinquefasciatus*. The *asterisk* (*) on the top of the *bars* indicate statistically significant differences ($p < 0.05$; one-way ANOVA with Dunnett's multiple comparison test)

detoxification enzymes has been detected in *Ae. aegypti* (Urmila et al. 2009). Muthusamy and Shivakumar 2015 reported metabolic detoxification enzymes mediated lambda cyhalothrin resistance in *Cx. quinquefasciatus* (Tan et al. 2012). Native PAGE electrophoresis revealed considerable increase in the number of the esterase bands in PerRes strain. Similarly, intensity of staining is also very high compare to that of susceptible strain (Fig. 3). High number and intensity of esterase banding pattern in native gel is commonly associated with pyrethroid resistance. Similarly high esterase activity has been associated with cypermethrin resistance in *Cx. quinquefasciatus*, *Blattella germanica*, and *Tetranychus urticae* (Sahgal et al. 1994; Anspaugh et al. 1994; Recep and Sibel 2012). Similarly, Somwang et al. (2011) reported that enzyme-based resistant mechanism involving pyrethroid resistant and susceptible *Aedes aegypti* strains.

The mixed function oxidases (MFOs) are commonly involved in pyrethroid (PY) and organophosphate (OP)

Table 5 Cross-resistance pattern of five insecticides against permethrin selection resistance strain of 4th instar *Cx. quinquefasciatus* mosquito larvae

Insecticides	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	RR*	*df*
Neem	375	1±0.5	36.3 (32.5–45.3)	1.22	1.1	3
Temephos	375	1.8±0.02	36.8 (34.4–48.6)	2.06	1.3	3
Dichlorvos	375	2.0	40.12 (38.8–54.6)	0.36	3.2	3
Deltamethrin	375	7.3±0.3	47.9 (41.15–64.8)	3.3	5	3
Cypermethrin	375	2.2±0.8	52.9 (44.1–74.7)	2.8	6.3	3

**RR* resistance ratio, determined by dividing the LD50 of resistant strain by LD50 of susceptible strain, *LD50* lethal dose concentration that kills 50 % of the exposed larvae, *C.L* confidence limit

Table 6 Cross resistance pattern of five insecticides against permethrin selection resistance strain of adult *Cx. quinquefasciatus* mosquito

Insecticides	n^a	Slope±S.E.	LC_{50} (95%CL ppm)	χ^2	RR*	*df*
Neem	300	1.2±0.8	29.7 (34.8–46.2)	3.01	1.25	2
Temephos	300	2.0	38.7 (34.8–46.2)	2.17	7.7	2
Dichlorvos	300	11.6±0.1	34.1 (31.1–41.02)	2.8	6.6	2
Deltamethrin	300	1.3±0.05	22.9 (21.7–25.4)	1.18	8	2
Cypermethrin	300	1±0.5	27.4 (25.9–30.6)	0.21	8.8	2

**RR* resistance ratio, determined by dividing the LD50 of resistant strain by LC50 of susceptible strain, *LC50* lethal dose concentration that kills 50 % of the exposed larvae, *C.L* confidence limit

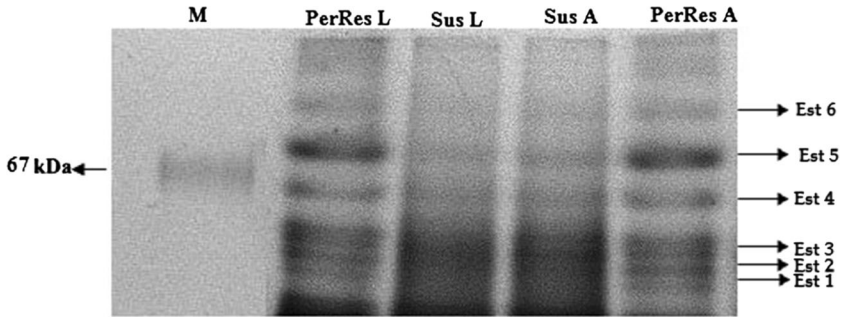

Fig. 3 Native PAGE profile of esterase isoenzymes from permethrin-selected resistance strain larvae and adult *Cx. quinquefasciatus* compare with susceptible strain. *M* marker, *PerRes L* permethrin resistance strain larvae, *PerRes A* permethrin resistance strain adult, *Sus L* susceptible larvae, *Sus A* susceptible adult

insecticide resistance to a lesser extent, and elevated levels of these enzymes are usually associated with the enhancement of resistance (Miller 1998; Scott et al. 1998). Our results showed that resistance to the PY, permethrin, was due to the MFO enzymes as demonstrated by the altered or incipiently altered MFO levels. The MFOs may also have played a role in the resistance to permethrin. Similar studies done by Paeporn et al. (2004) found increase of MFO and esterase levels in *Ae. aegypti* strains, selected for permethrin resistance. Rodríguez et al. (2000, 2007) also reported that MFOs and esterases were important in resistance to OP and Malathion insecticides in *Culex quinquefasciatus* populations.

Elevated glutathione S-transferase (GST) has been primarily associated with organochlorine resistance in insect species including houseflies and mosquitoes (Hemingway et al. 2004). GSTs were overproduced organochlorine resistant strain of *Ae. aegypti* (Grant et al. 1991). Our results showed no changes of GST activity. These results suggest that detoxification by GSTs may not be involved in permethrin resistance. Fonseca-González et al. (2009) reported that identification of specific resistance mechanism can be carried out through enzymatic measurements or molecular tools, and this information is required for the identification of alternative insecticide. Our results suggest that esterase and MFO detoxification enzymes are major plays involved in permethrin resistance. Our result shows cross-resistance study revealed that high resistance to cypermethrin (RR=6.3, 8.8-folds). The finding of the cross-resistance study is agreed with Cheng Guilin and Yanchou (1995) who found cross resistance to several other pyrethroids in pyrethroid selected *Helicoverpa armigera*. It has been clearly observed that permethrin resistance in larval stages is expressed in adult stages also. Tikar et al. (2009) similarly reported that temephos induced resistance and also cross resistance to other insecticides in *Ae. aegypti* mosquitoes. The high level of expression in adult stages shows the need to design the resistance management strategy. The cross-resistance patterns to pyrethroid compounds exhibited increased but fluctuating trend of cross-resistance to all compounds of this chemical group. It could be stated that continues selection pressure of permethrin against *Cx. quinquefasciatus* can lead to development of resistance. Our data provided into account for pest management measures implemented by all economic actors of a region in order to develop efficient vector control strategies.

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