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INSECTICIDES SUSCEPTIBILITY, ENZYME ACTIVITY AND EXPRESSION OF RESISTANT GENE (GST) IN TWO POTENTIAL MALARIA VECTORS, *ANOPHELES JAMESII* AND *ANOPHELES BARBIROSTRIS* FROM MIZORAM, INDIA

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A B S T R A C T

Anopheles jamesii and *Anopheles barbirostris* are the two dominant and potential vectors of malaria in Mizoram. These mosquito populations are continuously being exposed directly or indirectly to different insecticides including the most effective pyrethroids and Dichloro-diphenyl-trochloroethane. Therefore, there is a threat of insecticide resistance development. We subjected these vectors to insecticides bioassay by currently using pyrethroids *viz.* deltamethrin and organochlorine *viz.* DDT. An attempt was also made to correlate the activities of certain detoxifying enzymes such as α- esterase, β-esterase and glutathione-S transferase (GST) with the tolerance levels of the two vectors. The results of insecticide susceptibility tests and their biochemical assay are significantly correlated ($P<0.05$) as there is elevation of enzyme production in increasing insecticides concentrations. Characterization of GSTepsilon-4 gene resulted that *An .jamesii* and *An. barbirostris* able to express resistant gene.

Keywords: *Index, Anopheles*, Control, Malaria, Disease, Mosquitoes, Resistance.

INTRODUCTION

Mosquitoes (Diptera: Culicidae) and mosquito-borne diseases have been threatening human and animals. There are 38 genera of mosquitoes worldwide wherein three genera (*Anopheles, Aedes*and *Culex*) were the most important one transmitting dengue fever, yellow fever, malaria, filariasis, chikungunya and encephalitis (Adityaa *et al*., 2006). No part of the world is free from vector borne diseases. Mosquito-borne parasitic diseases are endemic in many areas of the world, causing more than 3.2 billion people to be at risk (WHO 1998). There are 444 formally named species and 40 unnamed members of species complexes recognized as distinct morphological and/or genetic species of *Anopheles* in the world (Harbach 2004).

In India, 58 species has been described, six of which have been implicated to be main malaria vectors. Several other anophelines including *An. annularis, An. barbirostris,An. jamesii, An. nigerrimus, An. peditaeniatus, An. tessellatus*and *An. varuna*are potential vectors (Limrat *et al*., 2001, Perera *et al*., 2008). Each year 300 to 500 million cases of malaria are reported worldwide, resulting in 1.5 to 2.7 million deaths (Center for disease control and prevention 2004). India is on 18th position in the total reported malaria cases and on 21st position in reported malaria deaths (National vector borne disease control program 2013). In India, Mizoram alone contributed 5.73% of deaths due to malaria in 2007 and 10.44% in 2010 (NVBDCP 2013). Combat against malaria started since 1957 as the name National Malaria Control Program (NMCP) which was Government of India flagship program. In 1958, NMCP was changed to National Malaria Eradication Program that reflected the action in Mizoram that IRS (Indoor Residual Spray) of organochlorine insecticide, Dichloro-diphenyl-trochloroethane (1 kg of DDT 50% effective conc. dissolved in 10 L of water *i.e.* 5% conc.) was started since 1960s still today. Moreover, toward vector control and management distribution of 1% K-othrine, a synthetic pyrethroid (Deltamethrine 2.5% active ingredient v/v) for treated bed-nets all over Mizoram

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which was replaced by distributions of Long lasting insecticidal nets (Olyset net *ie.* Permethrine incorporated into polyethylene) since 2008. Insecticide resistance is increasingly becoming a problem for malaria vector control programs. Widespread use of the same insecticides in the agricultural sector has made the situation worse. Resistance may develop due to changes in the mosquitoes enzyme systems, resulting in more rapid detoxification or sequestration of the insecticide, or due to mutations in the target site preventing the insecticide-target site interaction (Vijayan *et al*., 1993). Insecticides that can be used in malaria control are increasingly becoming limited. The glutathione S-transferases (GSTs) are members of a large family of multifunctional intracellular enzymes involved in the detoxification of endogenous and xenobiotic compounds via glutathione conjugation, dehydrochlorination, glutathione peroxidase (GPx) activity or passive/sacrificial binding (Hayes and Wolf 1988, Mannervik and Danielson 1988, Pickett and Lu 1989, Yang *et al.,* 2001). In mosquitoes, the metabolic resistance based on GST is the major mechanism of DDT-resistance (Hemingway and Ranson 2000).The esterase-based resistance mechanisms have been studied extensively at the biochemical and molecular level in mosquitoes. Work is in progress on related and distinct esterase resistance mechanisms in a range of *Anopheles* and *Aedes* species. Broad-spectrum organophosphate resistance is conferred by the elevated esterases of *Culex* species. All these esterases act by rapidly binding and slowly turning over the insecticide: They sequester rather than rapidly metabolize the pesticide (Kadous *et al*., 1983). Introduction of inappropriate insecticides without a proper understanding of the prevailing resistance mechanisms may lead to enhanced vector resistance and disease control failure. Early detection and knowledge on the resistance status and the underlying mechanisms in vector mosquitoes are essential for effective long-term control of the vector. Therefore, the status of insecticide resistance and prevalence of different types of resistance mechanisms in *An. barbirostris*and *An. jamesii* populations from six administrative districts of Mizoram is reported in this paper.

MATERIALS AND METHODS

Collection of mosquito: The study covered a major part of the six districts in Mizoram (between April 2009 to May 2013) including Aizawl (23°44' N, 92°42' E), Serchhip (23°16' N, 92°44' E), Mamit (23°55' N, 92°29' E), Lunglei (22°52' N, 92°43' E), Lawngtlai (22°18' N, 92°41' E) and Kolasib (23°13' N, 92°40' E) with the altitudinal variation of 54 - 1150 m (figure 1). The water bodies (ponds, ditches, pools, river beds, tree holes, rock holes, tanks and containers) were surveyed and subsequently sampled, collection of immature mosquitoes was also made on the same day (8:00 am – 3:00 pm)by the scoop-net method (WHO 1975), with a larval net of a fine mesh net mounted to an iron handle (25 cm diameter), plastic tub of different sizes, plastic dipper and dropper (21 - 38°C; 25 - 98% RH). Adults were collected at dusk and midnight (4:00 – 8:00 pm; 12:00 – 2:00 am) using electrical mosquito bat (commercially available), hand collection (WHO 1975)which consisted of a 250 ml glass jar and cotton moisten with chloroform kept at the base of the jar and CDC (Center for Disease Control) light trap from both indoor and outdoor.

Identification of mosquito: Morphological identification of mosquito was done on adult female taking color pattern of wing, palpi and leg as identification characters using dissecting light microscope and hand lens. The identification keys followed the illustration of Das *et al*., (1990), Reuben *et al*., (1994), Nagpal and Sharma (1995), Oo *et al*., (2005).

Insecticidal Bioassay: Susceptibility tests was carried out in three replicates using DDT (50% effective concentration) and Deltamethrine (2.5% active ingredient w/w) obtained from Department of Health Services, Govt. of Mizoram.

Maintenance of mosquito and preparation of test concentrations: Bioassay was conducted on field collected population. Larvae collected from the field were immediately carried to laboratory (25±3° C temp, 50-85% RH). Two-three day old female F1 mosquitoes were reared from collected material and used for subsequent experiments.

Susceptibility assay: Insecticide bioassays were conducted by means of tarsalcontact exposure to insecticide-impregnated papers as per World Health Organization protocol (1998). A rectangle of Whatman-No.1 filter papers (12 cm × 15 cm) was used for insecticide impregnation. DDT of different concentrations (0.25% - 4%) and deltamethrin (0.004 - 0.25 %) of 0.7 ml each were mixed with an equal volume of acetone (0.7ml) and the mixture was spread uniform on the filter paper (Perera *et al*., 2008). Batches of 30

early adults female mosquitoes were exposed to insecticide impregnated papers for one hour, dead mosquitoes were counted after a recovery period of 24 hours. At least five replicates for each insecticide were carried out with each population. Papers impregnated with the carrier (oil) and acetone was used as controls. Results were used only if the mortality in the controls was <20% and the mortalities were adjusted for using Abbott's formula. World Health Organization classification was used to interpret the results (WHO 2005).

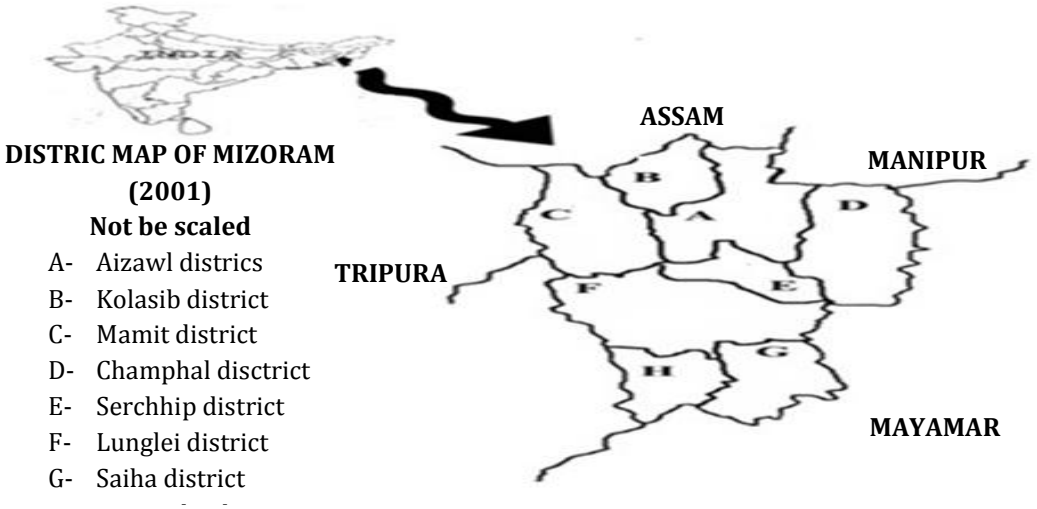

Figure 1. Location and landscape of Mizoram showing different districts.

Preparation of mosquito for quantitative enzyme assays: Enzyme assays were done as per WHO protocol (1998). Different concentration of insecticides treated mosquitoes which were alive after treated (stored at -20°C) was homogenized in -20°C cryo-box. 200 µl distilled water was added to it. It was spun at 14,000 rpm for 30 seconds; the supernatant was used as enzyme samples then stored at -20°C.

Protein assay: Quantification of the total protein of the early fourth instar larva was done according to the standard procedure of Lowry et al. (1951). A known concentration of bovine serum albumin (BSA) was used as the standard protein.

Standard (α-and β-) Napthol assay: α-napthol and β-napthol of 200µl/ml stock concentration was pipette in 100, 200, 400, 500 and 800 µl into test-tubes. The volume was made to 1 ml by addition of 0.02M PBS (pH 7.2) to each test-tubes and the blank contained 1 ml of 0.02M PBS (pH 7.2). 50 ml of Fast blue stain was added to each test-tube and incubated at room temperature for 5 minutes. Optical density was read at 570 nm. Two standard curved were made for α-napthol and β-napthol (WHO 1998).

Naphthyl Acetate assay for Esterase: 200µl of α-/β-Naphthyl Acetate was added to 20µl of homogenate and incubated at room temperature for 15 minutes. The blank contained 20µl of distilled water. 50 µl of Fast blue stain was added and further incubated for another 5 minutes. 2860 µl of 0.02M Phosphate buffer (pH 7.2) was added to increase the volume required by spectrophotometer used. OD was then read at 570 nm (WHO 1998).

Assay for Glutathione-S-Transferase: 10 µl of homogenate was mixed with 200 µl of chlorodinitrobenzene-Reduced Glutathione (CDNB-GSH) and incubated for 20 minutes. The blank contained 10 µl of distilled water. 2940 µl of of 0.02M Phosphate buffer (pH 7.2) was added to increase the volume required by spectrophotometer used and mixed thoroughly. It was incubated for 20 minutes in room temperature. OD was then read at 340 nm (WHO 1998).

Extraction of total RNA, cDNA synthesis and Reverse Transcriptase (RT)-PCR of *Anopheles* β-Actin: Total RNA was extracted from *An. Barbirostris* and *An. jamesii* using TRIzol reagent (SIGMA, USA), according to the manufacturer's instructions. Then mRNA was reverse transcribed into cDNA using Revert Aid™ First strand cDNA synthesis kit (Fermentas) following the manufacturer's protocol. *Anopheles* β-actin primer AF: 5'- ATG TAC GTC GCC ATC CAG GC -3 and β-actin AR; 5'-CGA TGG TGA TGA CCT GTC CGT -3' (Senthil Kumar *et al*., 2008) was used as a house keeping gene for

quantitative standardization of the cDNA sample. PCR condition consisted of initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds of primer annealing, 72°C for 30 seconds as primer extension and final extension at 72°C for 1 minute in Thermal Cycler™PCR (Eppendorf, Germany).

Expression of *Anopheles* Glutathione-S-Transferaseepsilon-4 gene: Primers (AGSTe4F 5'- TAC ACG GCC AAA CTC AGC -3' and AGSTe4R 5'- CGG TAC AGA TTG TCG ATC -3') to obtained the partial expression of *Anopheles* GSTe4 gene was designed from NCBI database (figure 2). 25 µl PCR reaction included *Taq* polymerase buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (0.25mM), primer (0.1pM each), *Taq* polymerase (0.5 U) and cDNA template.

The volume was made to 25µl with DEPC water. The concentration of cDNA template used for PCR was referred from standardized β-actin PCR result. PCR condition consisted of initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds for primer annealing, 72°C for 30 seconds as primer extension and final extension at 72°C for 1 minute.

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diruse4       ATGCCGAACATCAAGCTGTACACGGCCAAACTCAGCCCTCCGGGACGAGCGGTGGAGCTG 60
gambiaeE4     ATGCCAAACATTAAGCTGTACACGGCCAAACTCAGCCCACCGGGCCGGTCGGTCGAGCTG 60
              ***** ***** ************************* ***** **  **** ******

diruse4       ACGGGGAAGGCGCTGGGACTGGAGTTCGACATCTCCCCGATCAATCTGATCGCCGGAGAT 120
gambiaeE4     ACAGCAAAGGCGCTCGGGCTGGAGCTCGACATCGTGCCGATCAATCTGCTCGCGCAGGAA 120
              ** *  ******** ** ****** ********  ************ ****   **

diruse4       CACCTGCGGGAGGAGTTCCGGAAGCTGAATCCTCAGCACACGATCCCGCTGATCGACGAC 180
gambiaeE4     CATCTGACGGAAGCGTTCCGGAAGCTGAACCCGCAGCACACCATCCCGCTGATCGACGAC 180
              ** ***  *** * *************** ** ******** ******************

diruse4       GCCGGTACGATCGTGTACGAAAGCCACGCGATCATCGTGTACTTGGTGACGAAGTACGGC 240
gambiaeE4     AACGGGACGATCGTGTGGGACAGCCACGCCATCAATGTGTATCTGGTGAGCAAGTACGGC 240
              *** **********  ** ******** ****  *****  ******  *********

diruse4       GCGGACGATAGCCTCTATCCGTCGGACGCGGTGACGCGCTCCAAGGTCAACGCG 294
gambiaeE4     AAGCCCGAGGGCGACAGTTTGTATCCGTCGGATGTGGTGCAACGGGCGAAGGTTAACGCG 300
              * ** *** **  * ********** * ****  **  * ***** ******

diruse4       GCGCTACACTTCGATTCGGGTGTTCTGTTCGCCCGGCTGCGATTCTATTTGGAACCAATT 354
gambiaeE4     GCGCTACACTTCGATTCGGGCGTTCTGTTTGCCCGGTTCCGGTTCTATTTGGAACCAATA 360
              ************************************************************

diruse4       CTGTACTACGGATCGACCGAAACACCGCAGGAGAAGATCGACAATCTGTACCGGGCGTAC 414
gambiaeE4     CTGTACTACGGAGCGACCGAGACACCGCAGGAAAAGATCGACAATCTGTACCGCGCGTAC 420
              ************************************************************

diruse4       GAGCTGCTGAACGCCACGCTGGTCGACGATTACATCGTGGGAAGCCGGTTGACGCTGGCC 474
gambiaeE4     GAGCTGCTGAATGACACGCTGGTCGACGAGTACATCGTGGGCAACGAGATGACACTGGCC 480
              *********** * *************** *********** * *  * **** ******

diruse4       GATCTGAGCTGTGTTGCAAGCATCGCCTCGATGCATGCCATCTTCCCGATCGATGCCGGC 534
gambiaeE4     GATCTGAGCTGCATCGCCAGCATTGCTTCGATGCATGCGATTTTCCCGATCGATGCCGGC 540
              ***********  * ** ***** ** *********** ** ******************

diruse4       AAGTATCCGAAGCTGTTGGCCTGGGTCGAGCGTATCGCGAAGTTGCCCTACTATGCGGCG 594
gambiaeE4     AGTATCCGAGGCTGGCCGGTTGGGTCAAACGCCTTGCCAAGCTGCCGTACTACGAGGCA 600
              ********** ****   *  ****** * **  * ** *** **** ***** * ***

diruse4       ACGAATCAGGCCGGTGCGGAAGAACTGGCCCAGCTGTATCACGCCAAGCTAGCGGAGAAC 654
gambiaeE4     ACGAATCGGGCCGGTGCGGAAGAGCTCGCTCAGCTGTACCGTGCCAAGTTGGAGCAAAAC 660
              ******* *************** ** ** ******** *  ****** * * * * ***

diruse4       CGTGCTAAAGCAAAGTGA 672
gambiaeE4     CGCACCAACGCCAAGTGA 678
              **********
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Figure 2. CLUSTAL W alignment of GSTe4 complete coding sequences of *An.gambiae* (GenBank Accession NO.AY070254.1) and *Ae. aegypti* (GenBank Accession NO. AY819709.1). The highlighted region show the sequences selected for GSTe4 primer.

RESULTS

The results of the bioassay with the two insecticides against most dominant and potential vectors of malaria are provided in table 1. The LC_{50} values for the two species indicate differential tolerance levels. Insecticides susceptibility screening against two species of *Anopheles* showed that the level of tolerance against DDT was higher in *An. barbirostris*as compared to *An. jamesii.* In contrast *An. jamesii* showed a 1.24 fold increase in tolerance against deltamethrin compared to *An. barbirostris.* The results of the biochemical analysis on insecticides treated samples showed a similar pattern to bio-assay and there was a significant increase in enzymes production in increasing insecticides concentrations (Table2). In DDT treated samples, the amount of GST enzyme production was highest in *An. Barbirostris* adults (0.420±0.02) and a correlation was found between susceptibility tests on different concentrations of DDT and enzyme elevation (r=0.953; P<0.05). In case of carboxylesterases assay, the elevation of α- and β-esterase was significantly higher (P<0.05) in *An. jamesii* as compared to *An. barbirostris.* In deltamethrin treated samples, GST enzyme production was significantly higher in *An. Barbirostris* (0.320±0.02) than *An. Jamesii* (0.253±0.02).

Table 1. Insecticidal bioassays (Deltamethrin and DDT) against field collected *An. barbirostris*and *An. jamesii*.

Species	Insecticides used	Concent-ration in mg/l	Percent mortality	LC_{50}	LCL	UCL
An. barbirostris	DDT	0.25	8	1.607	1.361	1.937
		0 .50	21			
		1.0	36			
		2.0	52			
		4.0	78			
An. jamesii		0.25	12	1.340	1.128	1.615
		0.50	28			
		1.0	39			
		2.0	57			
		4.0	81			
An. barbirostris	Deltamethrin	0.004	48	0.0051	0.0048	0.0052
		0.006	58			
		0.008	69			
		0.010	78			
		0.025	96			
An. jamesii		0.004	42	0.0063	0.0041	0.0078
		0.006	49			
		0.008	56			
		0.010	63			
		0.025	88			

Table 2. Activity of esterases, glutathione S-transferasesin*An .jamesii*and *An. barbirostris* population.

Species	Insecticides used	Biochemical assay		
		General Esterase (α) (α-naphthol/min/mg protein)	General Esterase (β) (β-naphthol/min/mg protein)	Glutathione-S- transferase (µmoles/min/mg protein)
		Mean±SE	Mean±SE	Mean±SE
An. jamesii	Deltamethrin	0.102±0.03	0.072±0.02	0.253±0.02
An. barbirostris		0.076±0.02	0.094±0.03	0.320±0.02
An. jamesii	DDT	0.110±0.03	0.105±0.02	0.375±0.02
An. barbirostris		0.105±0.02	0.084±0.17	0.420±0.02

There was a significant correlation of GST enzyme elevation against increasing concentrations of deltamethrin (P<0.05). Moreover, the level of α- esterase enzyme elevation was significantly higher in *An. jamesii*. The standardized β-actin partial gene qRT-PCR gave the optimum band intensity for field collected *Anopheles*

species (figure 3) and different volumes of cDNA concentrations; *An. jamesii*-1.2µl and *An. barbirostris*-0.8µl were used for template to obtain similar band intensity. It was observed that *An. Jamesii* and *An. barbirostris* were able to express GSTe4 gene (figure 4) and therefore confirmed GST enzyme production in the biochemical assay.

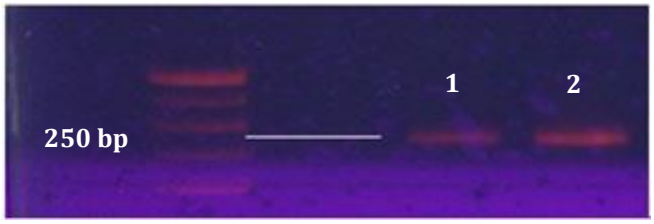

Figure 3. 1.5% agarose gel showing standardized β-actin gene qRT-PCR. 100 bp DNA marker was used. 1-*An.jamesii*; 2. *An. barbirostris.*

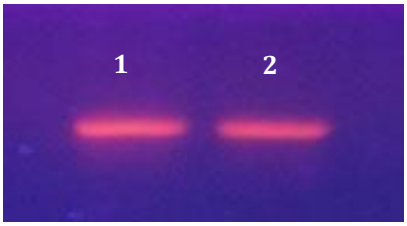

Figure 4. Expression of mosquito GSTe4 gene.1 – *An. jamesii*; 2 – *An. barbirostris.*

DISCUSSION

Chemical insecticides play a major role in vector control. However, the continuous and indiscriminate use of insecticide in a population will lead to the development of physiological resistance in the insects (Ganesh et al. 2003). The present results clearly suggest the differential effect of the same class of insecticides on two species belonging to different habitats. Earlier, Revanna and Vijayan (1993) and Vijayanet al. (1993) had shown differential susceptibility status in a few species of *Culex* mosquitoes from the same district. Bansal and Singh (1996) studied the susceptibility levels of some anophelines, such as *An. culicifacies*, *An. annularis, An. Stephensi* and *An. Subpictus* from Rajasthan, India and found that all these species were resistant to DDT and dieldrin, but were susceptible to fenitrothion and permethrin. The present study has also revealed a significant 1.1-fold increase in the GST enzyme activity in *An. barbirostris*, which could be correlated with the 1.2-fold increase in the DDT tolerance compared to *An. jamesii.* Prior to 1977, DDT was the insecticide used for malaria vector control programs in Sri Lanka. DDT resistance in *An. Culicifacies* and *An. subpictus* was first detected in 1969 in Sri Lanka (Perera *et al*., 2008). Vector resistance to DDT declined slowly after cessation of its usage, but increased again after 1983 due to a GST-based resistance mechanism, which was first selected by exposure to DDT (Perera *et al*., 2008). The present studies also suggest that DDT which was using since 1960s and there is a possibilities of resistance in the tested species as there is a significant elevation in GST enzyme activity in increasing insecticides concentration. High resistance levels of DDT in a population probably are due to increased levels of GST enzymes (Perera *et al*., 2008). Moreover, DDT was introduced in 1950s in India for IRS and was continued up to 1970. First report of DDT-resistance appeared in 1958and later widespread resistance was reported (Bansal and Singh 1996). There was a significant increase in esterase activity ($P<0.05$) in *An. jamesii,* which could be correlated with the DDT tolerance status. This may suggest species specific biochemical mechanism for detoxification. Insecticide resistance can be due to selection of changes in insect enzyme systems, leading to rapid detoxification or sequestration of insecticide or due to alterations of the insecticide target site preventing the insecticide-target site interaction. Increased metabolic capacity is usually achieved by increased activity of monooxygenases, GSTsor esterases. Metabolic enzyme genes usually have greater plasticity than insecticide target site genes. Increased enzyme activity can be brought about by gene amplification, up regulation, coding sequence mutations or by a combination of these mechanisms. P^{450s} can mediate resistance to all classes of insecticides. GSTs can mediate resistance to organophosphates, organochlorines and pyrethroids. Esterases can provide resistance to organophosphates, carbamates and pyrethroids which are rich with ester-bonds (Li et al. 2007). High genetic diversity has caused broad substrate specificity in insect metabolic enzymes. Isolation and characterization of candidate genes/gene families which are over-expressed in these vector populations will aid future vector control programs. However, induction of GST activity has been reported not only after exposure to organophosphates and organochlorides but also against pyrethroid (Kostaropoulos *et al*., 2001). Reports correlating the elevated levels of GST with resistance to pyrethroids do exist for *Triboliumcastaneum* (Reidy *et al*., 1990) and *Aedesaegypti* (Grant and Matsumura 1989). Therefore, the significantly higher level of GST

activity might play a role in pyrethroid tolerance in *An. barbirostris*and *An. jamesii*along with esterase activity. Indian scenario depicts prevalence of DDT-resistance co-existing with susceptibility to synthetic pyrethroids. The quick reversion of deltamethrin-resistance to susceptibility could be due to the contemplated nature of the recessive resistance gene. This further indicates that deltamethrin and synthetic pyrethroids excel other groups of insecticides in vector control as their useful life can be enhanced if used judiciously for vector control (Ganesh et al. 2003).GST-based resistance has been detected by elevated levels of GST activity in strains of insects resistant to organophosphates (Fournier *et al*., 1992), organochlorines (Grant and Hammock 1992) and pyrethroids (Kostaropoulos *et al*., 2001). In addition to these, there was a significant elevation of GST enzymes production in increasing deltamethrin concentrations but insignificant correlation was found in elevation of general esterase (*α*- and *β*- esterase) against deltamethrin. Thus, GST alone detoxification was responsible for slightly tolerant against deltamethrin but pyrethroid susceptibility in *An. Barbirostris* and *An. Jamesii* was found as there was insignificant correlation in level of esterase activity. As the mosquito populations of Mizoram area are exposed to DDT and deltamethrin insecticides in their respective habitats, tests conducted on the tolerance level and the enzymes involved in detoxification mechanisms are important. The present studies in respect of bioassay and biochemical estimations have revealed the probable mechanism developed by the local malaria vectors to combat the insecticides. Further, the study of enzymes involved in the detoxification mechanism will help us to introduce appropriate control measures such as combinations of insecticides and synergists for a better and effective control program of malaria.

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