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Application of synthetic insecticide and change in detoxifying enzyme levels in *Culex quinquefasciatus* Say

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Abstract
The mosquito populations are increasing day by day in Chennai, one of the filarial endemic regions in Tamil Nadu, India due to the unplanned growth of cities, increased and improper usage of insecticides, development of resistance in target insects etc. Continuous and extensive uses of chemical insecticides lead to selection of resistant mosquitoes in the environment. The early detection of resistance in vector mosquitoes will help the local government to plan and select appropriate alternative control measures or insecticides for effective control. Quantitative metabolic enzymes assay have been commonly used in the detection of insecticide resistance because it is very sensitive and gives results rapidly even at low frequencies. Elevation in detoxifying enzyme levels indicates the status of insecticide resistance. The present study compares the detoxifying enzyme levels of *Culex quinquefasciatus* Say of Chennai with laboratory population. The results shows the samples collected from the Chennai corporation shows 1.85, 1.79, 1.71 and 1.48 fold increase in α and β esterases, GST and MFO levels respectively. The % remaining activity of AChE in Propoxur inhibited fraction was 89.84 in field population indicates the organophosphate and carbamate resistance. The study highlights rise of multiple insecticide resistance in *Cx. quinquefasciatus* of Chennai and the urgent need to rapidly implement resistance management strategies by improving vector control measures using alternative ecofriendly techniques.

Keywords: Cx. quinquefasciatus, insecticide resistance, α and β esterases, glutathione-S-transferase, mixed function oxidases, acetylcholinesterase

1. Introduction
The rapid urbanization in most of the developing countries has resulted in breeding of the culicine species, especially the filarial vector *Culex quinquefasciatus* Say (Diptera: Culicidae). *Culex quinquefasciatus* is one of the major domestic pests in urban areas and carry *Wuchereria bancrofti*, the lymphatic filarial worm and many arboviruses [1]. National Health Mission Tamil Nadu reported that In Tamil Nadu, 13 districts are said to be endemic to filariasis, and Chennai is one among them. As per the reports of Directorate of Public Health and Preventive Medicine Chennai, in 2005 the microfilarial rate was 0.38% and dropped to 0.07% in 2011 and rose again to 0.11% in the year 2015. Last few years a multi fold increase in mosquito numbers reported in Chennai corporation area. In Chennai, pyrethrum and malathion is used during the fogging operations and temephos for spraying as larvicide. The use of synthetic insecticides to control insect vectors has led to selection of resistant insect population and hence the control measures fails to work properly. The major metabolic enzymes involved in resistance in mosquitoes include Cytochrome P450 mediated monooxygenase, non-specific esterases, acetylcholinesterases [2] and glutathion-s-transferase [3]. Quantitative metabolic enzymes assay have been commonly used in the detection of insecticide resistance because it is very simple, sensitive and gives results rapidly even at low frequencies[4,5].The elevated levels of esterases contribute the resistance of mosquitoes towards organophosphates, carbamates and pyrethroids [6]. Glutathione –S- transferase involved in the resistance towards organophosphates, organochlorines and pyrethroids [7-9]. The increased levels of mixed function oxidases are responsible for the resistance in mosquitoes to organophosphates, organochlorines, carabamates and pyrethroids; the widely using four major classes of insecticides [10]. The insensitive acetylcholinesterase plays a major role in organophosphate and carbamate resistance [11, 12].
The present study is to investigate the potential resistance mechanisms involving carboxylesterases, mixed function oxidases, glutathione-S-transferase and insensitive

Acetyl cholinesterase in *Cx. quinquefasciatus* from Chennai, Tamil Nadu, India. A better understanding of the prevailing insecticide resistance mechanisms could serve as a justification for changes in control measures and provide baseline data for population monitoring in accordance with appropriate insecticides.

2. Materials and Methods

2.1 Collection and rearing of mosquitoes: Mosquito larvae were collected from Chennai corporation area. The larvae were collected from insecticide regularly spraying area (RTS) and scarcely spraying area (UTS). The collected larvae were fed with dog biscuit and yeast powder in the ratio 3:1. Larvae were identified using taxonomical keys and emerged adults were also identified. The 4th instar larvae of F1 generation were used for the analysis. Susceptible laboratory population (LS) were collected from CDRL (Communicable Disease Research Laboratory), Irinjalakkuda, Thrissur and reared in the laboratory without exposing to synthetic insecticides.

2.2 Biochemical test: Individual fourth instar larvae were homogenized in 200µl distilled water on ice. The sample for acetylcholinesterase assay (2X25µl) were removed before spinning as being membrane bound, much of this enzyme may be pelleted and lost during centrifugation. Spin the remainder homogenate at 14000g for 30secs in a microfuge.

2.2.1 Protein estimation: Using the protocol of Bradford [13]. To 10µl sample was taken and added 300 µl Bradford reagent and incubated for 30mins. Absorbance was read at 570nm using Synergy/HT microplate reader, US.

2.2.2 Non-specific esterase microassay: The protocol followed by WHO [14]: techniques to detect insecticide resistance mechanisms (field and laboratory manual). 2 X 20µl replicates of homogenate were taken and placed in the separate wells of a microplate and then 200µl of α- Naphthyl acetate to one replicate and 200µl of β- Naphthyl acetate to the second replicate were added (1ml 30mM α/β Naphthyl acetate in 99ml 0.02M phosphate buffer pH 7.2). After 15 minutes incubation at room temperature, 50µl of RR fast blue stain solution (15ml 1% RR Fast blue in 35ml 5%SDS) was added. For Blank 20µl distilled water was used instead of homogenate, and all other reagents were added as in the case of test (200µl α/ β NA and 50µl of fast blue stain solution). Absorbance were read at 570nm using Synergy/HT microplate reader, US.

2.2.3 Glutathione-S-Transferase assay: To 10µl of homogenate, added 200µl working solution of GSH/CDNB (2.5ml 10mM reduced glutathione+ 125µl 63mM chlorodinitrobenzene). Kept at room temperature for 20 mins and read at 340nm using Synergy/HT microplate reader, US.

2.2.4 Mixed function oxidases assay: Activities were measured according to Brogdon *et al*., [15]. By using a micropipette, 100 µL homogenate was transferred to a microtiter plate. 3,3' 5,5'-tetramethylbenzidine (TMBZ) solution (200 µL) was then added into each well in the microtiter plate, followed by 25 µL of 3% hydrogen peroxide. The reactions were incubated for 5 minutes at room temperature. The colour intensities were then read using Synergy/HT microplate reader, US at wavelength 630nm to quantify the enzyme activity and it was expressed as optical density.

2.2.5 Acetyl cholinesterase assay: To 2 × 25µl homogenate and added 145µl 0.1M Triton phosphate buffer (pH-7.8) and 10µl 0.01M dithiobis 2-nitrobenzoic acid (DTNB). Then it was added 25µl of 0.01M Acetylthiocholine iodide (ASCHI) to one replicate and 25µl of ASCHI + Propoxur (0.1M) to other replicate. After 1hour incubation at room temperature, absorbance read at 405nm Synergy/HT microplate reader, US.

2.3 Data analysis: The mean of the enzyme levels of field strains were compared with that of laboratory strain and resistance ratio determined as follows: Resistance ratio (RR) of enzyme level= Mean enzyme level of field strain/ Mean enzyme level of laboratory strain. RR values > 1 indicated resistance, while values ≤ 1 were considered susceptible. (Dhang *et al*., [16]).All levels of statistical significance were determined at $p < 0.05$, and not significant where $p>0.05$, by t-test.

3. Results and Discussion

Fig 1: shows the data regarding the & β esterase levels in the field population and laboratory population. From the graph it is clear that the carboxyl esterase levels were high in the mosquito populations which were collected from regularly insecticide spraying areas. There was a significant increase in α &β esterase activity ($p<0.05$; paired t test) and the alteration in the activity of α &β esterase levels indicating the detoxification levels are higher in field population where insecticide application occurs regularly. Esterase based resistance to organophosphorus and carbamate insecticides are common in almost all insects. The esterase either produce broad spectrum insecticide resistance through rapid binding and slow turnover of insecticide or narrow spectrum resistance through metabolism of a very restricted range of insecticides containing a common ester bond. It has been reported that resistance to organophosphate insecticides has been associated with the carboxyl esterase activity changes in many insects and the nature of changes varies widely according to the sensitivity and differences in strains [17]. Elevated esterase activity accounts for resistance to organophosphates, carbamate and pyrethroid insecticides [4, 18].

Glutathione S-transferases (GSTs) are a large group of multifunctional enzymes present mostly in aerobic organisms, plants, and animals. Their primary role is to detoxify hydrophobic toxicants such as drugs, herbicides, and insecticides by catalyzing the nucleophilic attack of the tripeptide glutathione (GSH) on the electrophilic center of substrates [19]. As a result of the reaction they produce water soluble and excretable than the non-GSH conjugated substrates. They have been implicated in resistance to organophosphates, organochlorines, and pyrethroid insecticides [20].

Fig 2: shows the data obtained in the GST levels of laboratory strain (LS), sample collected from insecticide untreated areas (UTS) and sample collected from regularly treating areas (RTS). The values are represented as 0.095, 0.124, 0.162 nanomoles/min/mg of protein and it reveals an elevated level of GSTs in the field population as compared with that of the laboratory population. The resistance ratio was calculated

using the formula of Dhang *et al*., and the value higher than one indicates the resistance status of field population. The mosquitoes collected from regularly insecticide spraying area having the resistance ratio as compared with mosquitoes of scarcely insecticide spraying areas.

P450s are Phase I detoxification heme-thiolate enzymes catalyzing various reactions, but are best known for their monooxygenase activity, introducing reactive or polar groups into xenobiotics or endogenous compounds[21,22].The mixed function oxidase system is responsible for the resistance towards organophosphates, DDT, pyrethroids and growth regulators [13]. The metabolic detoxification of mixed function oxidase (MFO) may cause the development of cross resistance. The elevated levels of MFO in the present study suggested that the detoxification by this enzyme could be implicated in the cross resistance with DDT, pyrethroids and organophosphates.

Acetylcholinesterase (AChE), responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both organophosphate and carbamate insecticides. Selection of a modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism. In natural populations of mosquitoes, high level of resistance to carbamate and organophosphates is provided by insensitive acetylcholinesterase. The value for the well with propoxur divided by that without propoxur multiplied by 100 gives the % remaining activity in propoxur inhibited replicate rate. Populations with more than 70% remaining activity after inhibition can be characterized as homozygous resistance (RR) with respect to altered AChE mechanism. Populations with 30-70% and less than 30% remaining activity can be categorized as heterozygous (RS) and homozygous susceptible (SS) respectively [11]. Results of assay conducted to identify the insensitivity of AChE to insecticide inhibition by propoxur are presented in Table: 1. In this experiment the LS having the value 20.5 and UTS, 28.36 and both are homozygous susceptible whereas the RTS strain having the value 89.84 and it is homozygous resistant. The present results have provided a strong evidence on the role of insensitive acetylcholinesterase in the development of organophosphate and carbamate resistance in Chennai strain of *Cx. quinquefasciatus.*

The resistance ratio of all enzyme levels are higher than one, it indicates that the field population is resistant towards the insecticides used for the control measures. Fig: 3 shows the data regarding the resistance ratio of enzyme levels of field strains with that of laboratory strain.

a. Tables and figures

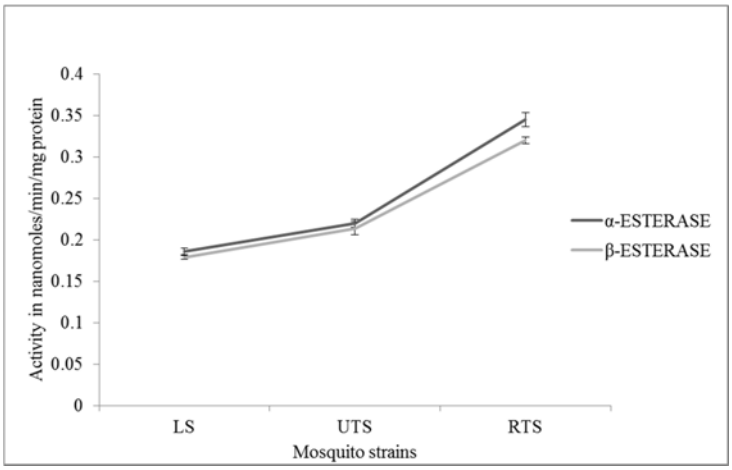

Fig 1: α & β Esterases Levels of Field and Laboratory Opulations of *Culex quinquefasciatus*

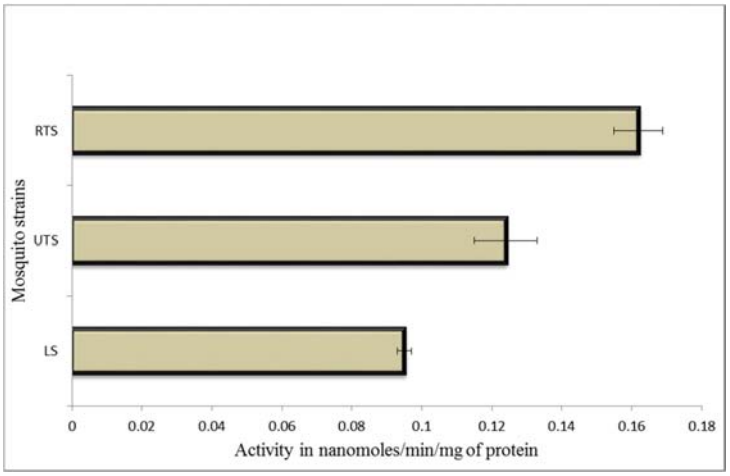

Fig 2: Glutathione S Transferase Levels of Laboratory and Field Populations of Cx. Quinquefasciatus

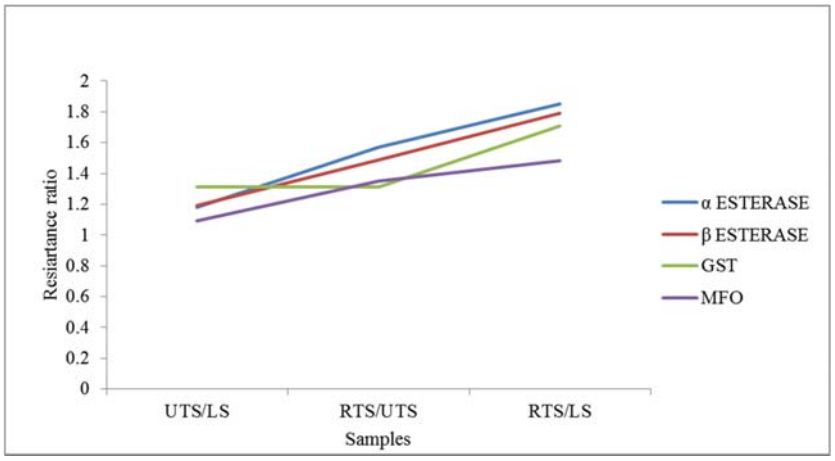

Fig 3: Resistance Ratio of Enzyme Lvels of Field Population with That of Laboratory Population

Table 1: Data on the mixed function oxidase activity and percentage remaining activity of AChE in propoxur inhibited fraction of laboratory and field population of *Cx. quinquefasciatus*

Strain	MFO (Absorbance 630nm)	% remaining activity of AChE in Propoxur inhibited fraction
LS	0.42±0.01	20.5
UTS	0.46±0.04	28.36
RTS	0.62±0.02	89.84

4. Conclusion

The biochemical analysis showed significant increase of esterase, glutathione S transferase activities and monooxygenase content in the field populations of *Cx. quinquefasciatus.* In conclusion, the presence of elevated enzyme levels indicated the multiple resistance mechanism in the field populations of *Cx. quinquefasciatus*. It may be an obstacle for the proper control of mosquito populations and vector borne diseases in the Corporation of Chennai. The development of resistance warrants the need of detailed evaluation of the efficacy of insecticides against the larvae of *Cx. Quinquefasciatus* which may help in reformulating control strategies, including rescheduling application of insecticide, or replacing the larvicide with other suitable compound sunder the vector control program.

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6. References

1. Ogoma SB, Lweitoijera DW, Ngonyani H, Furer B, Russel TL *et al*. Screening mosquito house entry points as a potential method for integrated control of endophagic filariasis, arbovirus and malaria vectors. PLoS Negl Trop Dis, 2010; 4(8):e773. doi: 10.1371/journal.pntd.0000773
2. Roberts DR, Andre RG. Insecticide resistance issues in vector. Am J Trop Med Hyg, 1994; 50:21-34.
3. Grant DF, Matsumura F, Gutatathione S. transferase in *Aedes aegypti* larvae: Purification and properties. Insect Biochem, 1988; 18:615-622.
4. Brogdon WG. Biochemical resistance detection: an alternative to bioassay. Parasitol. Today, 1989; 5:56-60.
5. Lee HL. A rapid and simple biochemical method for the detection of insecticide resistance due to elevated esterase activity in mosquito larvae of *Cx. quinquefasciatus*. Trop. Biomed, 1990; 7:21-28.
6. Peiris HTR, Hemingway J. Characterization and inheritance of elevated esterases in organophosphorus and carbamate insecticide resistant *Culex quinquefasciatus* (Diptera: Culicidae) from Sri Lanka. Bull. Entomol. Res, 1993; 83:127-132.
7. Hemingway J, Miyamoto J, Herath PRJ. A possible novel link between organophosphorus and DDT insecticide resistance genes in *Anopheles*: supporting evidence from fenitrothion metabolism studies. Pest. Biochem. Physiol, 1991; 39:49-56.
8. Zayed AB, Szumlas DE, Hanafi HA, Fryauff DJ, Mostafa AA. Use of bioassay and microplate assay to detect and measure insecticide resistance in field populations of *Culex pipiens* from filariasis endemic areas of Egypt. J. Am. Mosq. Control Assoc, 2006; 22:473-482.
9. Che-Mendoza A, Penilla RP, Rodrı´guez DA. Insecticide resistance and glutathione S-transferases in mosquitoes: a review. Afr. J. Biotechnol, 2009; 8:1386-1397.
10. Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). Bull. World Health Organization, 2001; 91:265-272.
11. Bourguet D, Capela R, Raymond M. An insensitive acetylcholinesterase in *Culex pipiens* (Diptera: Culicidae) from Portugal. J. Econ. Entomol. 1996; 89:1060-1066.
12. Pethuan S, Jirakanjanakit N, Saengtharatip S, Chareonviriyaphap T, Kaewpa D. Biochemical studies of insecticide resistance in *Aedes*(*Stegomyia*)*aegypti* and *Aedes*(*Stegomyia*) *albopictus* (Diptera: Culicidae) in Thailand. Trop. Biomed, 2007; 24: 7-15.
13. Bradford MM. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal

Biochem, 1976; 72:248-254.
14. World Health Organization. Techniques to detect insecticide resistance mechanism (field and laboratory manual) Geneva. 1998.
15. Brogdon WG, McAllister JC, Vuvule J. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. J. Am. Mosq. Control Assoc. 1997; 13:233-237.
16. Dhang CC, Ahmad NW, Lim LH, Benjamin S, Sofian A, zirun M. Biochemical detection of Temephos resistance in *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) from Dengue endemic areas of Selangor state, Malasya. Proc.3rdCongr. Trop. Med. Parasitol, 2008, 6-20.
17. Oppenoorth FJ. Biochemical and genetic in insecticide resistance. In: Comprehensive Insect Physiology Biochemistry and Pharmacology pergamon press, 1985, 731-773.
18. Hemingway J, Karunaratne SH. Mosquito carboxylesterase: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Med Vet Entomol, 1998; 12:1-12.
19. Armstrong RN. Glutathione S-transferases: reaction mechanism, structure, and function. Chem. Res. Toxicol, 1991; 4:131-140.
20. Fournier D, Bride JM, Hoffmann F, Karch F. Acetyl cholinesterase. Two types of modifications confer resistance to insecticide. J. Biol. Chem, 1992; 267:14270-14274.
21. Ranson H, Paton MG, Jensen B, McCarroll L., Vaughan A., Hogan JR *et al*., Genetic mapping of genes conferring permethrin resistance in the malaria vector, *Anopheles gambiae*. Insect Mol. Biol, 2004; 13, 379–386.
22. Scott JG. Cytochromes P450 and insecticide resistance. Insect Biochem Mol Biol, 2012; 29(9):757-777.