Esterases are responsible for malathion resistance in *Anopheles stephensi*: A proof using biochemical and insecticide inhibition studies

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ABSTRACT

**Background & objectives:** Increase in prevalence and intensity of insecticide-resistance in vectors of vector-borne diseases is a major threat to sustainable disease control; and, for their effective management, studies on resistance mechanisms are important to come out with suitable strategies. Esterases are major class of detoxification enzymes in mosquitoes, which confers protection against insecticides in causing resistance. This study was aimed at biochemical characterization of esterases responsible for malathion resistance in *Anopheles stephensi* mosquitoes, along with its validation through biochemical techniques and native-PAGE assays.

**Methods:** Laboratory maintained susceptible and resistant *An. stephensi* mosquitoes were used for assessing the activity and effect of α- and β-esterases on malathion. Bioassay, synergist bioassay, biochemical assay and native-PAGE were employed to characterize the role of esterases in conferring malathion-resistance.

**Results:** Notably significant (*p < 0.0001*) enhancement in α- and β-esterases activity was observed with 2-fold increase in resistant *An. stephensi* compared to susceptible *An. stephensi*. Native-PAGE depicted two major bands ‘a’ (*Rf* = 0.80) and ‘b’ (*Rf* = 0.72) in susceptible *An. stephensi*, while one intense band ‘b’ (*Rf* = 0.72) was visible in resistant *An. stephensi*. Inhibition assay revealed complete inhibition of α- and β-esterases activity in presence of 1 mM malathion in susceptible strain compared to observed partial inhibition in resistant strain on native-PAGE.

**Interpretation & conclusion:** This study provides a better understanding on the role of esterase enzyme (carboxylesterase) in conferring malathion-resistance in *An. stephensi* mosquitoes, as evident from the native-PAGE assay results. The study results could be used in characterizing the resistance mechanisms in vectors and for suggesting alternative chemical insecticide based resistance management strategies for effective vector-borne disease control.

**Key words** *Anopheles stephensi*; esterases; malathion; native-PAGE; triphenyl phosphate

INTRODUCTION

Insecticide based vector control is crucial for management of vector-borne diseases in public health programme. However, the continuous and unrestricted use of the insecticides leads to development of insecticide resistance in vectors. *Anopheles stephensi* is a major urban malaria vector in India, responsible for about 12% of malaria cases annually; it is also an important malaria vector in Pakistan and Iran. The insecticide resistance data for *An. stephensi* is meager in India. It has been reported resistant to DDT, dieldrin and malathion in Chennai (Tamil Nadu), Belgaum and Dharward (Karnataka), and Banaskantha and Amerli districts (Gujarat) in a study carried out by Roop Kumari et al in 1998. However, in a recent review on insecticide resistance carried out by Raghavendra et al, this species was reported resistant only to malathion in three districts, namely Gandhinagar, Jamnagar, Surat (Gujarat); and double resistant to DDT+malathion in seven districts, namely northwest Delhi, north Goa, Kutch (Gujarat), Ramanagar (Karnataka), Kolkata (West Bengal), Bikaner, Jodhpur (Rajasthan), and to malathion+deltamethrin in one district, i.e. Dakshina Kannada (Mangalore) in India. At present, IRS is not targeted for the control of *An. stephensi*, as a strategy for vector control in India, except in Rajasthan where this species is reported as primary vector of malaria. *An. stephensi* has been reported completely susceptible to malathion in Iran.

To date, four types of insecticide resistance mechanisms have been reported in mosquitoes, *i.e.* point mutations in target site genes to insecticides, elevation in enzyme levels or mutations in the coding regions of detoxification enzyme, changes in cuticle architecture, and behavioural changes. The detoxifying enzyme based resistance occurs mainly due to qualitative or quantitative changes in three main enzymes: Esterases, glutathione-S-transferases and monoxygenases, a cytochrome P450
super family enzyme\(^9\). In mosquitoes showing metabolic resistance mechanism(s), it is important to measure levels of specific detoxification enzyme that confer the resistance, and also to infer cross-resistance. Esterases are major family of enzymes that are responsible for insecticide resistance in disease vectors and agriculture pests\(^10\). Non-specific and general esterases are reported responsible for organophosphates (OPs)\(^11\), carbamate\(^12\) and pyrethroids resistance\(^5,8\). In a study carried out in Mysore, India, Ganesh et al\(^13\) reported that elevated levels of β-esterase are responsible for conferring resistance to organophosphates (malathion) in An. stephensi. Carboxylesterases are most abundant protein family in the insects. Insect carboxylesterases play important physiological role in lipid metabolism and xenobiotic metabolism\(^14\). They are frequently implicated for the resistance in insects to OPs, carbamates and pyrethroids through quantitative or qualitative change in the enzyme or combination of these mechanisms\(^15\).

In the present study, the susceptibility status of laboratory reared An. stephensi populations to malathion and synergistic effect of carboxylesterase specific synergist, triphenyl phosphate (TPP) with malathion were determined. Synergist bioassays can not provide definitive proof of the resistance mechanisms; and needs to be combined with other assays, such as electrophoresis to provide better biochemical characteristics of resistance in an insect population\(^16\). Quantitative microplate biochemical assays are performed to assess the levels of α- and β-esterases and native-polyacrylamide gel electrophoresis (PAGE) for localization of α- and β-esterases in susceptible- and resistant-An. stephensi. This study would provide a better understanding of the role of esterase enzyme in malathion-resistance and provide additional evidence to show esterase mediated malathion metabolism through native-PAGE in Indian An. stephensi. Based on literature search, this appears first such study on An. stephensi mosquitoes, which provides information on the OP resistance mechanism using native-PAGE.

**MATERIAL & METHODS**

**Mosquito strains**

The mosquito strains used in this study are maintained at the insectaria of the National Institute of Malaria Research, New Delhi, India. Insecticide susceptibility assays were ascertained quarterly, each year since 2011 following WHO method\(^17\).

*Anopheles stephensi*\(_{BB}\)

Black Brown (BB) skin colored An. stephensi mosquitoes, collected from district Sonipat, Haryana, India, were established in the year 1996. This strain is found to be susceptible to DDT, malathion and deltamethrin in the range of 95–100, 92–100 and 98–100 respectively.

*Anopheles stephensi*\(_{GOA}\)

An. stephensi mosquitoes collected from Goa, India were established in the year 2009. This strain is found to be resistant to DDT, malathion and deltamethrin in the range of 12–60, 10–80 and 54–92 respectively.

**Chemicals, insecticides and equipment**

For biochemical assays, analytical grade chemicals purchased from Sigma Chemicals Co. (USA), and for protein estimation, reagents from Bio-Rad Laboratories, Inc. (USA) were used. Malathion (5%) insecticide impregnated papers were purchased from the Vector Control Research Unit (VCRU), University Sains Malaysia, Malaysia (www.usm.my). Technical grade malathion (96%) were ingratiated from the Hindustan Insecticides Ltd, India. NanoQuant Infinite® M200 PRO ELISA reader (Tecan Group Ltd., Switzerland) with inbuilt Magellan 7.2 software, and SCIE-PLAS electrophoresis apparatus (England) were used in the study.

**Insecticide susceptibility assay**

Three to five days old sugar fed female An. stephensi\(_{BB}\) (n=116) and An. stephensi\(_{GOA}\) (n=129) mosquitoes were exposed in replicates (20–25 mosquitoes per replicate) for 1 h to 5% malathion impregnated paper along with control replicates by using standard WHO method\(^17\) and kit provided by VCRU. Then mosquitoes were transferred to holding tubes and kept in climatic chamber maintained at 27±2°C and 80±10% relative humidity for 24 h. Then, dead mosquitoes were scored and percent mortality calculated as follows.

\[
\text{% Mortality} = \left( \frac{\text{Total No. of dead mosquitoes}}{\text{Total mosquitoes exposed}} \right) \times 100
\]

If, the mortality in control replicates was found between 5 and 20%, it was corrected using Abbott’s formula\(^18\), and if the morality in control replicates was >20%, the test was rejected.

\[
\text{Corrected % mortality} = \left( \frac{\text{(% Test mortality} - \text{% Control mortality})}{(100 - \text{% Control mortality})} \right) \times 100
\]

**Synergist bioassay**

For studying synergistic effect of a specific synergist carboxylesterase, i.e. TPP, the 3–5 days old sugar fed fe-
male susceptible *An. stephensi*$_{BB}$ (n = 119) and resistant *An. stephensi*$_{GOA}$ (n = 147) mosquitoes were pre-exposed to malathion (5%) impregnated paper$_{11,19}$ for 1 h and then exposed to the malathion (5%) insecticide impregnated WHO papers for 1 h. Mortality was scored after 24 h holding period as described in insecticide susceptibility assay.

**Interpretation of insecticide susceptibility and synergist data**

Insecticide susceptibility status was designated on the basis of WHO$_{17}$ criteria: > 98% mortality–Susceptible, 91 to 97% mortality–Possible resistance, and < 90% mortality–Resistant. For determining the synergistic effect, the difference in percent mortality after exposure to malathion alone and TPP + malathion was noted.

**Esterase activity assay**

The adult non-blood fed 1–3 days old live or –80°C/Liquid N$_2$ stored female susceptible and resistant *An. stephensi* mosquitoes were used for 96 well microplate assays. Individual mosquitoes were homogenized in 50 µl of distilled water in 1.5 ml centrifuge tube on ice and made up to a final volume of 200 µl. Homogenate was centrifuged at 14,000 rpm for 30 sec at 4°C. The supernatant was used for α- and β-esterase activity assays. Esterase activity assays were performed as described by Penilla et al$_{9}$ with minor modifications in 96 well microplate. For α-esterase assay, 200 µl α-naphthyl acetate (NA) solution (100 µl of 30 mM α-NA in acetone in 10 ml of 0.02 M sodium phosphate buffer, pH 7.2) was added to 10 µl of homogenate in a well. Similarly, for β-esterase assay, 200 µl β-NA solution (100 µl of 30 mM β-NA in acetone in 10 ml of 0.02 M sodium phosphate buffer, pH 7.2) was added to 10 µl of homogenate in another well, simultaneously. The reactions were incubated for 15 min at room temperature and to stop the reaction, 50 µl of o-dianisidine stain (a mixture of 22.5 mg o-dianisidine in 2.25 ml distilled water and 5.25 ml of 5% sodium lauryl sulphate in 0.1 M sodium phosphate buffer, pH 7.0) was added to each well. Control wells contained 10 µl distilled water in place of homogenate, 200 µl of α-NA or β-NA solution and 50 µl of o-dianisidine stain. End point enzyme activity was measured at 570 nm in ELISA reader.

The total protein of the individual mosquitoes was estimated following Bradford method using Bio-Rad reagent, following manufacture’s protocol. The activities of α- and β-esterase of the individual mosquitoes were expressed as mmoles of product formed/min/mg protein based on the α- and β-naphthol standard curves respectively. The activity data was compared between susceptible and resistant strains using Mann-Whitney U-test.

**Esterase microplate inhibition assay**

A pooled homogenate of five mosquitoes from the susceptible *An. stephensi*$_{BB}$ and resistant *An. stephensi*$_{GOA}$ population were prepared separately in 1.5 ml centrifuge vials containing 50 µl of 0.02 M sodium phosphate buffer (pH 7.2) and made up to a final volume of 1.0 ml, and centrifuged at 14,000 rpm for 30 sec at 4°C. Resistant and susceptible *An. stephensi* mosquito homogenates (10 µl) were incubated individually in 96 well microplates with 10 µl of different concentrations of technical malathion (96%) (serial dilution of 10 mM to 0.001 mM malathion in sodium phosphate buffer, pH 7.2) for 15 min at room temperature. After incubation, α- and β-NA assay was performed as described in esterase activity assay and the end point enzyme activity was measured at 570 nm in ELISA reader. The activities of α- and β-esterase were expressed as mmoles of product formed/min/mg protein.

**Esterase native polyacrylamide gel electrophoresis**

Native-PAGE was performed for determining α- and β-esterase profile of the susceptible and resistant strains of *An. stephensi* following Gopalan et al$_{21}$ method with minor modifications, i.e. 8% resolving and 5% stacking gel. Single mosquito from susceptible *An. stephensi*$_{BB}$ and resistant *An. stephensi*$_{GOA}$ was homogenized in 150 µl of 0.02 M sodium phosphate buffer (pH 7.2) and centrifuged at 14,000 rpm for 30 sec at 4°C in individual vials, and the protein was estimated from the supernatant. Volume of homogenate equivalent to 8 µg of protein was loaded on the gel and electrophoresed initially at 50 V for 30 min and increased to 75 V for 3 h with continuous cooling at 4°C to localize the enzymes. After electrophoresis, the gels were incubated separately in petri dishes containing 0.1 M sodium phosphate buffer (pH 6.0) at 4°C for 10 min. After incubation, the buffer in the petri dishes were replaced with 0.1 M sodium phosphate buffer, pH 6.0 containing 1 mM α- or β-NA (30 mM stock in acetone) substrate solution for 20 min at 37°C and stained with 0.025% o-dianisidine (in DD H$_2$O) to localize α- and β-esterase activity on the gel, washed with DDW and analyzed.

**Esterase inhibition on native-PAGE**

The malathion inhibition effect on α- and β-esterase activity was then assessed by using native-PAGE. Pooled homogenate of five mosquitoes each from the susceptible *An. stephensi*$_{BB}$ and resistant *An. stephensi*$_{GOA}$ population were prepared in 1.5 ml centrifuge vials in 50 µl of 0.02 M
sodium phosphate buffer (pH 7.2), made up to 750 µl with 0.02 M sodium phosphate buffer (pH 7.2), and centrifuged at 14,000 rpm for 30 sec at 4°C. The protein was estimated from the supernatant using Bio-Rad reagent. Volume of homogenate equivalent to 8 µg of protein was loaded on the gel and electrophoresed as described in previous section. Gels were pre-incubated with 0.1 M sodium phosphate buffer (pH 6.0) for 10 min followed by incubation in 1 mM malathion (dissolved in 0.1 M sodium phosphate buffer pH 6.0) for 20 min at room temperature before detecting the esterase activity. Control gels were processed without malathion incubation.

RESULTS

Adult susceptibility and synergist assay

The malathion-susceptible *An. stephensi* _BB_ showed 100% mortality while malathion-resistant *An. stephensi* _GOA_ reported 82% mortality. The TPP synergistic assay revealed increase in the malathion susceptibility in the resistant line from 82 to 97% showing synergism of carboxylesterase, thereby indicating the possible involvement of this enzyme in conferring malathion resistance. The average control % mortality in control exposures with susceptible *An. stephensi* _BB_ was 8.7% while in resistant *An. stephensi* _GOA_, it was nil.

Esterase activity assay

The mean value of α- and β-esterase activity (mmol/min/mg) of *An. stephensi* _BB_ (susceptible strain) and *An. stephensi* _GOA_ (resistant strain) are shown in Table 1. There was a significant increase in α- and β-esterase activity of resistant *An. stephensi* _GOA_ (1.85 and 2.18 mmol/min/mg protein) compared to the α- and β-esterase activity of susceptible *An. stephensi* _BB_ (0.87 and 0.88 mmol/min/mg protein) (p < 0.0001; Mann-Whitney U-test). The α- and β-esterase activity increased by 2.12 and 2.47 times in resistant strain compared to susceptible strain. The susceptibility threshold of α- and β-esterase activity in susceptible population was 2 mmol/min/mg. The proportion of population showing activity beyond this susceptibility threshold was considered resistant. About 30% of resistant *An. stephensi* _GOA_ population showed activity beyond this threshold (Fig. 1).

Table 1. Mean α- and β-esterses activity (mmol/min/mg) in *An. stephensi* _BB_ and *An. stephensi* _GOA_.

| Mosquito strain (n) | Activity (mmol/min/mg) ± SD | α-esterase | β-esterase |
|--------------------|----------------------------|------------|------------|
| *An. stephensi* _BB_ (n = 47) | 0.87 ± 0.10 | 0.88 ± 0.14 |
| *An. stephensi* _GOA_ (n = 47) | 1.85 ± 1.3 | 2.18 ± 1.87 |

SD = Standard deviation; n = Total number of mosquitoes tested.

Esterase microplate inhibition assay

Dose dependent inhibition of α- and β-esterases activity with technical malathion (96%) in susceptible *An. stephensi* _BB_ (Fig. 2a) and resistant *An. stephensi* _GOA_ (Fig. 2b) were observed. However, the strains showed differential inhibition activity and >90% inhibition was observed beyond 1.2 mM malathion concentration. The activities of α- and β-esterase in susceptible and resistant strains were respectively ~1.3 and >2 mmol/min/mg.

Esterase native polyacrylamide gel electrophoresis

The α- and β-esterase activity profiles were localized on native-PAGE (Fig. 3). In *An. stephensi* _BB_ two major bands ‘a’ [Retention factor (Rf) = 0.80] and ‘b’ (Rf = 0.72) were observed based on its mobility. In *An. stephensi* _GOA_ only one band ‘b’ (Rf = 0.72) was observed which was common to both the strains. The intensity of ‘b’ was relatively more in resistant strain than in susceptible strain (Fig. 3).
Fig. 3: The α- and β-esterases activity in An. stephensi BB [Two bands observed ‘a’ (R_f = 0.80) and ‘b’ (R_f = 0.72)]; and An. stephensi GOA [One intense band observed ‘b’ (R_f = 0.72)] detected on native-PAGE.

Esterase inhibition on native-PAGE

The α- and β-esterases activity inhibition were studied in presence of inhibitor malathion in An. stephensi_BB and An. stephensi_GOA on native-PAGE assay (Fig. 4). The α- and β-esterases bands of An. stephensi_BB were completely inhibited by malathion (Fig. 4a) however, the intensity of ‘b’ in An. stephensi_GOA decreased (Fig. 4a) compared to uninhibited samples (Control) (Fig. 4b).

DISCUSSION

Involvement of elevated carboxylesterase activity has been observed in many insecticide-resistant insects of agriculture and public health importance viz. multi-insecticide resistant peach-potato aphids to organophosphates, carbamates and pyrethroids22, chloropyrifos resistant Culex species 23, organophosphate resistant Lygus hesperus 24, rice brown plant hopper Nilaparvata lugens Stal 25,

Fig. 2: Inhibition of α- and β-esterases activity (mmol/min/mg) with different concentrations of malathion in susceptible An. stephensi_BB and resistant An. stephensi_GOA.

(b) Malathion

(a) Control

Fig. 4: Inhibition of α- and β-esterases activity in An. stephensi_BB and An. stephensi_GOA with (a) Malathion; and (b) without Malathion (Control).
rice green leafhopper *Nephotettix cincticeps* Uhler26 and in German cockroaches27. Involvement of malathion specific carboxylesterase has been reported in *An. culicifacies sensu lato* from India1 and Sri Lanka28, *An. arabiensis* from Sudan29, *An. stephensi* from Pakistan30 and India (K. Raghavendra, personal communication).

The synergist study on malathion-resistant *An. stephensi*GOA showed strong synergism to 10% TPP, indicating the involvement of carboxylesterase mediated mechanism of malathion-resistance. The malathion susceptibility increased from 82% in malathion alone exposures, to 97% with TPP and malathion exposure indicating involvement of carboxylesterase in conferring malathion-resistance.

In the present study, microplate biochemical assays showed 2.12 and 2.47 times elevated levels of α- and β-esterases, respectively in resistant *An. stephensi*GOA strain compared to the levels in susceptible *An. stephensi*BB strain and supported increased synergism with TPP, thereby substantiating the involvement of carboxylesterase in conferring malathion-resistance. Similar results have been reported in peach-potato aphids (*Myzus persicae*)21, *Culex quinquefasciatus*31-32, *Cx. pipiens*33 and in *An. culicifacies*GOA for organophosphates.

The esterase activity was also analyzed through native-PAGE by staining with α- and β-NA substrates. Two major bands were observed in the *An. stephensi*BB namely, ‘a’ (*Rf* = 0.80) and ‘b’ (*Rf* = 0.72), while in malathion-resistant *An. stephensi*GOA only one intense band ‘b’ was seen. Gopalan et al31 have identified intense carboxylesterase in malathion selected line of *Cx. quinquefasciatus*. In a similar study by Ganesh et al33, increased levels of carboxylesterase activity were found on native-PAGE in deltamethrin tolerant *An. stephensi* larvae. In this study, native-PAGE also illustrated complete inhibition of esterases by malathion at 1.0 mM concentration in susceptible *An. stephensi*BB; however, in resistant *An. stephensi*GOA esterase inhibition was relatively less at this concentration, which further suggested involvement of esterases in conferring malathion-resistance.

**CONCLUSION**

The study showed that levels of esterases are higher in resistant *An. stephensi*GOA strains compared to susceptible *An. stephensi*BB. The results indicated unequivocal evidence for major involvement of malathion carboxylesterase (MCE) mediated malathion-resistance mechanism in Indian strain of *An. stephensi*. This information could be of immense use in suggesting alternative chemical insecticide based resistance management strategies for effective disease vector control.

**Conflict of interest**

The authors declare no conflict of interest.

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