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## Biochemical Assay Baseline Data of Permethrin Resistance in *Anopheles stephensi* (Diptera, Culicidae) from Iran

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**Abstract:** Bioassays were performed on 1-2 days old sugar fed adult mosquitoes of field and susceptible Beech strains of *An. stephensi* using WHO standard kit with some modifications. The  $KT_{50}$ s of exposure to 0.08% permethrin impregnated papers were  $40 \pm 1$ ,  $43 \pm 1$  and  $44 \pm 2$  for susceptible, Kazeroun and Bandar Abbas strains, respectively. Biochemical assays of esterases, monooxygenases and glutathione S-transferases were undertaken on *An. stephensi* from Kazeroun and Bandar Abbas and the results were compared to those of the susceptible Beech strain. The mean alfa-esterase activity of Kazeroun, Bandar Abbas and Beech strains were  $6.5 \times 10^{-2}$ ,  $7.1 \times 10^{-2}$  and  $6.1 \times 10^{-2}$ ; the mean beta-esterase activity in those strains were  $7.2 \times 10^{-3}$ ,  $3.2 \times 10^{-3}$  and  $2.4 \times 10^{-3}$ ; the mean content of  $p^{450}$  in the above-mentioned strains were  $8 \times 10^{-3}$ ,  $4.6 \times 10^{-3}$  and  $3.4 \times 10^{-3}$ ; the mean GST activity were 8.7, 10.5 and 3.6, respectively. Although according to the results of this study, *An. stephensi* from Iran is not considered to be resistant to pyrethroids, the results of bioassays and the mean activity of all enzyme groups in field strains were significantly different from those of the susceptible strain. The results of bioassays were significantly different from those performed some 15 years ago on the same strains. Hence, insecticide resistance monitoring techniques should be put in place and also resistance management strategies and measures should be considered implementing in the area to prevent or postpone insecticide resistance development.

**Key words:** *An. stephensi*, biochemical assays, permethrin, resistance

### INTRODUCTION

*Anopheles stephensi* is a serious urban vector of malaria in many countries in the Middle East and the Indian subcontinent. There are reports of pyrethroid resistance or tolerance in *An. stephensi* in the region (Ladonni, 1988; Vatandoost, 1996; WHO, 2000; Ganesh *et al.*, 2003). Laboratory selection of *An. stephensi* with pyrethroids lead to resistance in as few as 5 generations (Kumar *et al.*, 1991; Chakravorthy and Kalyanasundaram, 1992). In some strains of *An. stephensi* DDT-resistance produced cross-resistance to pyrethroids and selection of DDT-resistant *An. stephensi* by pyrethroids produced higher levels of resistance to both insecticides (Omer *et al.*, 1980; Ladonni, 1988; Vatandoost *et al.*, 1996). In strains from Iran and Pakistan, however, kdr-type cross-resistance was not observed (Malcolm, 1990; Enayati, 1992). When the KDR site-insensitivity resistance mechanism is present, cross-resistance between DDT and pyrethroids occurs. In the DUB-S strain of *An. stephensi* and a strain from Pakistan, DDT and pyrethroid susceptibility was not

fully restored by synergists like dichlorophenyl ethanol (DMC), piperonyl butoxide (PBO) and S,S,S-tributylphosphorothiolate (DEF), suggesting that a site insensitivity mechanism was present (Omer *et al.*, 1980; Ladonni, 1988; Vatandoost, 1996; Enayati *et al.*, 2003).

Monooxygenases, esterases and glutathione S-transferases are the three major enzyme groups involved in metabolic insecticide resistance (Oppenoorth *et al.*, 1977; 1979; Hemingway and Karunaratne, 1998; Scott, 1999). Monooxygenase involvement in pyrethroid resistance has been well studied in different vectors (Scott and Georgiou, 1985, 1986; Ladonni, 1988; Kumar *et al.*, 1991; Anspaugh *et al.*, 1994; Vatandoost, 1996; Liu and Scott, 1998; Kasai and Scott, 2000; Valles *et al.*, 2000). Highly elevated esterases, due to gene amplification in *Culex*, are only important in OP and carbamats resistance (Karunaratne *et al.*, 1993; Vaughan *et al.*, 1995; De Silva *et al.*, 1997; Small *et al.*, 1998; Hemingway and Ranson, 2000; Paton *et al.*, 2000). However, the elevated E4 and FE4 esterases in aphid *M. persicae* contribute at least marginally to permethrin resistance (Devonshire and

Moore, 1982; Devonshire *et al.*, 1998). In some other agricultural insect pests e.g. *Helicoverpa armigera*, *H. punctigera* and *Bemisia tabaci* some permethrin hydrolysing esterases have been characterised (Gunning *et al.*, 1996; 1997; 1999). Although for the new generations of  $\alpha$ -cyano-pyrethroids, esterases are more important resistance route, they have not been as well studied as monooxygenase-based pyrethroid resistance. The route of glutathione S-transferase-based pyrethroid resistance in insects has only recently been determined (Vontas *et al.*, 2001; 2002). In this study the susceptibility status of the field collected strains of *An. stephensi* from Iran and their potential biochemical resistance mechanisms are studied.

## MATERIALS AND METHODS

**Mosquito strains:** Field strains of *An. stephensi* were collected from Bandar Abbas and Kazeroun, southern Iran and reared to the first generation in insectary under standard condition and used for bioassays and biochemical assays. Beech, a pyrethroid susceptible strain originated from India in 1940 and kept in the insectary without being exposed to insecticides.

**Bioassays:** World Health Organisation (WHO) standard kits for adult mosquito susceptibility tests with some modifications were used for bioassay experiments. Permethrin impregnated papers (0.08%) were prepared locally using pure olive oil, acetone and analytical grade permethrin. Groups of 25, 1-2 days old adults were placed into holding tubes and held for 30 min to control possible mortality due to handling. During this holding period, mosquitoes were provided with cotton pads moisturized with 10% sucrose solution. Exposure tubes containing insecticide impregnated papers were attached to the holding tubes and mosquitoes were gently transferred to the exposure tubes by blowing through the holding tubes. After a fixed exposure time, mosquitoes were transferred back to the holding tubes, provided with 10% sucrose and kept at 25°C and 70-80% relative humidity. Thirty min after exposure, the knockdown rates were scored and the data were subjected to probit analysis.

**Biochemical assays:** Biochemical assays for general esterases, glutathione S-transferases and monooxygenases were performed on adult mosquito according to Hemingway (1998) with some modifications. Adult mosquitoes were individually homogenised in 200  $\mu$ L of distilled water in a flat-bottomed microtitre plate on ice. The plate was spun at 3000 rpm for 15 min in a Beckman Culter centrifuge (Beckman) at 4°C and the supernatant was used as the source of enzymes in reaction mixtures. In each biochemical assay, blank

replications (all components of the reaction mixture except for the enzyme source) were provided.

**Monooxygenases assay:** This assay measures the total amount of haem containing protein using a haem-peroxidase assay. As cytochrome P<sup>450</sup>s make up the bulk of the proteins in non-blood-fed insects, results can crudely be expressed as equivalent units of cytochrome P<sup>450</sup> within the insect (Brogdon *et al.*, 1999b). The reaction mixture in each well of the microtitre plate contained 20  $\mu$ L of insect homogenate, 80  $\mu$ L of 0.0625 M potassium phosphate buffer pH 7.2, 200  $\mu$ L of 3, 3', 5, 5' tetramethyl benzidine (TMBZ) solution (0.01 g TMBZ dissolved in 5 mL methanol plus 15 mL of 0.25 M sodium acetate buffer pH 5.0) and 25  $\mu$ L of 3% hydrogen peroxide. The plates were incubated at room temperature for 2 h and absorbance was read at 450 nm as an endpoint in a Biotek ELX 808U plate reader. The values were compared with a standard curve of purified cytochrome C and were reported as equivalent units of cytochrome P<sup>450</sup>/mg protein corrected for the known content of cytochrome C and P<sup>450</sup>.

**General esterases:** General esterases activities with the substrates  $\alpha$ - and  $\beta$ -naphthyl acetate were determined. Reaction mixtures contained 20  $\mu$ L of insect homogenate in duplicate in adjacent microtitre plate wells (assigned  $\alpha$  and  $\beta$ ) and 200  $\mu$ L of  $\alpha$ - or  $\beta$ -naphthyl acetate solution (120  $\mu$ L of 3.0 mM  $\alpha$  or  $\beta$ -naphthyl acetate dissolved in 12 mL 0.02 M phosphate buffer pH 7.2), respectively. The reaction mixtures were incubated at room temperature for 30 min before the addition of 50  $\mu$ L of fast blue solution (0.023 g fast blue dissolved in 2.25 mL distilled water and 5.25 mL of 5% SDS 0.1 M sodium phosphate buffer pH 7) to each well. Plates were incubated at room temperature for 5 min and then absorbance was read at 570 nm as an endpoint value. The resulting optical densities (OD) were compared with standard curves of ODs for known concentrations of the products  $\alpha$ - and  $\beta$ -naphthol, respectively to convert the absorbances to product concentrations. The enzyme activities were reported as nmol of product formed/min/mg protein.

**Glutathione S-transferases:** Glutathione S-transferase activity was measured using a reaction mixture of 10  $\mu$ L of the homogenate plus 200  $\mu$ L of reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) solution (10 mM reduced glutathione dissolved in 0.1 M phosphate buffer pH 6.5 and 3 mM CDNB originally dissolved in 1 mL methanol). The increase in absorbance was measured at 340 nm for 5 min. The enzyme activity was reported as  $\mu$ M/min/mg protein using the extinction co-efficient corrected for the path length of the solution in the microtitre plate well.

# RESULTS

**Bioassays:** The results of bioassays performed on adult *An. stephensi* using 0.08% permethrin impregnated papers were subjected to probit analysis and summarised in Table 1. The bioassays shows that there are statistically significant differences between the  $KT_{50}$  of Bandar Abbas

and Kazeroun field strains with that of Beech ( $p<0.05$ ). Also the results show that the order of  $KT_{50}$  of the strains is Bandar abbas>Kazeroun>Beech.

**Biochemical Assays:** Monooxygenase, general esterase and glutathione S-transferase assays were carried out on adults of Bandar Abbas, Kazeroun and Beech strains of

Table 1: The results of probit analysis on the bioassay results performed on adults of different strains of *An. stephensi* from Iran compared with a susceptible strain Beech

Strains of <i>An. stephensi</i>	$\alpha$	$\beta$	$KT_{50} \pm SD$	$KT_{50}$	Resistance ratio
Beech	-8.95	7.8	40 $\pm$ 1	57	1
Kazeroun	-9.6	8.94	43 $\pm$ 1	59	1.075
Bandar Abbas	-8.42	8	44 $\pm$ 2	63	1.1

Table 2: Descriptive analysis of the results of biochemical assays performed on field and susceptible strains of *An. stephensi* from Iran. GST: Glutathione transferases, Alfa: Alfa esterases, Beta: Beta esterases,  $P^{450}$ : Cytochrome  $P^{450}$ . 1: Beech susceptible strain, 2: Kazeroun strain, 3: Bandar Abbas strain

95% Confidence interval for mean									
	N	Mean	Std. deviation	Std. error	Lower bound	Upper bound	Minimum	Maximum	
GST	1.00	93	3.62390	5.00841	0.51935	2.59243	4.65537	0.000	16.030
	2.00	93	10.54629	5.39187	0.55911	9.43585	11.65674	1.244	28.742
	3.00	93	8.67253	6.96781	0.72253	7.23752	10.10753	0.004	24.705
	Total	279	7.61424	6.52431	0.39060	6.84533	8.38315	0.000	28.742
Alfa	1.00	93	6.12E-02	1.57369E-02	1.63E-03	5.79386E-02	6.44206E-02	0.0392	0.1242
	2.00	93	7.14E-02	1.88097E-02	1.95E-03	6.75372E-02	7.52848E-02	0.0462	0.1827
	3.00	93	6.54E-02	2.82230E-02	2.93E-03	5.95936E-02	7.12185E-02	0.0229	0.1843
	Total	279	6.60E-02	2.19164E-02	1.31E-03	6.34160E-02	6.85818E-02	0.0229	0.1843
Beta	1.00	93	2.39E-03	3.327821E-03	3.45E-04	1.707411E-03	3.078124E-03	0.00000	0.01406
	2.00	93	3.26E-03	3.773553E-03	3.91E-04	2.484952E-03	4.039260E-03	0.00064	0.01358
	3.00	93	7.26E-03	1.399397E-02	1.45E-03	4.376274E-03	1.014032E-02	0.00002	0.09140
	Total	279	4.30E-03	8.814172E-03	5.28E-04	3.265613E-03	5.343167E-03	0.00064	0.09140
$P^{450}$	1.00	93	3.46E-03	1.595058E-03	1.65E-04	3.135541E-03	3.792537E-03	0.00091	0.00620
	2.00	93	4.62E-03	8.026919E-03	8.32E-04	2.966281E-03	6.272530E-03	-0.00026	0.04531
	3.00	93	7.97E-03	7.314638E-03	7.58E-04	6.460855E-03	9.473719E-03	-0.00019	0.03060
	Total	279	5.35E-03	6.597802E-03	3.95E-04	4.572673E-03	6.127815E-03	-0.00026	0.04531

Table 3: One way ANOVA analysis of the results of biochemical assays performed on field and susceptible strains of *An. stephensi* from Iran. GST: Glutathione transferases, Alfa: Alfa esterases, Beta: Beta esterases,  $P^{450}$ : Cytochrome  $P^{450}$ . 1: Beech susceptible strain, 2: Kazeroun strain, 3: Bandar Abbas strain

Dependent variable	(1) 1 = India, 2 = Kazeroun 3 = Bandar Abbas		(J) 1 = India 2 = Kazeroun 3 = Bandar Abbas	Mean difference (1-J)	Std. Error	Sig.	95% Confidence interval	
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	Lower bound Upper bound							
GST	1.00	2.00	3.00	-6.92240*	0.85805	0.000	-8.93341	-4.91138
					-5.04863*	0.85805	0.000	-7.05964
	2.00	1.00	3.00	6.92240*	0.85805	0.000	4.91138	8.93341
					1.87377	0.85805	0.074	-0.13724
	3.00	1.00	2.00	5.04863*	0.85805	0.000	3.03762	7.05964
					-1.87377	0.85805	0.074	-3.88478
Alfa	1.00	2.00	3.00	-1.023E-02*	3.17E-03	0.004	-1.7651E-02	-2.8120E-03
					-4.226E-03*	3.17E-03	0.376	-1.1646E-02
	2.00	1.00	3.00	1.023E-02	3.17E-03	0.004	2.81200E-03	1.76507E-02
					6.005E-03	3.17E-03	0.140	-1.4145E-03
	3.00	1.00	2.00	4.226E-03	3.17E-03	0.376	-3.1929E-03	1.16459E-02
					-6.005E-03	3.17E-03	0.140	-1.3424E-02
Beta	1.00	2.00	3.00	-8.693E-04	1.26E-03	0.769	-3.8202E-03	2.081556E-03
					-4.866E-03*	1.26E-03	0.000	-7.8164E-03
	2.00	1.00	3.00	8.693E-04	1.26E-03	0.769	-2.0816E-03	3.820234E-03
					-3.996E-03*	1.26E-03	0.004	-6.9471E-03
	3.00	1.00	2.00	4.886E-03*	1.26E-03	0.000	1.914635E-03	7.816426E-03
					3.996E-03*	1.26E-03	0.004	1.045296E-03
p <sup>450</sup>	1.00	2.00	3.00	-1.155E-03	9.29E-04	0.428	-3.3334E-03	1.022703E-03
					-4.503E-03*	9.29E-04	0.000	-6.6813E-03
	2.00	1.00	3.00	1.155E-03	9.29E-04	0.428	-1.0227E-03	3.333436E-03
					-3.348E-03*	9.29E-04	0.001	-5.5260E-03
	3.00	1.00	2.00	4.503E-03*	9.29E-04	0.000	2.325178E-03	6.681318E-03
					3.348E-02*	9.29E-04	0.001	1.169812E-03

\* The mean difference is significant at 0.05 level

*An. stephensi*. All raw data were analysed using formulas and standard curves in Microsoft Excel software and reported as the equivalent units of cytochrome P<sup>450</sup> for monooxygenase and activity rates for the latter two enzymes. The mean alfa-esterase activity of Bandar Abbas, Kazeroun and Beech strains were  $6.5 \times 10^{-2}$ ,  $7.1 \times 10^{-2}$  and  $6.1 \times 10^{-2}$ ; the mean beta-esterase activity in those strains were  $7.2 \times 10^{-3}$ ,  $3.2 \times 10^{-3}$  and  $2.4 \times 10^{-3}$ ; the mean content of p<sup>450</sup> in the above-mentioned strains were  $8 \times 10^{-3}$ ,  $4.6 \times 10^{-3}$  and  $3.4 \times 10^{-3}$ ; the mean GST activity were 8.7, 10.5 and 3.6, respectively. Analysis of variance of the data showed that the differences between the enzymes activities of the field and Beech strains were statistically significant ( $\alpha < 0.001$ ) (Table 2 and 3).

## DISCUSSION

Monitoring insecticide resistance and its underlying mechanisms are crucially important in vector control as well as insecticide resistance management. Classic bioassay is a powerful, cheap and relatively easy method of monitoring and measuring insecticide resistance, however, it lacks adequate sensitivity and does not give a clear picture of mechanisms involved in insecticide resistance. When bioassay is undertaken along with more sensitive biochemical assays, more information and solid evidence come out about the insecticide resistance status and its possible mechanisms. In this study, possible mechanisms of insecticide resistance were investigated using bioassays based on WHO standards and biochemical assays according to Hemingway (1998).

Although the results of bioassays performed on the field strains and the susceptible strain showed statistically significant differences, based on WHO criteria, the results revealed that there is neither a resistance nor even a tolerance to permethrin in the field strains of *An. stephensi* collected from southern Iran. However, it is obvious that long term use of pyrethroid insecticides will eventually lead to resistance and in fact pyrethroid resistance in *An. stephensi* has already been documented from many countries in the Middle East and Indian subcontinent (Ladonni, 1988; Kumar *et al.*, 1991; Chakravorthy and Kalyanasundaram, 1992; Vatandoost, 1996; Enayati *et al.*, 2003; Ganesh *et al.*, 2003). The results of the bioassays showed a clear and statistically significant increase compared to the results of a study on the same strains performed some 15 years ago (Enayati, 1992). This implies that a trend of building up pyrethroid resistance has been started and developed over the years and sooner or latter may lead to insecticide resistance.

Comparative analysis of the results of biochemical assays performed on the field strains and the susceptible strain Beech revealed that esterases, monooxygenases

and glutathione S-transferases were at higher levels in the field strains. The increase in P<sup>450</sup> quantity, beta esterases and glutathione S-transferases activities in the field strains were about three times as much as those in Beech susceptible strain. Therefore, biochemical assays confirm that esterases, monooxygenases and possibly glutathione S-transferases are involved in pyrethroid resistance in the field strains of *An. stephensi* from Iran. Involvement of these enzymes in pyrethroid resistance in mosquitoes is well documented in previous studies on the same species as well as other mosquitoes (Omer *et al.*, 1980; Ladonni, 1988; Kumar *et al.*, 1991; Chakravorthy and Kalyanasundaram, 1992; Enayati, 1992; Vatandoost, 1996; Vulule *et al.*, 1996, 1999; Enayati and Ladonni, 1997; Enayati *et al.*, 2003; Ganesh *et al.*, 2003).

The activity of beta esterase and the quantity of P<sup>450</sup> in Bandar Abbas strain is much more than that of the susceptible strain as well as Kazeroun strain. However, the activities of alfa esterase and glutathione transferase in Kazeroun strain are only slightly more than those of the Bandar Abbas strain. This might be because the population of *An. stephensi* from Bandar Abbas has been under more insecticide selection pressure than the Kazeroun population. These findings are in accord with the results of other studies that esterases and oxidases are mainly involved in pyrethroid resistance (Oppenoorth *et al.*, 1977; 1979; Scott and Georghiou, 1985; 1986; Ladonni, 1988; Kumar *et al.*, 1991; Anspaugh *et al.*, 1994; Vatandoost, 1996; Hemingway and Karunaratne, 1998; Liu and Scott, 1998; Scott, 1999; Kasai and Scott, 2000; Valles *et al.*, 2000).

A quick look at the levels of the enzymes in the field and susceptible strains shows that the order of importance of the enzymes in conferring insecticide resistance is beta esterases, glutathione transferases and P<sup>450</sup>. Involvement of esterases, glutathione transferases and cytochrome oxidases in pyrethroid resistance have been identified in various mosquito species and strains (Jianhua and Jialiang, 1987a, b; Ladonni, 1988; Kumar *et al.*, 1991; Enayati, 1992; Sahgal *et al.*, 1994; Vatandoost, 1996; Vulule *et al.*, 1996, 1999; Enayati and Ladonni, 1997; Kasai *et al.*, 1998; Brogdon *et al.*, 1999a, b; Kasai and Scott, 2000; Enayati *et al.*, 2003; Enayati and Hemingway, 2006).

There is a great deal of coordination between the results of bioassays and biochemical assays, each confirming the results of the other. The resistance ratios obtained by bioassays shows slower increase compared with that of biochemical assays. This is at least partly because biochemical assays are rather more sensitive and give a more clear picture of resistance before it is too late. In other words, when resistance is confirmed by bioassays, the gene frequency is at such a level that no resistance management strategies can easily be put in

place with success. The indications that biochemical assays give at much higher levels, helps the authorities and experts in implementing vector control and resistance management strategies in time and with higher chance of success.

Although no resistance to insecticide has been reported for *An. stephensi* from Iran, but the trend of increase in the level of enzymes responsible for insecticide resistance should be a cause for concern as this means that the process of building up tolerance and finally resistance to insecticides has already been started. In fact detection of a 3-fold increase in the activity or amount of the esterases, glutathione transferases and P<sup>450</sup> in the field strains compared to the susceptible strain should encourage the authorities and the experts to think of implementing resistance management strategies such as rotation, mosaics, mixture, change or any other methods in order to prevent or postpone the process of insecticide resistance development.

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