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Microplate assay of elevated esterase activity in individual pyrethroid-resistant mosquitoes

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Abstract. Involvement of esterase-mediated hydrolysis as a mechanism of pyrethroid-resistance in three species of mosquitoes, *viz., Aedes aegypti, Culex quinquefasciatus* and *Anopheles Stephensi* was investigated by microplate assay of β-esterases in individual larva and adult female and male mosquitoes. Assuming an absorbance value of 0·4 and above at 555 nm as the threshold level of elevated esterase activity which confers resistance, frequency distributions of such individual test mosquitoes were constructed in resistant and susceptible populations. The results indicate the involvement of ester hydrolysis of Pyrethroids as a predominant mechanism of pyrethroid-resistance in the larvae of *Culex quinquefasciatus* but not in *Aedes aegypti.* However, a marginal role of esterases is indicated in the larvae of *Anopheles stephensi.*

Keywords. Non-specific esterases; pyrethroid-resistance; microplate assay; *Aedes aegypti; Culex quinquefasciatus; Anopheles stephensi,*

1. Introduction

Synthetic Pyrethroids are known to excel other insecticides in mosquito abatement programmes primarily due to their outstanding insecticidal potency coupled with marvellous ecological compatibility. However, laboratory studies have established that intense selection pressure for many generations could select the mosquito larvae for resistance to synthetic Pyrethroids, such as permethrin and deltamethrin, in Indian strains of yellow fever mosquito, *Aedes aegypti* L., tropical house mosquito, *Culex quinquefasciatus* Say and urban malaria vector, *Anopheles stephensi* Liston (Kumar *et al* 1991; Thomas *et al* 1991). Efforts to identify the molecular mechanisms of such acquired pyrethroid-resistance centres around two possibilities of detoxification of Pyrethroids by microsomal oxidation and ester hydrolysis (Yamamoto *et al* 1969; Ishaaya and Casida 1980). Our earlier studies have elucidated a preeminent role of monooxygenase -mediated degradation of deltamethrin in conferring deltamethrin-esistance in the larvae of *Ae. aegypti, Cx quinquefasciatus* and *An. Stephensi* (Kumar *et al* 1991). Since the role of esterases in pyrethroid-resistance in mosquitoes has not been established unlike in other insects (Jao and Casida 1974; Riskallah 1983) the possibility of ester hydrolysis as a mechanism of permethrin- and deltamethrin-resistance in strains of *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* has now been investigated. This was accomplished by estimating levels of non-specific β-esterases in single larva/adult mosquitoes using the microplate assay method of Brogdon and Dickinson (1983) with certain modifications.

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2. Materials and methods

2.1 *Materials*

Stock of *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* originated from field-collected engorged female adults from Delhi, which were maintained in the cloth cages in an insectary at 28 ± 2° C and 80 ± 5% RH with a photoperiod of 14 h of artificial daylight and 10 h of darkness (Kumar *et al* 1991). Mosquito larvae and adults were drawn from the strains of *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi.*

2.1a *Susceptible:* Field-collected strains with larval LC_{50}-0·000645, LC50-0·004606, and LC_{50}-0·003624 ppm to permethrin and 0·000118, 0·000121 and 0·001194 ppm to deltamethrin for *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* respectively.

2.1b *Permethrin-R:* Selected with permethrin at the larval stage for 25 generations exhibiting 99-, 175-and 37- fold resistance to permethrin (LC_{50}-0·063554, LC_{50}-0·806358 and LC_{50}-0·133454 ppm) for *Ae. aegypti, Cx, quinquefasciatus* and *An, stephensi* respectively.

2.1c *Deltamethrin-R:* Selected with deltamethrin for 40 generations at the larval stage showing 703-, 1449- and 60-fold resistance to deltamethrin (LC_{50}-0·082965, LC_{50}-0·175350 and LC_{50}-0·071722 ppm) for *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* respectively.

2.2 *Chemicals*

2.2a *Permethrin:* 3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate – *cis : trans* isomers :: 20 : 80, obtained from Roussel-Uclaf, India.

2.2b *Deltamethrin:* (s)-α-cyano-3-phenoxybenzyl cis-(lR)-3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropane carboxylate, supplied by Roussel Uclaf, India, 98·8% pure,

2.3 *Microplate assay of* β*-esterases (carboxylesterases-EC* 3. 1. 1. 1)

Early fourth instar larvae and 3-day old, unfed adult females and males were used for enzyme assay. The non-specific esterase activity was determined by the microplate assay of single mosquitoes as described by Brogdon and Dickinson (1983),

Individual mosquitoes (larva/adult female/adult male) were homogenized in 1 ml of 0·05mol/l potassium phosphate buffer (pH 6·8) in all glass grinders. Aliquots of 90 µl were used for each assay in triplicate. To each of the 90 µl of the homogenate, was added 90 µl of β-naphthyl acetate (56 mg/10 ml 2-propanol/90 ml buffer) using an ELISA plate and the preparation was incubated at room temperature (30–32°C) for 10 min. A 90 µl aliquot of + 0-dianisidine tetrazotised (100 mg/100 ml water) was then added and the absorbances (O. D.) were read using an interference filter of 555 nm in a BIO-RAD Enzyme Immunoassay Reader-2550.

For each strain, more than 200 larvae and approximately 50 adult females and 50 males were assayed, where each larva/adult female/adult male had 3 replicates.

The wells in the microtitre plate, which served as controls, had 90 µl of buffer solution.

In order to avoid size variations between individual larva/adult mosquitoes, the enzyme activity was calculated at O. D. 555 nm/mg protein/min. Protein estimations of the individual mosquito were simultaneously carried out during the enzyme assay using the method of Lowry *et al* (1951).

Esterase activity as frequency distributions of susceptible and resistant individual mosquitoes were plotted against absorbance (O. D.) values at 555 nm/mg protein/min and the frequency of resistant larva or adult mosquitoes with elevated esterase activity was scored.

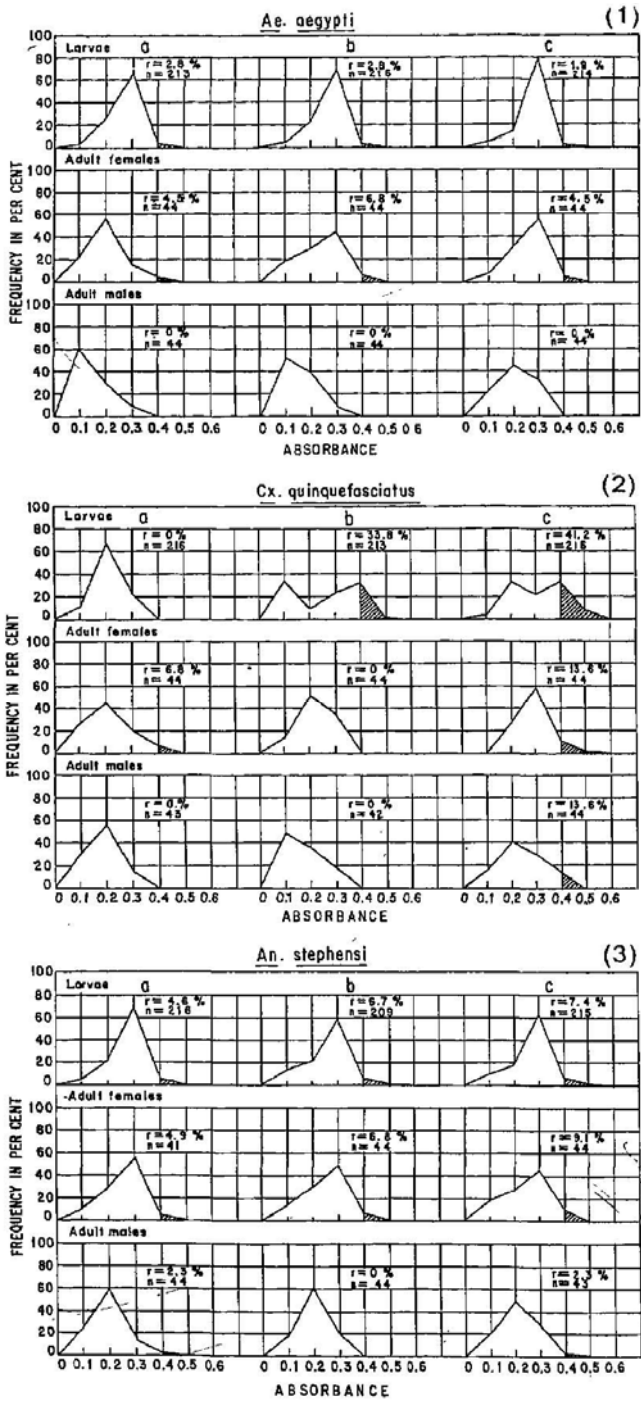
3. Results

Frequency of test mosquitoes for different absorption values at 555 nm showing β-esterase levels of larva/adult mosquitoes from different populations of the pyrethroid-resistant and susceptible strains are plotted as frequency distributions and presented in figures 1–3. On comparison of the esterase profile of the susceptible populations, a threshold level of elevated esterases of absorbance value 0·4 and above was considered as resistance threshold in all the three species of mosquitoes.

In the larvae of *Ae. aegypti,* the esterase profiles of the susceptible and pyrethroid-resistant strains were identical with a single peak at absorbance value of 0·3 (figure 1). Also, the frequency of the larvae beyond threshold resistance did not differ in resistant and susceptible strains as it ranged between 1·9 to 2·8%. In the adult males of *Ae. aegypti,* no elevated esterase activity was observed either in the susceptible or resistant strains. However, the esterase profiles of adult females displayed a peak at 0·3 in pyrethroid-resistant strains as compared to a peak at 0·2 in the susceptible strain. Also, the frequency of the elevated esterase activity in females of the permethrin-R strain was slightly higher than that of its susceptible counterpart (figure 1).

In the case of *Cx. quinquefasciatus,* though the esterase profiles of susceptible larvae revealed only one peak at absorbance value of 0·2, the pyrethroid-R strains showed the presence of an additional peak at 0·4, probably corresponding to the resistant heterozygote (figure 2). The frequency of the larvae beyond the cut-off level of 0·4 was 33·8% and 41·2% in the permethrin- and deltamethrin-R strains, respectively. Unlike in the larvae, the adult females and males of *Cx. quinquefasciatus,* in general, had less esterase activity. The frequency of elevated esterases was almost doubled in females and showed 13·6-fold increase in males of deltamethrin-R strain as compared to its susceptible counterpart. However, permethrin-R strain did not show the presence of elevated esterase individuals either among male or female mosquitoes (figure 2).

The esterase profiles of the larvae of *An. stephensi* showed just one peak at 0·3 in all the strains (figure 3). The elevated esterase activity in the larvae of pyrethroid-R strains showed a marginal increase as the frequency increased from 4·6 % in the susceptible strain to 6·7% in the permethrin-R and 7·4% in the deltamethrin-R strains. In the case of adult males, the esterase levels did not show any distinction between the susceptible and resistant strains. However, female adults *of An. stephensi* exhibited more esterase activity as compared to males (figure 3). The esterase



Figures 1–3. Frequency distributions of absorbance values at 555 nm as the activity of β-esterases in larvae and adult females and males of susceptible **(a),** permethrin-ressistant (b) and deltamethrin-resistant (c) strains of (**1**) *Ae. aegypti,* **(2)** *Cx quinquefasciatus* and (**3**) *An stephensi*

r, Frequency of individuals with elevated esterases; *n,* number of individuals tested.

profiles displayed only one peak at 0·3. The elevated esterase level reached 6·8% and 9·1% in the females of permethrin- and deltamethrin-R strains, respectively, as against 4·9% in case of the parental strain.

4. Discussion

Microplate assay is considered as an excellent tool to identify resistance mechanism and to provide information on the frequency of resistant individuals present in a population (Brogdon 1989). The detection of resistance and its mechanism even at low frequency by this method essentially makes monitoring for the early onset of insecticide resistance in the field, a practical reality (Cordón-Rosales *et al* 1990). It is well documented that microplate assay can detect the presence of elevated esterases that degrade β-naphthyl acetate and confers resistance to a large number of organophosphorus insecticides in mosquitoes such as *Ae. aegypti* (Field *et al* 1984), *Cx. tritaeniorhynchus* (Takahashi and Yasutomi 1987), *Cx. quinquefasciatus* (Hemingway *et al* 1985; Pasteur and Georghiou 1989; Pieris and Hemingway 1990; Bisset *et al* 1991), *An. crucians, An. albimanus* and *An. pseudopunctipennis* (Brogdon *et al* 1988). Similarly, a linear relationship between high esterases activity and pyrethroid-resistance has been established in insects other than mosquitoes such as *Oncopeltus fascial us* (Jao and Casida 1974), *Trichoplusia ni* (Ishaaya and Casida 1980), *Spodoptera littoralis* (Riskallah 1983) and *Musca domestica* (JingLi and Kun 1988).

The β-esterase profiles of *Ae. aegypti* larvae and adults clearly indicate absence of hydrolytic detoxification in permethrin- and deltamethrin-resistance. Similarly, Hemingway *et al* (1989) found that pyrethroid-resistance in a Puerto Rican strain of *Ae. aegypti* was not associated with a quantitative change in esterase activity. Therefore, microsomal oxidation appears to be the main mechanism of pyrethroid-resistance in *Ae. aegypti* as our earlier studies have demonstrated a significant linear increase in monooxygenase activity in deltamethrin-resistant strains of *Ae. aegypti* (Kumar *et al* 1991).

On the contrary, the present data amply indicate a significant role of ester hydrolysis in permethrin- and deltamethrin-resistant strains of *Cx. Quinquefasciatus* as evident from the esterase profiles of larvae and adult female mosquitoes. The presence of two peaks in the frequency distribution of larval populations of resistant strains with regard to elevated non-specific esterases, correspond to susceptible homozygote and resistant heterozygote populations, respectively. Therefore, esterase-mediated detoxification has a predominant role in pyrethroid -resistance in *Cx. quinquefasciatus,* in addition to oxidative detoxification by monooxygenases as demonstrated by Kumar *et al* (1991). It may be of interest to note that only the larvae of *Cx. quinquefasciatus* could select for 1,449-fold deltamethrin- and 175-fold permethrin-resistance as compared to 703-fold deltamethrin- and 99-fold permethrin-resistance in *Ae. aegypti* and 60-fold deltamethrin and 37-fold permethrin-resistance in *An. stephensi* under identical selections. This may evidently be due to their ability to detoxify Pyrethroids by oxidation and ester hydrolysis at equal pace. Though the involvement of both the mechanisms are strongly indicated in this mosquito, it is known that the relative significance of these two mechanisms vary depending on the compound, species and strains (Ishaaya and Casida 1980).

A marginal involvement of the non-specific esterase mechanism in pyrethroid-

resistant *An. stephensi* is also indicated. This established the fact that monooxygenase-mediated detoxification is the major factor resulting in the development of deltamethrin-resistance in the larvae of *An. stephensi* (Kumar *et al* 1991). However, biochemical and bioassay evidences have shown that elevated esterases produced fenitrothion and deltamethrin cross-resistance in the larvae and adults of a multi resistant strain of *An. albimanus* (Brogdon and Barber 1990).

In conclusion, the present data amply demonstrate a predominant role of β-esterases in conferring high levels of permethrin- and deltamethrin-resistance in *Cx. Quinque-fasciatus.*

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