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* GOA compared to susceptible *An. stephensi* BB. native-PAGE depicted two major bands 'a' (Rf = 0.80) and 'b' (Rf = 0.72) in susceptible *An. stephensi* BB, while one intense band 'b' (Rf = 0.72) was visible in resistant *An. stephensi* GOA. 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 programme¹. However, the continuous and unrestricted use of the insecticides leads to development of insecticide resistance in vectors². *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 Iran³. 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 malaria⁴. *An. stephensi* has been reported completely susceptible to malathion in Iran⁵.

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 changes⁶. The detoxifying enzyme based resistance occurs mainly due to qualitative or quantitative changes in three main enzymes: Esterases, glutathione-S-transferases and monooxygenases, 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)\(^11\), carbamates\(^12\) and pyrethroids resistance\(^5,8\). In a study carried out in Mysore, India, Ganesh \textit{et al}\(^13\) reported that elevated levels of \(\beta\)-esterase are responsible for conferring resistance to organophosphates (malathion) in \textit{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 \textit{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 \(\alpha\)- and \(\beta\)-esterases and native-polyacrylamide gel electrophoresis (PAGE) for localization of \(\alpha\)- and \(\beta\)-esterases in susceptible- and resistant-\textit{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 \textit{An. stephensi}. Based on literature search, this appears first such study on \textit{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 insectariums of the National Institute of Malaria Research, New Delhi, India. Insecticide susceptibility assays were ascertained quarterly, each year since 2011 following WHO method\(^17\).

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

Black Brown (BB) skin colored \textit{An. stephensi} mosquitoes, collected from district Sonepat, 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.

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

\textit{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\(^\circledR\) 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 \textit{An. stephensi}\(_{BB}\) (n=116) and \textit{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, \textit{i.e.} TPP, the 3–5 days old sugar fed fe-
male susceptible *An. stephensi*<sub>BB</sub> (n = 119) and resistant *An. stephensi*<sub>GOA</sub> (n = 147) mosquitoes were pre-exposed to TPP (10%) impregnated paper<sup>11,19</sup> 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<sup>17</sup> 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<sub>2</sub> 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 <i>et al</i><sup>21</sup> method with minor modifications, i.e. 8% resolving and 5% stacking gel. Single mosquito from susceptible *An. stephensi*<sub>BB</sub> and resistant *An. stephensi*<sub>GOA</sub> 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 7.0) to localize α- and β-esterase activity on the gel, washed with DDW and analyzed.

**Esterase inhibition on native-PAGE**

Native-PAGE was performed for determining α- and β-esterase profile of the susceptible and resistant strains of *An. stephensi* following Gopalan <i>et al</i><sup>20</sup> method with minor modifications, i.e. 8% resolving and 5% stacking gel. Pooled homogenate of five mosquitoes each from the susceptible and resistant strains using Mann-Whitney U-test. Pooled homogenate of five mosquitoes each from the susceptible *An. stephensi*<sub>BB</sub> and resistant *An. stephensi*<sub>GOA</sub> 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. stephensiBB showed 100% mortality while malathion-resistant An. stephensiGOA 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. stephensiBB was 8.7% while in resistant An. stephensiGOA, it was nil.

Esterase activity assay
The mean value of α- and β-esterase activity (mmol/ min/mg) of An. stephensiBB (susceptible strain) and An. stephensiGOA (resistant strain) are shown in Table 1. There was a significant increase in α- and β-esterase activity of resistant An. stephensiGOA (1.85 and 2.18 mmol/min/mg protein) compared to the α- and β-esterase activity of susceptible An. stephensiBB (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. stephensiGOA population showed activity beyond this threshold (Fig. 1).

Table 1. Mean α- and β-esterases activity (mmol/min/mg) in An. stephensiBB and An. stephensiGOA

<table>
<thead>
<tr>
<th>Mosquito strain (n)</th>
<th>Activity (mmol/min/mg) ± SD</th>
<th>α-esterase</th>
<th>β-esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. stephensiBB (n = 47)</td>
<td>0.87 ± 0.10</td>
<td>0.88 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>An. stephensiGOA (n = 47)</td>
<td>1.85 ± 1.3</td>
<td>2.18 ± 1.87</td>
<td></td>
</tr>
<tr>
<td>SD = Standard deviation; n = Total number of mosquitoes tested.</td>
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</tbody>
</table>

Esterase microplate inhibition assay
Dose dependent inhibition of α- and β-esterases activity with technical malathion (96%) in susceptible An. stephensiBB (Fig. 2a) and resistant An. stephensiGOA (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. stephensiBB two major bands ‘a’ [Retention factor \(R_i = 0.80\)] and ‘b’ \(R_i = 0.72\) were observed based on its mobility. In An. stephensiGOA only one band ‘b’ \(R_i = 0.72\) 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).
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 pyrethroids$^{22}$, chloropyrifos resistant $Culex$ species$^{23}$, organophosphate resistant $Lygus$ hesperus$^{24}$, rice brown plant hopper $Nilaparvata$ lugens Stal$^{25}$,
rice green leafhopper *Nephotettix cincticeps* Uhler and in German cockroaches. Involvement of malathion specific carboxylesterase has been reported in *An. culicifacies sensu lato* from India and Sri Lanka, *An. arabiensis* from Sudan, *An. stephensi* from Pakistan and India (K. Raghavendra, personal communication).

The synergist study on malathion-resistant *An. stephensi* 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* strain compared to the levels in susceptible *An. stephensi* 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*) Culex quinquefasciatus, *Cx. pipiens* and in *An. culicifacies* 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* strain namely, ‘a’ (Rf = 0.80) and ‘b’ (Rf = 0.72), while in malathion-resistant *An. stephensi* only one intense band ‘b’ was seen. Gopalan et al. have identified intense carboxylesterase in malathion selected line of *Cx. quinquefasciatus*. In a similar study by Ganesh et al., 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*; however, in resistant *An. stephensi* 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* strains compared to susceptible *An. stephensi*. 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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