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RESEARCH ARTICLE

Biochemical Monitoring of Detoxifying Enzyme Levels in Field Population of Mosquitoes: *Culex quinquefasciatus* Say and *Aedes aegypti* (L.)

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ABSTRACT

The major cause of resistance mechanism in mosquitoes is the detoxification and degradation of insecticides by overproduction of various metabolic enzymes. Quantitative metabolic enzyme assays of carboxylesterases (α and β), mixed function oxidases (MFO), and glutathione S-transferases (GST) have been commonly used in the detection of insecticide resistance due to its sensitive nature even at low frequencies. For the present study, larval strains of *Culex quinquefasciatus* Say and *Aedes aegypti* (L) were collected from the Cochin Corporation, Kerala, India, and were assayed to organophosphate temephos and carbamate propoxur. The resistance ratio of median lethal time for temephos and propoxur from the field population of *C. quinquefasciatus* and *A. aegypti* is higher than the laboratory population. Elevated levels of α and β esterase enzyme were observed with the ratio of 1.6 and 1.54 for *C. quinquefasciatus* and 1.51 and 1.47 for *A. aegypti*. In *Culex* mosquitoes, 1.71, and in *Aedes,* 1.64 fold increase in GST enzyme level and 1.38 and 1.3 fold increase for the MFO level determined. The study results revealed the urgent needs of improving the vector control methods by introducing alternative techniques and strategies against mosquitoes.

Keywords: *Aedes aegypti*, *Culex quinquefasciatus*, insecticide resistance, propoxur, temephos.

INTRODUCTION

Mosquito-transmitted disease continues to be a major source of illness and death in many parts of the world. Intensifying globalization and climatic changes are increasing the risk of contracting arthropod-borne illnesses.[1] Mosquito-borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates. They act as vectors for the transmission of malaria, dengue fever, yellow fever, filariasis, schistosomiasis, Japanese encephalitis, etc, causing millions of deaths in every year.[2]

Culex quinquefasciatus Say (Diptera: Culicidae) is one of the major domestic pests in urban areas and carry *Wuchereria bancrofti*, the lymphatic filarial worm, and many arboviruses. Lymphatic filariasis is the second leading cause of permanent and long-term disability in the world. In India, filariasis is endemic in 250 districts from 17 states and 6 union territories, also about 553 million people at risk of infection.[3]

Aedes aegypti (L.) is generally known as a vector for an arbovirus responsible for dengue fever, which is endemic to Southeast Asia, the Pacific island area, Africa, and America. This mosquito is also the vector of yellow fever in Central, South America, and West Africa. Dengue fever has become an important public health problem; the number of reported cases continues to increase, especially with more severe forms of the disease such as dengue hemorrhagic fever and dengue shock syndrome or with unusual manifestations such as central nervous system involvement.[4]

Since 1950s, chemical insecticides have been extensively used to control such vectors. Large-scale use of insecticide leads to the development of resistance in target organism, and it in turn limits the vector control options and thus considered as a serious public health issue in the disease control strategies. Not only the resistance but also the cross-resistance lead to decrease the effectiveness due to the relation of chemical compounds present

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in different insecticides. Improving the knowledge about resistance and cross-resistance mechanisms will be successful to minimize or prevent the further development of resistance.[5] In addition, data on insecticide use for vector control programs such as the mode of usage and the concentration of insecticides help to design management systems to reduce the risks of development of resistance. The expertise hands, pesticide registration guidelines, and pesticide procurement practices will also help to reduce the risks toward human health and environment.[6]

The sequestration or biodegradation of insecticides by the enzymes present in insects leads to the development of metabolic resistance.[7,8] Target-site insensitivity and metabolic resistance are known as the most common and highest levels of resistance mechanisms in mosquitoes. Esterases, monooxygenases, and glutathione S-transferases (GST) are the enzyme groups mainly involved in insecticide metabolism. Enhancements of esterases, cyt p450 activities, and their relation to insecticide resistance have been reported in different mosquito species.[9-11]

Temephos is being used as a larvicide in various regions of the world, including India, to control the population of *C. quinquefasciatus* mosquito. It is considered as a basic larvicide for immature stages of mosquitoes and its continuous usage leads to the development of resistance by increasing the detoxification enzyme levels.[12] Propoxur (Baygon) is a carbamate insecticide used against many of the household pests including mosquitoes. Chemical control is the main method of vector control adopted in urban areas to keep the mosquito population within the acceptable level. The present study was carried out to assess the current susceptibility status of larvae of *C. quinquefasciatus* and *A. aegypti* collected from selected sites of Corporation of Cochin, Kerala.

MATERIALS AND METHODS

Test organism

C. quinquefasciatus and *A. Aegypti* were the test mosquitoes selected for the present study. Three strains of both *C. quinquefasciatus* and *A. Aegypti* were used in this investigation: Laboratory strain (LS), strain from untreated area (UTS), and strain from regularly insecticide-treated area (RTS). Susceptible laboratory population were collected from Communicable Disease Research Laboratory, Irinjalakuda, Thrissur, and reared in the laboratory without exposing to synthetic insecticides. Collected larvae were fed with yeast and dog biscuit in the ratio 3:1. Emerged adults were identified and the fourth instar larvae of F_1 generation used for all the assays.

Study area

The field strains used in this study were collected from Cochin Corporation, Kerala state, India (10° 00' N and 76° 25' E) in 2014 and 2015. The Cochin Municipal Corporation manages 94.88 km^2 of city limits of Kochi metropolitan city. This study site was selected because temephos have been used as larvicide for the control of mosquitoes for more than 5 years in Cochin Corporation.

Larvicidal bioassay

Larval bioassays were conducted, using 0.01 ppm temephos (analytical grade: Temephos PESTANAL SIGMA-ALDRICH) and 0.01 ppm propoxur (analytical grade: Propoxur PESTANAL SIGMA-ALDRICH) by the standard method developed by the WHO.[13] The time taken for 50% death of larvae was noted and the median lethal time (LT_{50}) in minutes was used to calculate resistance ratio (RR).[14]

Biochemical microplate assays

Non-specific esterase microassay

Insecticide resistance mechanisms (field and laboratory manual) were detected using the WHOdeveloped protocol.[15] Take 2 µl × 20 µl of homogenate as separate replicate, add 200 µl of α-naphthyl acetate to one replicate, and 200 µl of β-naphthyl acetate to the second replicate. Kept in room temperature for 15 min. Add 50 µl of RR fast blue stain solution to each replicate (15 ml 1% RR fast blue in 35 ml 5% SDS). Blank was taken with 20 µl distilled water. Measure the absorbance at 570 nm using Synergy/HT microplate reader, US.

GST/chlorodinitrobenzene (CDNB) assay

To 10 µl homogenate, add 200 µl working solution of GST/CDNB (2.5 ml 10 mM reduced glutathione

[GSH] + 125 µl 63 mM CDNB). A blank was carried out using 10 µl of distilled water. Leave at room temperature for 20 min and read at 340 nm using Synergy/HT microplate reader, US.

Mixed function oxidases (MFO) assay

MFO assay was conducted using Brogdonprotocol.[16] The color intensities were then read using Synergy/HT microplate reader, US at wavelength 630 nm to quantify the enzyme activity, and it was expressed as optical density.

Data analysis

Bioassay data from three replicates were pooled and analyzed. LT_{50} values for the laboratory and field strains of both larvae were analyzed using a standard probit analysis.[14] The computed LTs for the field strain compared with the LS, and the RR was determined as follows:

RR of LT_{50} = LT_{50} of field strain/LT_{50} of LS.

RR values >1 indicated resistance, while values ≤1 were considered susceptible.[17]

The mean of the enzyme levels of field strains was compared with that of LS and RR determined by the equation:

RR of enzyme level = Mean enzyme level of field strain/mean enzyme level of LS.

RR values >1 indicated resistance, while values ≤1 were considered susceptible.[17] All levels of statistical significance were determined by *t*-test.

RESULTS

Larvicidal bioassay

The LT_{50} values of different mosquito strains (LS, UTS, and RTS) of *C. quinquefasciatus* and *A. aegypti* collected from Cochin Corporation during 2014 and 2015, tested against 0.01ppm concentration of temephos and propoxur, are tabulated in Table 1. LSs of both *C. quinquefasciatus* and *A. aegypti* showed the lowest LT_{50} values [Table 1] and also the lowest RR against temephos and propoxur, whereas strains from regularly insecticides spraying area have the highest resistance power [Figures 1 and 2]. Strains from the untreated area showed intermediate mode of insecticide resistance, the LT_{50} values stands between LS and RTS. When compare 2014 insecticide resistance with 2015 data, there exhibit significant ($P < 0.05$) increase in resistance. The observations proved that LSs are more susceptible and also the *C. quinquefasciatus* larvae showed more resistance than *A. aegypti,* against temephos and propoxur.

Biochemical microplate assays

Non-specific esterase microassay

Table 2 illustrated the RR of α and β esterase levels in different strains of both *C. quinquefasciatus* and *A. aegypti.* According to the results obtained, strains from regularly insecticides spraying area show the highest level of α and β esterase activity. Laboratory stains have the lowest level of both α and β esterase. Results also imply year wise (2014–2015) increase in α and β esterase level resistance in both *C. quinquefasciatus* and *A. aegypti* strains.

GST and MFO assay

Table 3 shows MFO and GST level and its RR in strains of *C. quinquefasciatus* and *A. aegypti.* The mean optical density of elevated MFO and GST activity was significant ($P < 0.05$). The lowest optical density of elevated MFO and GST activity was observed in the LSs, while highest activity and resistance were in the strains from regularly insecticide-treated area. The results showed the year wise (2014–2015) increase in MFO and GST activity and corresponding emergence of resistance in *C. quinquefasciatus* and *A. aegypti* mosquitoes.

DISCUSSION

Resistance adaptations of *C. quinquefasciatus* and *A. aegypti* toward temephos and propoxur insecticides were examined in the present study. The results reveal that strains collected from regularly insecticide applied area had the highest level of RR. When compared with *A. aegypti,* the resistance level is higher in *C. quinquefasciatus* and year-wise insecticidal resistance data showed a upward trend with time. The LT_{50} values of temephos and propoxur against LSs of *C. quinquefasciatus* and *A. aegypti* indicate their high susceptibility to insecticides. Due to

Table 1: LT_{50} (min) of selected mosquito strains tested against 0.01 ppm temephos and propoxur

Mosquito strains	LT_{50} (min) 95% CL (0.01 ppm temephos)	LT_{50} (min) 95% CL (0.01 ppm propoxur)
C. quinquefasciatus		
LS	37.02 (35.69–38.34)	44.42 (42.87–46.97)
UTS1	43.47 (42.88–45.29)	51.47 (49.88–53.29)
RTS 1	72.95 (69.69–75.27)	88.95 (86.99–90.47)
UTS 2	44.10 (42.61–45.55)	54.10 (52.61–56.56)
RTS2	85.69 (83.45–87.70)	90.79 (87.45–93.80)
A. aegypti		
LS	36.07 (34.75–37.35)	43.47 (42.88–45.29)
UTS1	42.31 (42.30–43.25)	52.31 (50.30–54.45)
RTS 1	65.32 (53.32–57.67)	79.32 (53.32–57.67)
UTS 2	43.51 (40.10–46.13)	55.63 (53.10–47.18)
RTS2	74.65 (73.11–76.18)	85.64 (83.12–87.68)

LS: Laboratory strain, UTS: Strain from untreated area, RTS: Strain from regularly insecticide spraying area, *C. quinquefasciatus: Culex quinquefasciatus, A. aegypti: Aedes aegypti,* LT_{50}: Median lethal time

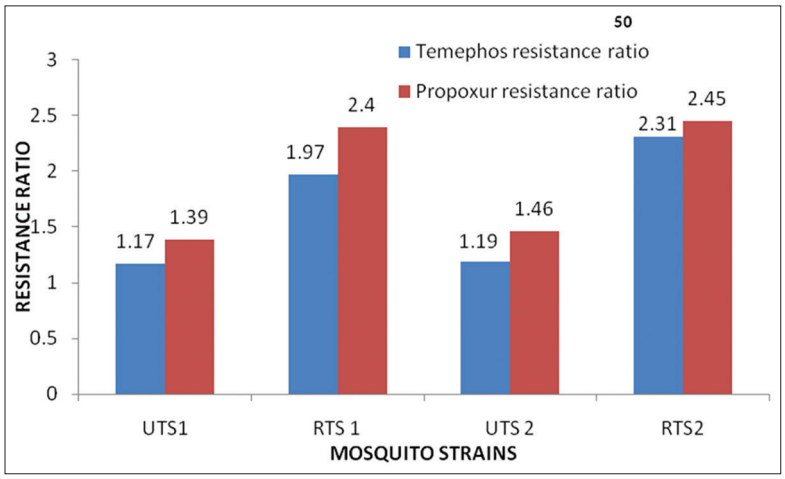

Figure 1: *Culex quinquefasciatus* resistance ratio of median lethal time

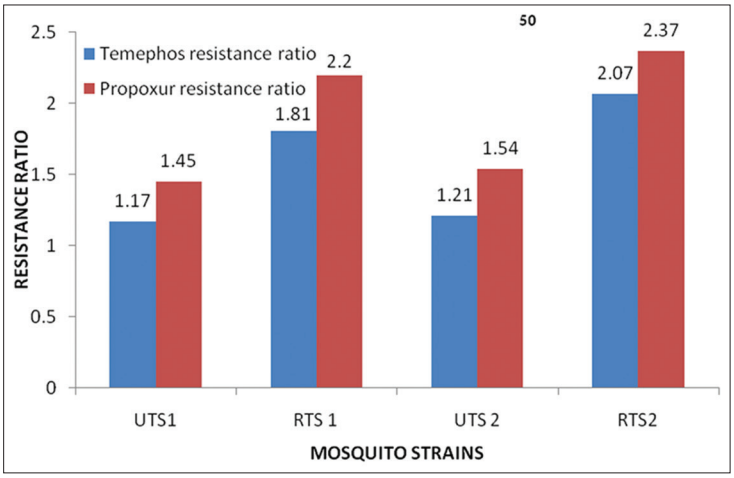

Figure 2: *Aedes aegypti* resistance ratio of median lethal time

the absence of previous insecticide exposure, the resistance mechanisms are not developed in the LSs. Whereas, the strains from regularly insecticide applying area experienced continuous exposure of insecticides, which leads to enhance their resistance machinery for better survival. Strains collected from the untreated area have moderate RR because of the much more stressed environment compared to the laboratory atmosphere. Mosquitoes quickly develop high level of insecticide resistance, mainly due to the variations of gene frequencies.[18]

The present investigation evaluated the enzyme-based insecticide resistance in *C. quinquefasciatus* and *A. aegypti* using biochemical assays such as non-specific esterase, GST, and MFO. Esterase-based resistance to organophosphate and carbamate insecticides is common in almost all insects. The esterase either produces 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. Results reveal that the carboxylesterase levels were high in the mosquito populations which were collected from regularly insecticide spraying areas.

Table 2: RR of α and β esterase levels of field population with that of laboratory population

Mosquito strains	α-esterase RR with LS	β-esterase RR with LS
C. Quinquefasciatus		
UTS1	1.14	1.12
RTS 1	1.5	1.45
UTS 2	1.15	1.17
RTS2	1.6	1.54
A. aegypti		
UTS1	1.13	1.10
RTS 1	1.36	1.38
UTS 2	1.16	1.15
RTS2	1.51	1.47

LS: Laboratory strain, UTS: Strain from untreated area, RTS: Strain from regularly insecticide spraying area, 1 = year 2014, 2 = year 2015, *C. quinquefasciatus: Culex quinquefasciatus, A. aegypti: Aedes aegypti,* LT_{50}: Median lethal time, RR: Resistance ratio

The significant increase and alteration in α and β esterase activity levels indicate the detoxification levels, which are higher in field population because of the regular and repeated insecticide application. It has been reported that resistance to organophosphate insecticides associated with the carboxylesterase activity changes in many insects and the nature of changes varies widely according to the sensitivity and differences in strains.[19] Elevated esterase activity accounts for resistance to organophosphates, carbamate, and pyrethroid insecticides.[20] The most frequently observed organophosphate-selected resistance mechanism in *Culex* and *Aedes* is the amplification of non-specific esterases[21-23] which is comparable with the present study.

Biochemical assays of GST and MFO indicate the high level of GST activities and monooxygenase content in the field populations of both *C. quinquefasciatus* and *A. aegypti*. The GSTs are members of a large family of multifunctional intracellular enzymes involved in the detoxification of xenobiotic compounds. Elevated levels of GST activity have been found to be associated to insecticide resistance in many insects. GSTs can produce resistance to a range of insecticides by conjugating reduced GSH to the insecticides or its primary toxic metabolic products. The majority of reports involve organophosphorous resistance, and recent works show resistance to pyrethroids and DDT (Dichlorodiphenyltrichloroethane). Where

Table 3: Data on mean enzymatic levels of MFO and GST in laboratory and field populations of *C. quinquefasciatus* and *A. aegypti*

Mosquito strain	MFO (absorbance 630 nm) Mean ± SD	RR with LS	GST (CDNB- ηmol/min/mg protein) Mean ± SD	RR with LS
C. quinquefasciatus				
LS	0.42 ± 0.02	-	0.095 ± 0.02	-
UTS 1	0.44 ± 0.01	1.05	0.118 ± 0.01	1.24
RTS 1	0.53 ± 0.02	1.26	0.152 ± 0.03	1.6
UTS 2	0.43 ± 0.01	1.02	0.122 ± 0.04	1.28
RTS2	0.58 ± 0.03	1.38	0.162 ± 0.02	1.71
A. aegypti				
LS	0.4 ± 0.01	-	0.090 ± 0.01	-
UTS 1	0.41 ± 0.02	1.03	0.110 ± 0.02	1.22
RTS 1	0.49 ± 0.02	1.23	0.134 ± 0.01	1.49
UTS 2	0.43 ± 0.01	1.08	0.116 ± 0.01	1.28
RTS 2	0.52 ± 0.02	1.3	0.148 ± 0.02	1.64

LS: Laboratory strain, UTS: Strain from untreated area, RTS: Strain from regularly insecticide spraying area. 1 = year 2014, 2 = year 2015*, C. quinquefasciatus: Culex quinquefasciatus, A. aegypti: Aedes aegypti,* RR: Resistance ratio, MFO: Mixed function oxidases, GST: Glutathione-S-transferases, CDNB: Chlorodinitrobenzene, SD: Standard deviation

conjugation of primary metabolite occurs, the GST mechanism often acts as secondary resistance mechanism in linkage disequilibrium with esterase and monooxygenase-based resistance mechanism.[24,25]

The enzymatic detoxifications due to MFO are associated with resistance to organophosphates, DDT, pyrethroids, and growth regulators.[17,26] The metabolic detoxification of MFO can induce 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.

Mosquitos are the major carriers of many vector-borne diseases; they progressively attain resistance toward insecticides, it will negatively affect various mosquito control programs. The detoxification status of *C. quinquefasciatus* and *A. aegypti* against temephos and propoxur reinforces the need for constant surveillance of mosquito populations susceptibility against the insecticides used in control programs as well as their effectiveness in the field. Frequent monitoring of insect resistance should be needed for detecting the resistance status and to develop new strategies for mosquito control.

CONCLUSIONS

The findings of the present study demonstrate the gradual procurement of high-level insecticide resistance among mosquito populations. The presence of elevated enzyme levels indicated the multiple resistance mechanism in the field populations of *C. quinquefasciatus* and *A. aegypti*. It may be an obstacle for the proper control of mosquito populations and vector-borne diseases. The present study suggests that the regular and continuous monitoring of resistance should be needful for confirming the efficacy of insecticides and also to choose the most effective insecticides for the eradication of various mosquito mediated diseases.

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