Original Article

Biochemical Basis of Cyfluthrin and DDT Resistance in *Anopheles stephensi* (Diptera: Culicidae) in Malarious Area of Iran

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Abstract

Background: *Anopheles stephensi* is a key urban malaria vector in the Indian subcontinent and Middle East including south and southeast of Iran. Wide application of insecticides resulted in resistance of this species to various insecticides in these regions. This study was conducted to reveal the role of metabolic mechanisms in the development of resistance in *An. stephensi* to DDT and cyfluthrin.
Methods: Field mosquito specimens were collected from Chabahar Seaport, southeast corner of Iran, in 2015. Insecticide susceptibility and enzyme assays were conducted as recommended by WHO**.**
Results: Mean enzyme ratios were 3.95 and 3.04 for α- esterases and 2.40 and 1.97 for β- esterases in the DDT and cyfluthrin- resistant populations correspondingly compared with the susceptible strain. The GSTs enzyme mean activity ratios were 5.07 and 2.55 in the DDT and cyfluthrin- resistant populations compared with the susceptible beech strain. The cytochrome p450s enzyme ratios were 1.11 and 1.28 in the DDT and cyfluthrin- resistant populations respectively compared with the susceptible beech strain.
Conclusion: Metabolic mechanisms play a crucial role in the development of DDT and cyfluthrin resistance in *An. stephensi*, therefore, further evaluation of the mechanisms involved as well as implementation of proper insecticide resistance management strategies are recommended.

Keywords: *Anopheles stephensi*, Insecticide, Resistance mechanisms, Malaria

Introduction

Malaria is still a major public health problem in southeast corner of Iran (1). There are seven *Anopheles* species as malaria vectors in Iran including *An. stephensi, An. culcifacies* s.l., *An. maculipennis* s.l., *An. sacharovi, An. superpectus* s.l., *An. dthali*, and *An. fluviatilis* s.l.. *Anopheles stephensi* is the most important malaria vector in southern region of the country (2-10).

Application of chemical insecticides is one of the most important interventions for malaria control, used in Iran during past decades. Different groups of insecticides including organochlorines (DDT, dieldrin and BHC), organophosphates (pirimiphos-methyl and malathion), carbamate (propoxur) and pyrethroids (lambdacyhalothrin and delthamethrin) in dif-

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ferent forms of application such as indoor residual spraying (IRS) and insecticide-treated nets (ITNS) for adult mosquito control and organophosphates for larviciding were used in malarious areas of the country (11-13).

Iran has embarked on the malaria elimination program since 2007 relying on application of chemical insecticides specially pyrethroid compounds for malaria vector control (14).

Anopheles stephensi is resistant to several insecticides including DDT, dieldrin, and malathion (12, 15-19). The first indication of pyrethroid resistance was reported from Chabahar Seaport, southeast of Iran in 2012 (13). Moreover, there are many reports on resistance of this species to different insecticide groups including pyrethroids from Iran neighboring countries including Pakistan, Afghanistan, the Indian subcontinent as well as the Middle East countries (14, 15, 18, 20-25).

Due to the importance of pyrethroids in malaria control program and the slow process of development of new insecticide compounds, monitoring and management of insecticide resistance are necessary (26, 27). Metabolic and target site insensitivity are two common resistant mechanisms in insects. In metabolic resistance, alteration in the levels or activities of detoxification enzymes such as esterases, glutathione S-transferases (GSTs), and cytochrome P450s may occur (24, 28). In target site insensitivity, mutations in the sodium channel, acetylcholinesterase and GABA receptor genes occur (29). Therefore, determination of resistance mechanisms in *An. stephensi* is essential for proper management of insecticide resistance through vector control interventions.

The aim of this study was to determine the possible involvement of enzymes groups' in DDT and pyrethroid insecticides resistance functioning in *An. stephensi*, the main malaria vector in southeast of Iran.

Materials and Methods

Mosquito collection and rearing

Anopheles stephensi larvae were collected from larval habitats using the standard dipper from Chabahar Seaport (25°25′N, 60°45′E) Sistan and Baluchestan Province, southeast of Iran (Fig. 1) during Apr to June 2015. The larvae specimens were transported in cool boxes to insectary of the Medical Entomology and Vector Control Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran and reared to adult stage under standard condition at 25 °C, 80% relative humidity with a 12h day/night lighting cycle. The adult mosquito specimens were identified to species level using the identification key (30).

Moreover, a pyrethroid susceptible strain (Beech strain) originated in India in 1940 and kept in the insectary without being exposed to insecticides used as a control in all experiments.

Adult susceptibility tests and selection

Six different insecticide impregnated papers including DDT 4%, lambdacyhalothrin 0.05%, deltamethrin 0.05%, cyfluthrin 0.15%, permethrin 0.75%, and etofenprox 0.5% supplied by WHO were used for evaluating the susceptibility status of *An. stephensi* populations from Chabahar. Two or three days old adult female mosquitos that were kept on 10% aqueous sucrose solution were used for susceptibility test procedure according to the WHO method (27). Then the mosquito populations with the lowest mortality rates were subjected to selection pressure of the two insecticides in the laboratory. The mosquito populations were exposed to the two insecticides in two separate lines over 18 and 19 generations throughout four and five selection phases. For both insecticides, mortality rate was calculated in different times and regression lines were plotted in each generation using Microsoft Excel (ver. 2013). A subset (40 specimens for each sample) of resistant and susceptible mosquito populations were placed in a 1.7ml tubes and kept in freezer (-80 °C). These frozen specimens were then transported in a cold chain to the Pesticide Biochemistry

Laboratory of Medical Entomology Department, School of Public Health, Mazandaran University of Medical Sciences, Sari, Iran for further biochemical assays.

Biochemical assays

Biochemical tests were performed according to the method described by Hemingway (1998) (31). The enzyme activities/contents of P450s, glutathione S-transferases (GSTs), and esterases using corresponding fresh buffer solutions were quantified. Frozen adult mosquito specimens were individually put in wells of flat-bottomed 96-well microtiter plate and manually homogenized using a steel pestle in 250µL cold distilled water at 4 °C. The plate was spun at 3000rpm for 20min in a Beckman Coulter (Beckman Inc., USA) centrifuge at 4 °C and the supernatant was used as the source of enzymes in reaction mixtures. In each biochemical assay, blank replications (all component of the reaction mixture except for the enzyme source) were provided. Preparation of all reaction mixtures was carried out on ice (31).

Total Protein assay

In order to minimize the error due to different size and protein contents of mosquitos and homogenizing process, total protein component of each specimen was measured using Bradford method in triplicate by adding 300 µL of Bio-Rad solution (diluted with distilled water by 1:4) to 10µL of the homogenate. After 5min incubation at room temperature, the absorbance was measured at 570nm in a Biotek ELX808 Ultra Microplate Reader (Biotek Inc, USA) (31). The value was changed into product concentration using a bovine serum albumin standard curve obtained with the same reagents and method.

Cytochrome P450s assay

This test quantifies the amount of hem containing protein in the specimens. In each well, the reaction cocktail comprised of 20µl of the mosquito homogenate in duplicate, 80µL of 0.0625M potassium phosphate buffer PH 7.2, 200µL of 3, 3', 5, 5' tetramethyl benzidine (TMB) solution (0.01g TMB dissolved in 5ml methanol plus 15ml of 0.25M sodium acetate buffer pH 5.0) and 25µL of 3% hydrogen peroxide. After 2h incubation in room temperature, the absorbance was measured at 450nm. The protein contents were described as correspondent units of cytochrome (EUC) P450sec/mg protein corrected for the known hem content of P450s and cytochrome C using a standard curve of purified cytochrome C (31).

Glutathione S-transferase assay

The reaction mixture contained 200µL of reduced glutathione plus 1-coloro-2, 4-dinitrobenzene (CDNB) added to 10µL of the mosquito homogenate in duplicate. The increase in absorbance was measured at 340nm for 5 min. The amount of conjugate produced/min/mg protein (mM) using the extinction coefficient of CDNB corrected for the path length of the solution in the microplate well was reported as enzyme activity (31).

General esterase assay

In this assay, the activity of α-esterase and β-esterase with the alfa and beta-naphthyl acetate as universal substrates were measured. To a reaction mixture of 200µL of alpha or beta-naphthyl acetate solution was added to 20µL of mosquito homogenate in duplicate. After 30min incubation at room temperature, 50µL of fast blue solution was added to each mixture. Plates were incubated at room temperature for another 5min and then absorbance was recorded at 570nm (19). The optical densities (OD) of solutions were converted to product concentration as µM of product formed/min/mg protein using standard curves of ODs for known concentrations of the products α- or β –naphthol (31).

In each biochemical assays, four blank replicates were set using the same materials of

each assay except for distilled water added instead of the mosquito homogenate. The ODs of the wells containing mosquito homogenates were adjusted by deducting with the average ODs of the blank replicates.

Data conversion and analyses

The activity/contents of the enzymes were measured and used for further analysis by Microsoft Excel. The data then were transformed into the actual enzyme activity values using standard curves. Mean values of the enzyme activities of all populations were compared using ANOVA in conjunction with the Tukey's statistical test using SPSS ver. 19 software (Chicago, IL, USA) (P< 0.05). Enzyme ratios (ER) were computed by dividing the mean activities of each resistant population with those of the Beech susceptible strain (24).

Results

Selection process

Susceptibility tests showed that *An. stephensi* Chabahar strain was susceptible to permethrin and etofenprox, resistant candidate to deltamethrin and resistant to DDT, cyfluthrin, and lambda cyhalothrin. This strain showed the highest resistance to DDT 4% and cyfluthrin 0.15% respectively among the insecticides tested (Fig. 2). Populations with resistance ratio (RR) to Cyfluthrin of 11.6 and to DDT of 2.05 RR in comparison with the susceptible strain were chosen for insecticide selection process. This process continued for four and five phases throughout 18 and 19 generations respectively to achieve resistance ratio of 28.75 for the population exposed to DDT (R1) and 6.8 for the population exposed to cyfluthrin (R2).

Biochemical assays

Activities of α- and ß-esterases, glutathione-S-transferase (GST) and the contents of cytochrome P450s were tested for the R1 and R2 *An. stphensi* populations are summarized in Table 1 and Fig. 3. The cytochrome P450s enzyme ratios were 1.11 and 1.28 fold in the DDT and cyfluthrin- resistant populations compared with the susceptible strain. Although the median activities of the resistant population were 2.2 and 2.7 times more than the susceptible one, however, the mean activity/content of P450 enzymes in the resistant and the susceptible strains was not significant (P< 0.05). The enzyme ratios for esterases with α-naphthyl acetate were 3.95 and 3.04 and with β-naphthyl acetate were 2.4 and 1.97 in the DDT and cyfluthrin- resistant populations correspondingly compared with the susceptible strain. The GSTs enzyme ratios were 5.07 and 2.55 in the DDT and cyfluthrin- resistant populations compared with the susceptible Beech strain. The enzyme ratios for both the esterases and GST enzymes were higher in the DDT-resistant population than the cyfluthrin- resistant population (Fig. 3). Statistical analysis showed that the activity/content of the two esterases and GST enzymes of the selected populations and the beech susceptible strain were significantly different (P< 0.05) (Fig. 3).

Table 1. Details of enzyme activities and enzyme ratios (ER) measured in *Anopheles stephensi* resistant populations from southeastern Iran. Beech, susceptible, R1, DDT resistant, and R2, the Cyfluthrin resistant population

Enzyme	Population	N	Median	Mean±SE	Enzyme Ratio
	Beech	53	1.014e-005	2.365e-005±4.899e-006	1
P450	R1	58	2.183e-005	2.626e-005±2.035e-006	1.11
	R2	69	2.753e-005	3.050e-005±2.319e-006	1.28
GST	Beech	71	0.03485	0.03856±0.002610	1
	R1	66	0.15330	0.19590±0.01373	5.07

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Table 1. Continued …

	R2	69	0.08683	0.09857±0.006261	2.55
α-eseterase	Beech	80	0.0002753	0.0002825±1.307e-005	1
	R1	80	0.001022	0.001116±6.949e-005	3.95
	R2	80	0.0007664	0.0008604±5.001e-005	3.04
β-eseterase	Beech	80	0.0003146	0.0003397±1.588e-005	1
	R1	80	0.0007474	0.0008167±5.603e-005	2.4
	R2	79	0.0006188	0.0006718±3.299e-005	1.97

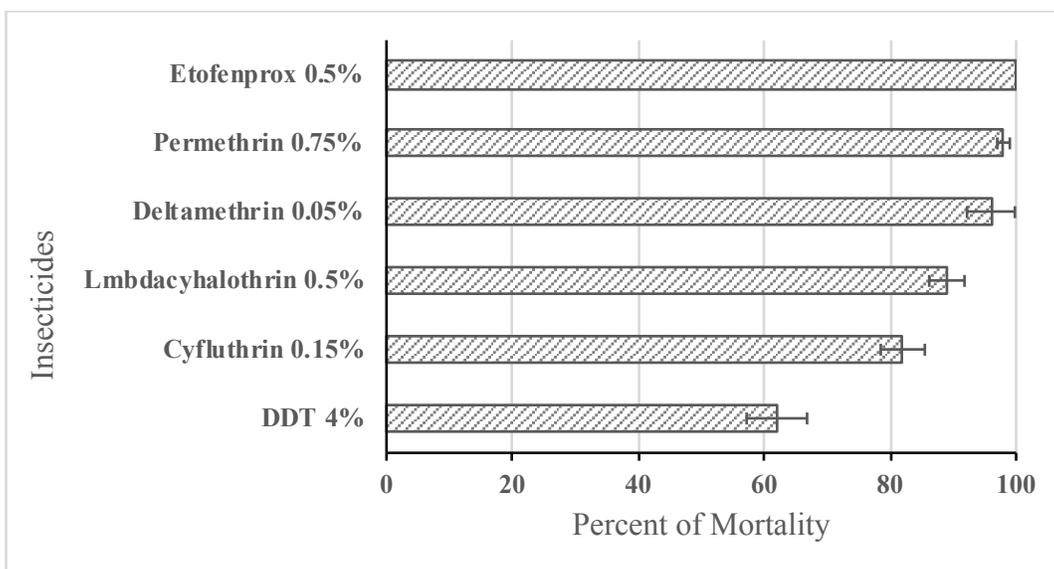**Fig. 1.** The map of the study area in Chabahar Seaport, Southeast of Iran

Fig. 2. Mortality rate (mean and SEM) of *Anopheles stephensi* Chabahar strain to six insecticides
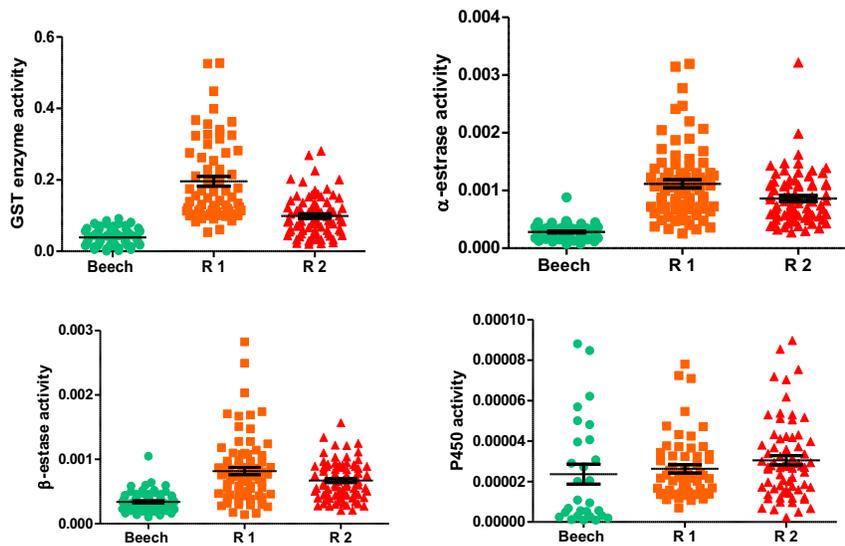
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Fig. 3. Mean and SEM activity profiles of P450, GST and α- and ß-Esterase enzymes in the DDT resistant (R1), the Cyfluthrin resistant (R2), and the susceptible Beech-strain of *Anopheles stephensi*

Discussion

This study revealed that *An. stephensi* from Chabahar District, southeast of Iran is resistant to pyrethroids including cyfluthrin and lambda cyhalothrin, DDT and tolerant to deltamethrin. Therefore, far various modes of resistance including modification or overexpression of detoxification enzymes, target site insensitivity, as well as behavioral adaptations have been developed and documented in insects (32, 33). Resistance to insecticides might be due to increased monitoring, misuse of insecticides, geographical extension of resistance, and new resistance genes (34) leading to decrease in the effectiveness of vector control programs. Resistance to pyrethroids in *An. stephensi* has been reported in several countries in the Eastern Mediterranean Region, notably Afghanistan, and Oman (12, 15, 16, 24, 34, 35). In addition, there are reports on DDT resistance in Yemen (34, 35), DDT and pyrethroid resistant in *Anopheles* mosquito of Iran (13, 15).

There have been reports of resistance to three of the four insecticides classes in *An. stephensi* mosquitoes in Afghanistan (24, 34).

Resistances to all four classes of insecticide have been reported in *An. stephensi* from Somalia and Sudan, including widespread resistance to DDT and an increasing frequency of resistance to pyrethroids (34, 36). Unfortunately, most of the new resistance reports are to pyrethroid compounds that are the only insecticides used for long lasting insecticide nets (LLINs).

This study showed that biochemical mechanisms are driving the resistance in this field population. This was shown by measuring the activities of the enzymes which could be responsible for the insecticide resistance in *An. stephensi* from Chabahar. The differences between activities of three enzyme groups including alpha and beta esterases, and GSTs in the Chabahar population were higher than those of the susceptible Beech strain, esterases and GSTs could all be involved in insecticide resistance in this population. Our result showed that in order α-esterase, β-esterase and GST enzyme have played the highest role in resistance to the DDT resistant populations (Ta-

ble 1). This order was α-esterase, GST enzyme, and then β-esterase for the cyfluthrin resistant population. Accordingly, almost similar situation has been reported in the field populations of *An. stephensi* in Afghanistan, a neighboring country sharing border line with Chabahar (24). Esterases and cytochrome P450s are involved in pyrethroid resistance in *An. stephensi* (21, 37), *An. gambiae* (38), *An. albimanus* (39, 40), and *An. minimus* (41). Moreover, esterases are involved in organophosphate (OP) resistance with cross-resistance to pyrethroids (21, 40, 42, 43). Rising enzyme activities in many insects have been reported including mosquitoes which are resistant to various insecticides from different parts of the world (17, 28, 42, 44-47). As biochemical mechanisms are involved in insecticide resistance in the Chabahar population, using of synergists in formulation of pyrethroid insecticides should be evaluated.

Different forms of resistance mechanisms have been reported in different species of *Anopheles* so that in some species only metabolic resistance has currently been reported. For example in the study on *An. funestus s.s* in Uganda just enzymatic resistant have been reported (48), whereas both metabolic and target-site insensitivity have been found as resistant mechanisms in *An. gambiae s.s.* in Africa (48, 49).

In this study the target-site insensitivity (kdr) mutations as a potential resistance mechanism in the *An. stephensi* populations were not examined. These mutations have been shown in *An. stephensi* from Afghanistan (24) and might be present in Chabahar population. Therefore this molecular assay is highly recommended to test the presence of kdr resistance mechanism in this population.

Conclusion

The *An. stephensi* Chahbahar population is becoming resistant to deltamethrin. This in-

secticide is currently used in malaria elimination program against malaria vectors including *An. stephensi* in the region. Although this insecticide may still be useful to combat *An. stephensi* in the area, surveillance of the susceptibility of populations by bioassay as well as biochemical and molecular assays are recommended to prevent building up of deltamethrin resistance levels. Insecticide resistance management strategies are also recommended to suspend or to slow the rate of resistance development to deltamethrin in Chahbahar District.

This study showed enzyme elevation and enzymatic resistance in the resistant population. Therefore conducting biochemical assays along with bioassay can be helpful for monitoring and management of resistant phenomena. Biochemical assays can be involved in routine malaria program for better monitoring and management of resistance in vector populations. Moreover, using other insecticides with different mode of action can be helpful for vector resistant management.

The main resistance mechanism in *An. stephensi* from the study area is metabolic and different enzyme groups play various roles in the resistance. Therefore, continuous surveillance of the susceptibility of populations and monitoring of insecticide resistance in the malaria vectors is crucial for successful control measures in Iran.

Acknowledgements

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