DETERMINATION OF INSECTICIDE SUSCEPTIBILITY IN *CULEX QUINQUEFASCIATUS* SAY ADULTS BY RAPID ENZYME MICROASSAYS

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Abstract. Rapid enzyme microassays for the detection of resistance due to organophosphate and carbamate in individual field-collected strains of *Culex quinquefasciatus* adults were conducted. These tests allowed accurate differentiation by eye, on the basis of color changes of susceptible and resistant individuals. Two separate tests were conducted for the biochemical assays. In the insensitive acetylcholinesterase (AChE) test, acetylthiocholine iodide (ACTH) and 5, 5−dithiobis−(2−nitrobenzoic acid) (DTNB) were used as substrate and coupling agent respectively. The resulting yellow chromophore indicated AChE activity. Test results showed that the color intensity decreased as increasing concentrations of propoxur were added, thereby confirming the susceptibility of the enzyme to inhibitor. Assay of non-specific esterase however, indicated elevated levels which were correlated with degree of malathion resistance. Electrophoretic data revealed the presence of 2 esterase bands in all strains. It was concluded that such a pattern was not contributory to malathion resistance in adults.

INTRODUCTION

The widespread global use of chemical insecticides has in the past decades successfully curbed insect vectors and the diseases they transmit. However, the emergence of insecticide resistance in these vectors has necessitated the development of resistance detection techniques. The present WHO standardized bioassay which is based on insect survivorship following exposure to an insecticide, has been widely used for the past 2 decades and this test gives an indication of development and trends of resistance. However, several shortcomings of the technique have prompted the development of biochemical assay methods. Biochemical techniques are essentially based on the detection and quantification of enzymes known to be responsible for resistance.

Brogdon and Dickson (1983) first developed such tests using microplate assay system to measure acetylcholinesterase (AChE) and non-specific esterase in mosquito homogenates. Using the esterase tests, Lee (1990) demonstrated that malathion resistance in Malaysian *Culex quinquefasciatus* larvae was due to high levels of non-specific esterase. Although adults of this mosquito are known to be highly resistant to malathion (Lee, unpublished data), no biochemical studies have been conducted to elucidate the underlying mechanism(s). This study aims to examine the existence of resistance by using rapid biochemical tests.

MATERIALS AND METHODS

Adult mosquito collection

Adult *Cx. quinquefasciatus* collected from 4 localities in Kuala Lumpur, Malaysia were used for the study. These localities were Tar College, Semerah Padi, Wangsa Maju and Kampong Malaysia. Female mosquitos were collected using bare-leg catch techniques between April and July 1991. Captured mosquitos were fed on mouse blood and allowed to lay eggs in a bowl of water 4 days post-feeding. Egg rafts were hatched in a tray of tap water containing ground monkey pellets. Pupae were collected into a bowl and introduced into a cage. Adult female mosquitos that emerged (F-1 generation) were used for the WHO bioassay test, microassay of AChE and non-specific esterases as well as starch gel electrophoresis. Standard laboratory-bred mosquitos were also used as controls. This strain which originated from Penang, Malaysia and has been main-

tained in the insectary for >10 years was highly susceptible to insecticides.

Bioassay

The WHO (1981) standard adult bioassay procedures were followed with some modifications. Sugar-fed, less than 7 days old adult female mosquitos were exposed to papers impregnated with 5% malathion inside an exposure tube. The insecticide-impregnated paper was obtained from the WHO test kit. Cumulative mortality counts were recorded every 5 minutes until about 90% mortality was achieved. Each bioassay experiment comprised 4 replicates and controls. The data were analyzed with a personal computer programmed with probit analysis as described by Raymond (1985).

Microassay of acetylcholinesterase

Homogenates from female mosquitos were screened for insensitive AChE using a modification of Ellman test (Brogdon *et al,* 1988). Whole bodies of individual less than 7 days old females were used for all experiments. Single mosquitos were homogenized in wells of a plastic spot plate with 100 µl of 0.05M potassium phosphate buffer (pH7.0) using a glass rod. Each homogenate was diluted to a final volume of 500 µl with the same buffer, transferred into microcentrifuge tubes and centrifuged at 8,000 rpm for 10 minutes. Fifty µl of the clear homogenate was then pipetted into a well of microplate. In this way, a total of 7 replicates (50 µl) could be obtained from each mosquito for testing. A 50 µl aliquot mixture of 10% acetone-buffer solution of acetylthiocholine (ACTH) plus various concentrations of propoxur was added into each test well. This was followed by the addition of a 50 µl aliquot of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). After incubation at room temperature (28°C), the intensity of the chromophore was scored visually and scanned by an immunoassay reader (Dynatech, MR 600) at 410 nm. As a positive control, 50 µl ACTH solution (without propoxur) was used.

Microassay of non-specific esterase

A substract solution was first prepared by mixing 0.5 ml α-naphthyl acetate in acetone (6 g/l) with 50 ml phosphate buffer (0.02M; pH 7.0). A coupling reagent consisting of 150 mg of Fast Blue B salt in 15 ml water and 35 ml aqueous sodium dodecyl sulphate was also prepared. Each individual adult was similarly homogenized in 0.5 ml buffer using a glass rod and centrifuged. With a micropipette, 50 µl of the homogenate was transferred to a well in a microplate. Fifty µl of the substrate solution was then pipetted into each well and left for 60 seconds. The coupling reagent (50 µl) was then added. Immediately a deep purple color developed which turned to blue after standing for 10 minutes. The reaction was stopped by the addition of 50 µl 10% acetic acid into each well. The intensity of the final color, indicative of esterase activity, could be differentiated by eye and was assigned the following scores : 0 = colorless/very faint blue; 1 = faint/light blue; 2 = greenish blue; 3 = dark blue. The intensity of the final color was also scanned by an immunoassay reader at 450nm in order to determine quantitatively the color intensity.

Starch gel electrophoresis (SGE)

SGE was conducted to determine the pattern of esterase bands in the adult females. A 10% gel was prepared by dissolving 20g of hydrolyzed starch (Sigma) in 200ml buffer solution by heating. The gel was then poured into a perspex mould and allowed to set at room temperature for at least 3 hours. The mosquito females were homogenized in 2 drops of water using a glass rod and a microtiter plate on ice. A piece of Whatman No. 3 paper (0.5cm × 1.0cm) was placed in each sample well and wetted with the homogenized adults. Electrophoresis was conducted using a LKB apparatus. The voltage was adjusted to 200V for a 4-hour run or 80V for a 17-hours run at constant voltage. At the end of electrophoresis, the gel was sliced and stained with Fast Blue B (Merck); using 1% α-naphthyl acetate as substrate.

RESULTS

Bioassay

Table 1 shows the susceptibility of laboratory and field strains of *Cx. quinquefasciatus* adults to malathion. The LT50 value for the laboratory strain was 20.17 minutes and the value for field strains ranged from 77.14 to 199.29 minutes

Table 1

Malathion susceptibility of strains of adult female *Culex quinquefasciatus* as estimated by a modified WHO bioassay.

Locality	Lethal time (minutes)			
	LT50 (95% CL#)	*Ratio	LT99 (95% CL#)	*Ratio
Laboratory	20.17 (19.02-21.29)	1.00	43.64 (39.28-50.24)	1.00
Tar College	77.14 (77.79-79.55)	3.82	168.10 (154.31-187.05)	3.85
Semerah Padi	112.37 (108.01-116.94)	5.57	458.37 (394.03-554.01)	10.50
Kampong Malaysia	159.06 (153.41-164.99)	7.89	509.56 (450.06-594.67)	11.68
Wangsa Maju	199.29 (189.14-211.01)	9.88	1274.83 (1037.17-1645.07)	29.21

$$* = \frac{\text{LT50, 99 of field strain}}{\text{LT50, 99 of lab strain}}$$

\# = Confidence Limit

Table 2

Propoxur susceptibility of *Culex quinquefasciatus* adult as estimated by AChE microassay.

Propoxur conc (mg/l)	Mean absorbance at 410 nm ± SD				
	Laboratory	Tar College	Semerah Padi	Kampong Malaysia	Wangsa Maju
0	0.24±0.07	0.33±0.13	0.24±0.08	0.24±0.07	0.32±0.77
14	0.13±0.01	0.13±0.01	0.11±0.01	0.13±0.01	0.11±0.01
16	0.10±0.01	0.10±0.01	0.09±0.01	0.10±0.01	0.08±0.01
18	0.10±0.01	0.10±0.01	0.08±0.01	0.10±0.01	0.08±0.01
20	0.09±0.01	0.09±0.01	0.08±0.01	0.09±0.01	0.07±0.01
25	0.09±0.01	0.09±0.01	0.08±0.01	0.09±0.01	0.07±0.01
50	0.08±0.01	0.09±0.01	0.07±0.01	0.08±0.01	0.06±0.01

Table 3

Semi-quantitative determination of esterase in strains of *Culex quinquefasciatus* adults.

Locality	No. replicates	Av eye scores
Laboratory	77	1.2
Tar College	77	2.8
Semerah Padi	77	2.9
Kampong Malaysia	77	2.4
Wangsa Maju	77	2.6

(range of ratio : 3.82-9.88). The field strains hence exhibited some degree of resistance/tolerance to malathion. The descending order of susceptibility of these field strains was Tar College, Semerah Padi, Kampong Malaysia and Wangsa Maju.

AChE microassay

Table 2 shows the propoxur susceptibility of different strains of *Cx. quinquefasciatus* as estimated by the insensitive AChE assay. AChE activity of all strains was inhibited at different propoxur test concentrations ($p < 0.05$). Hence AChE in these strains was sensitive to inhibitors such as carbamates or organophosphates.

Non-specific esterase microassay

Eighty homogenate samples of *Cx. quinquefasciatus* adults were assayed for activity of esterases. The average visual scores of all the wells were recorded and the results are presented in Table 3. Bioassay results were also correlated between these 2 tests (Table 4).

An average eye score of 1.2 was obtained from laboratory strain of *Cx. quinquefasciatus* (LT50 = 20.17 minutes). The 4 field strains had higher LT50 values with corresponding high scores of 2.4-2.9. It could thus be concluded that a color reading of $\geqslant 2$ was indicative of malathion resistance. There is therefore a good correlation between the average eye score and LT50 values ($r = 0.7$).

The frequency distribution of esterase activity is shown in Fig 1-5. The absorbance value of laboratory *Cx. quinquefasciatus* was $\leqslant 0.1$ (Fig 1). Since bioassay results confirmed that this strain was highly susceptible, the resistance threshold of *Cx. quinquefasciatus* adult to malathion must be = 0.1. Fig 2-5 show that the frequency of the replicates were all distributed with 57.5-93.9% having absorbance of $\geqslant 2.0$.

Electrophoresis

Two distinct darkly stained bands of α-naphthyl acetate hydrolysing esterase were obtained from all strains.

DISCUSSION

The WHO bioassay kit has been used for decades to determine resistance in mosquitos, but as the problem becomes complicated; biochemical tests are required to supplement and complement bioassay results. Hence in this study, bioassay tests were supported with enzyme assays to attempt

Table 4

Esterase activity in correlation with malathion susceptibility of *Culex quinquefasciatus* adults.

Locality	LT50 (minutes)	Average eye score	Maximum absorbance	*No. of esterase bands
Laboratory	20.17	1.2	0.1	2
Tar College	77.14	2.8	$\geqslant$ 2.0	2
Semerah Padi	112.37	2.9	$\geqslant$ 2.0	2
Kampong Malaysia	159.06	2.4	$\geqslant$ 2.0	2
Wangsa Maju	199.29	2.6	$\geqslant$ 2.0	2

* Determined by electrophoresis

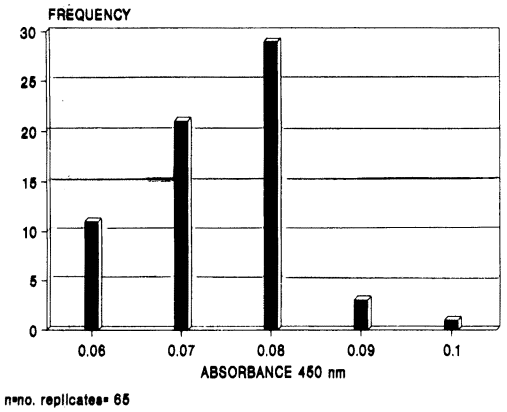

Fig 1—Non-specific esterase microassay of *Culex quinquefasciatus* adults (lab).

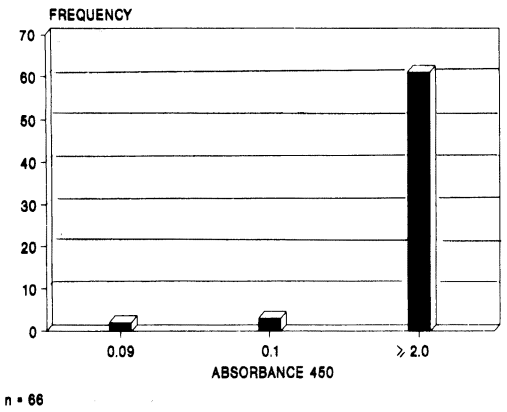

Fig 2—Non-specific esterase microassay of *Culex quinquefasciatus* (Tar College).

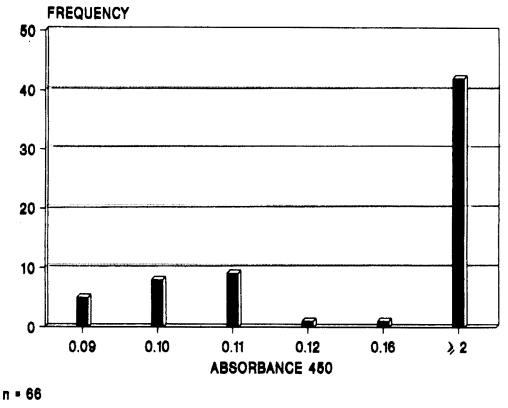

Fig 3—Non-specific esterase microassay of *Culex quinquefasciatus* (Wangsa Maju)

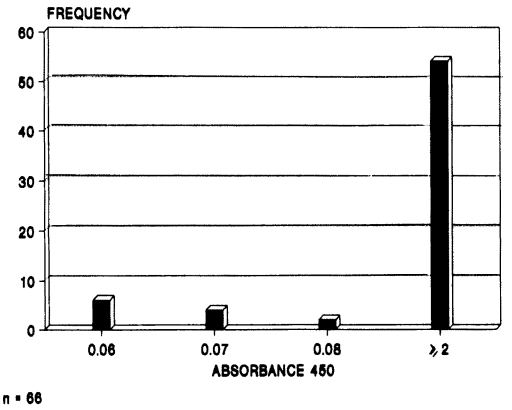

Fig 4—Non-specific esterase microassay of *Culex quinquefasciatus* (Semerah Padi).

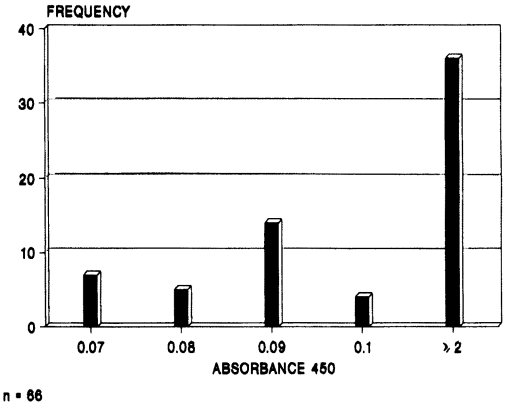

Fig 5—Non-specific esterase microassay of *Culex quinquefasciatus* (Kampong Malaysia).

to provide a more accurate and reliable picture. The existence of malathion resistance in Malaysian *Cx. quinquefasciatus* adults was confirmed by these tests and the underlying mechanism (elevated levels of esterase) was also elucidated. In fact, high level of resistance due to increased levels of esterase was noted in *Cx. quinquefasciatus* larvae in an earlier study (Lee, 1990).

Several other workers have also reported on the possibility of using these biochemical tests. Hemingway and Smith (1986) reported that it was possible to detect the presence of an altered AChE-type organophosphate and carbamate resistance in individual insects using the AChE assay. The level of AChE was indicated by intensity of yellow

coloration after an incubation period of 20-30 minutes. Using this technique, Hemingway *et al* (1986) were able to show that *Anopheles nigerrimus* and *Cx. quinquefasciatus* in Sri Lanka carried this type of resistance gene. Brogdon *et al* (1988) detected the presence of 2 mechanisms of resistance namely, insensitive AChE and elevated non-specific esterase in Guatemalan *An. albimanus*. However, Lee (1991a, 1991b) did not find the presence of insensitive AChE in *Aedes aegypti* larvae and suggested that resistance/tolerance to temephos was most likely due to elevated levels of non-specific esterase.

The importance of scanning the test wells with a reader is that this permits differentiation of susceptibility of mosquitos from any localities by the pattern of frequency distribution. Therefore it was determined that the maximum absorbance value of laboratory strain of *Cx. quinquefasciatus* adult in the non-specific esterase assay was 0.1. Lee (1990) had determined that the threshold absorbance value for *Cx. quinquefasciatus* was 0.09, while a value of 0.9 was reported for *An albimanus* by Brogdon *et al* (1988).

Electrophoretic studies were also conducted with the aim of demonstrating the esterase pattern of strains of the mosquito. Although Chen and Sudderuddin (1987) suggested that the level of insecticide tolerance was found to be related directly to the number of esterase bands, this did not seem to be the case in *Cx. quinquefasciatus* adults; as only 2 bands were observed in all the strains. The resistance mechanism hence could not be due to different isoenzyme but caused by the different levels of esterase.

Microplate assays of enzymes responsible for resistance in mosquitos are sensitive and simple techniques that may have widespread implications for future resistance detection methodology. We are presently attempting to adapt these tests for mass scale field use.

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