Determination of glutathione S-transferase e2 region (*GSTe2*) in DDT susceptible and resistant *Anopheles stephensi* populations: significance and application of nucleotide and amino acid comparison

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Abstract
Glutathione S-transferases (GSTs) are a major family of detoxification enzymes which possess a wide range of substrate specificities. Interest in insect GSTs has primarily focused on their role in insecticide resistance. In this study, following World Health Organization (WHO) routine susceptibility test, DNA was extracted from specimens of *Anopheles stephensi* collected from the Kazeroon district in the Fars province as control area and Saravan, Chabahar, Nikshahr districts in Sistan and Baluchistan province representing major malarious areas under insecticide treatment, in Iran. The (Glutathion S-transferase Epsilon class 2) *GSTe2* gene including exon I and II and the full sequence of intron I, belonging to *An. stephensi* specimens were then amplified. The size of the resulting amplicons from the control area and the insecticide treated areas were 492 and 489 bp, respectively. These fragments were purified and then sequenced from both ends. The comparison of total amplified fragments among Kazeroon and Nikshahr and/or other populations of the Sistan and Baluchistan province (Saravan and Chabahar) showed 98% and 97% similarities, including 9-11 nucleotide substitutions, none of which had led to any amino acid change, within these populations. Comparison of the nucleotide sequence of *GSTe2* in *An. stephensi* populations with that of the major world malaria vector, *Anopheles gambiae* revealed 86% homology, while amino acid similarity between the two species was approximately 90%. However, the main difference between the two susceptible and resistance groups in *An. stephensi* populations is related to their intron sequence with a distance of 8-9%, while this distance among resistance populations from the Sistan and Baluchistan province varied by approximately 0-4%. The results obtained from this study serve as a first report and baseline data regarding the structure of *GSTe2* gene, including exon I, exon II and intron I in susceptible and resistance field specimens of *An. stephensi.* However, the integration of these data into the malaria control program still remains a challenge in Iran and neighboring countries, especially Afghanistan and Pakistan.

Keywords: Glutathione S-transferase; *Anopheles stephensi*; *Anopheles gambiae*; DDT; Metabolic resistance.

INTRODUCTION

The glutathione transferases (GSTs) are a large family of multifunctional enzymes involved in the detoxification of a wide range of xenobiotics including insecticides (Enayati *et al*., 2005). GSTs can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugating glutathione (GSH; L-glutamyl-cysteinyl-glycine) to xenobiotic compounds with electrophilic centers (e.g. drugs, herbicides, insecticides), converting them from reactive lipophilic molecules into water-soluble non-reactive conjugates that may easily be excreted (Chen *et al*., 2003). In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides.

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There are at least two ubiquitously distributed distantly related groups of GSTs, classified according to their location with in the cell: microsomal and cytosolic. A third group of GSTs, the Kappa class, are located in mammalian mitochondria and peroxisomes (Lander *et al*., 2004; Morel *et al*., 2004) and are structurally distinct from the microsomal and cytosolic GSTs (Robinson *et al*., 2004). A single microsomal GST gene is present in the genome of the fruit fly *Drosophila melanogaster* whereas the mosquito *Anopheles gambiae* has three microsomal GST genes (Ranson *et al*., 2002; Toba and Aigaki, 2000). Microsomal GSTs have not been implicated in the metabolism of insecticides (Enayati *et al*., 2005). Insect cytosolic GSTs were initially assigned numbers according to their order of elution from the various purification procedures employed or isoelectric points (Prapanthadara *et al*., 1993; Clark *et al*., 1985).

Over 40 *GST* genes have been detected in the genomes of higher eukaryotes for which full genome sequence data are currently available (Holt *et al.*, 2002). These have been classified into at least 13 different classes based on their amino acid sequence identities, immunological properties and, where known, substrate specificities (Ortelli *et al.*, 2003). The majority of studies on insect GSTs have focused on their role in conferring insecticide resistance (Ding *et al*., 2003; Vontas *et al*., 2001) and, more recently, in protecting against cellular damage by oxidative stress (Zou *et al*., 2000; Singh *et al*., 2001). Elevated GST activity has been associated with resistance to all the major classes of insecticides (Vontas *et al*., 2001; Huang *et al*., 1998; Prapanthadara *et al*., 1993). Insect GSTs were recently classified into six classes (δ, ε, σ, θ, ω and ζ) by comparative analysis of the *D. melanogaster* and *An. gambiae* genomes (Chen *et al.*, 2003). The two largest GST classes in *An. gambiae* are the insect specific delta (δ) and epsilon (ε) classes (Ding *et al*, 2003).

In this study we analyzed and compared the nucleic acid and amino acid sequences of the *GSTe2* gene in specimens of *Anopheles stephensi* which were collected from the Saravan, Chabahar, Nikshahr districts in the Sistan and Baluchistan province (exposed to insecticide spraying), and the Kazeroon district in the Fars province (representing a control area) where insecticides have not been applied for the last 30 years. *GSTe2* encodes an enzyme that has the highest levels of Dichloro-Diphenyl-Trichloroethane (DDT) dehydrochlorinase activity (Enayati *et al*., 2005; Ortelli *et al*., 2003).

MATERIALS AND METHODS

Mosquito collection, morphological identification and susceptibility test: Specimens of *An. stephensi*, collected from Saravan, Chabahar, and Nikshahr districts in the Sistan and Baluchistan province (under insecticide treatment) and Kazeroon district in the Fars province (control area), were identified by using the morphological key to Iranian anophelines (Shahgoudian, 1960). Susceptibility tests were carried out in two replicates with standard WHO impregnated paperas of DDT 4%, dieldrin 0.4 %, malathion 5%, permethrin 0.25 %, lambda cyhalothrin (ICON) 0.1 %, deltamethrin 0.025 % on *An. stephensi* specimens, based on recommended procedures. Mortality counts (percent of death) were recorded after 24 h recovery period.

DNA extraction and PCR amplification of the *GSTe2* gene: DNA was extracted from collected specimens by using a slight modification of the Collins' method (Collins *et al.*, 1987). Application of PCR for the amplification of *GSTe2* including exon I and II and full sequence of intron I was carried out on all specimens by E2f (forward) (5′-ATCACCGAGAGCCACG-CAATCAT-3′) and E2r (reverse) (5′-GCCAC-CGTTCGCTTC CTCGTAGT-3′) primers which were designed on the basis of the *An. gambiae* genome. PCR conditions were optimized by changing the thermal cycling conditions (ramp time and annealing temperature). The optimal conditions for amplification of the *GSTe2* gene were as follows: 35 cycles of denaturation at 95ºC for 1 min , annealing at 62ºC for 1 min and extension at 72ºC for 1min with a 10 min extra extension in the last cycle. Specimens of *An. stephensi* from the Fars province and *An. gambiae* (LSTM strain) were used as positive and control DNA samples, respectively, while the negative control was a 24 µl PCR mixture plus 1 µl of ddH_2O instead of DNA. 5 µl of each of the amplification products was mixed with loading buffer and subjected to electrophoresis in 1.5% (w/v) agarose gel in TBE buffer containing ethidium bromide. The resulting fragments were visualized by a UV transilluminator (Uvitec, Uk). Selected PCR products were purified from the gel by using the QIAquick gel extraction kit (Qiagen) (country, Germany).

Sequence analysis: Sequencing of specimens was per-

formed on an AB1 sequencing machine (Applied BioSystem, USA) from both sides of the amplified fragments using forward and reverse primers. Sequence data, on arrival were double checked by comparison with the related signals and blast analysis. Gene Runner (Version 3.05, Hastings Software Inc., Hastings on Hudson, NY) and ClustalW (Thompson *et al*., 1994) programs were used for determination of amino acid sequences, detection of reading frames, and alignments.

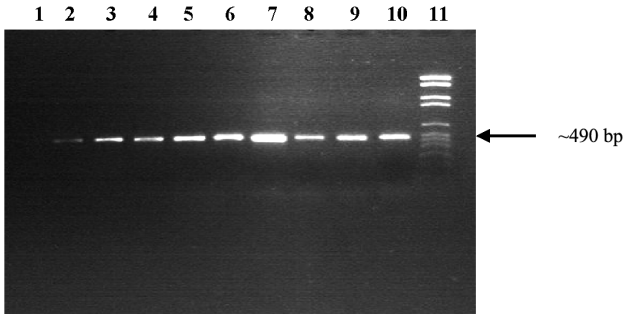

Figure 1. Amplification of the GSTe2 gene in *An. stephensi* specimens of Saravan, Chabahar, Nikshahr and Kazeroon districts. Lane1: Negative control, Lanes 2 and 3: Saravan specimens, Lanes 4 and 5: Chabahar specimens, Lanes 6 and 7: Nikshahr specimens, Lanes 8, 9 and 10: Kazeroon specimens, Lane 11: Molecular weight marker VI, Roche, Germany .

RESULTS

The WHO routine susceptibility test carried out in duplicate during this study showed that *An. stephensi* specimens collected in areas under insecticide treatment for vector control (Saravan, Chabahar and Nikshahr districts in the Sistan and Baluchistan province) were resistant to DDT (39% death in the presence of 4 % (g/m^2) DDT), while specimens from the control area with no insecticide application since the last 30 years (Kazeroon district in the Fars province) showed no resistance to DDT. Furthermore, resistance to the other tested insecticides dieldrin 0.4%, malathion 5%, permethrin 0.25%, Icon 0.1%, Deltamethrin 0.025% (g/m^2) were not detected in any of the examined *An. stephensi* specimens.

Amplification of the *GSTe2* region by E2f and E2r primers in *An. stephensi* specimens from the Sistan and Baluchistan and Fars provinces revealed 492 and 489 bp fragments on 1.5% (w/v) agarose gel (Fig. 1). These fragments were purified and sequenced from both ends. Sequence alignment of the *GSTe2* gene including exon I, exon II and intron I, in these *An. stephensi* specimens showed 98% and 97% similarities between the Kazeroon and Nikshahr populations and/or other populations of the Sistan and Baluchistan province (Saravan and Chabahar), respectively (Fig. 2). The main difference between these sequences is related to intron I region, so that the similarity percentage with respect to the coding sequence of the *GSTe2* gene increases to 99% without considering the intron sequence, between the Kazeroon population and three Sistan and Baluchistan populations (Saravan, Chabahar and Nikshahr).

Comparison of the nucleotide sequence of *GSTe2* from *An. stephensi* populations with the main world malaria vector, *An. gambiae* revealed 86% homology, while amino acid similarity between the two species was approximately 90%. Analysis of the intron I region of the *GSTe2* gene in the three populations of *An. stephensi* showed that the size of this region was 78 and 75 bp in specimens from the Kazeroon district and the three districts of Sistan and Baluchistan province, respectively. Comparisons of the sequence similarity percentages at intron I of the *GSTe2* gene among *An. stephensi* specimens of the Kazeroon, Saravan, Chabahar and Nikshahr districts are shown in Table 1.

Table 1. The nucleotide similarity percentage at intron I sequence of the *GSTe2* gene among *An. stephensi* specimens from Kazeroon, Saravan, Chabahar and Nikshahr districts.

	Nucleotide similarity (%)			
	Saravan	Chabahar	Nikshahr	Kazeroon
Saravan	-	100	96	91
Chabahar		-	96	91
Nikshahr			-	92
Kazeroon				-

Figure 2. Alignment of *GSTe2* gene including exon I, intron I and exon II in different specimens of *An. stephensi* which collected from Kazeroon district in Fars province and Saravan, Chabahar, Nikshahr districts in Sistan & Baluchistan province. Intron sequences in 5 specimens are bolded. The underlined sequences are priming sites.

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Saravan      ATCACCGAGAGCCACGCAATCATGATCTATCTGGTGACGAAGTACGGCAAGGATGACTCC 60
Chabahar     ATCACCGAGAGCCACGCAATCATGATCTATCTGGTGACGAAGTACGGCAAGGATGACTCC 60
Nikshahr     ATCACCGAGAGCCACGCAATCATGATCTATCTGGTGACGAAGTACGGCAAGGATGACTCC 60
Kazeroon1    ATCACCGAGAGCCACGCAATCATGATCTATCTGGTGACGAAGTACGGCAAGGATGACTCC 60
Kazeroon2    ATCACCGAGAGCCACGCAATCATGATCTATCTGGTGACGAAGTACGGCAAGGATGACTCC 60
             ************************************************************

Saravan      CTCTACCCGAAGGACCCAGTCAAGCAGGCTCGTGTGAACGCTGCCCTGCACTTCGAATCC 120
Chabahar     CTCTACCCGAAGGACCCAGTCAAGCAGGCTCGTGTGAACGCTGCCCTGCACTTCGAATCC 120
Nikshahr     CTCTACCCGAAGGACCCAGTCAAGCAGGCTCGTGTGAACGCTGCCCTGCACTTCGAATCC 120
Kazeroon1    CTCTACCCGAAGGACCCAGTCAAGCAGGCTCGTGTGAACGCTGCGCTGCACTTCGAATCC 120
Kazeroon2    CTCTACCCGAAGGACCCAGTCAAGCAGGCTCGTGTGAACGCTGCGCTGCACTTCGAATCC 120
             ************************************************ ***************

Saravan      GGTGTACTGTTTGCCCGGATGCGATTCATCTTTGTAAGTTGATTGATCGTCTGTTGCATC 180
Chabahar     GGTGTACTGTTTGCCCGGATGCGATTCATCTTTGTAAGTTGATTGATCGTCTGTTGCATC 180
Nikshahr     GGTGTACTGTTTGCCCGGATGCGATTCATCTTTGTAAGTTGACTGATCATCTGTTGCATC 180
Kazeroon1    GGTGTACTGTTTGCCCGGATGCGATTCATCTTTGTAAGTTCATTGATCATCTGTTGCATC 180
Kazeroon2    GGTGTACTGTTTGCCCGGATGCGATTCATCTTTGTAAGTTCATTGATCATCTGTTGCATC 180
             *************************************** * ***** ***********

Saravan      A---CGGCGTGAGTAAAATTATGTCTTATCCCACCAAACCCAACAAACCAGGAACGTATT 237
Chabahar     A---CGGCGTGAGTAAAATTATGTCTTATCCCACCAAACCCAACAAACCAGGAACGTATT 237
Nikshahr     A---CGGCGTGAGTAAAATTATGTCTTATCCCACCAAACCCAACCAACCAGGAACGTATT 237
Kazeroon1    ATCACAGCGTGAGTAAAATTATGTCTTATCCCACCAAACCCAACCAACCAGGAACGTATT 240
Kazeroon2    ATCACAGCGTGAGTAAAATTATGTCTTATCCCACCAAACCCAACCAACCAGGAACGTATT 240
             *   * ************************************ ***************

Saravan      CTGTTCTACGGAAAAACGGACTTGCCCGAGGATCGCGTTGAGTATGTGCAGAAATCGTAC 297
Chabahar     CTGTTCTACGGAAAAACGGACTTGCCCGAGGATCGCGTTGAGTATGTGCAGAAATCGTAC 297
Nikshahr     CTGTTCTACGGAAAAACGGACTTGCCCGAGGATCGCGTTGAGTATGTGCAGAAATCGTAC 297
Kazeroon1    CTGTTCTACGGCAAAACGGACTTGCCCGAGGATCGCGTTGAGTATGTGCAGAAATCGTAC 300
Kazeroon2    CTGTTCTACGGCAAAACGGACTTGCCCGAGGATCGCGTTGAGTATGTGCAGAAATCGTAC 300
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Saravan      CGCTTGCTGGAGGACACGCTGCTGGACGATTTTGTAGCCGGACCGGCCATGACGATTGCC 357
Chabahar     CGCTTGCTGGAGGACACGCTGCTGGACGATTTTGTAGCCGGACCGGCCATGACGATTGCC 357
Nikshahr     CGCTTGCTGGAGGACACGCTACTGGACGATTTTGTAGCCGGACCGGCCATGACGATTGCC 357
Kazeroon1    CGCTTGCTGGAGGACACGCTACTGGACGATTTTGTAGCCGGACCGGCCATGACGATTGCC 360
Kazeroon2    CGCTTGCTGGAGGACACGCTACTGGACGATTTTGTAGCCGGACCGGCCATGACGATTGCC 360
             ******************* ************************************

Saravan      GACTTTAGCTGCATCTCCACGATCTCTAGCATTATGGGCGTTGTTGCGCTGGACAAAGCG 417
Chabahar     GACTTTAGCTGCATCTCCACGATCTCTAGCATTATGGGCGTTGTTGCGCTGGACAAAGCG 417
Nikshahr     GACTTTAGCTGCATCTCCACGATCTCTAGCATTATGGGCGTTGTTGCGCTGGACAAAGCG 417
Kazeroon1    GACTTTAGCTGCATCTCCACGATCTCTAGCATTATGGGCGTTGTTGCGCTGGACAAAGCG 420
Kazeroon2    GACTTTAGCTGCATCTCCACGATCTCTAGCATTATGGGCGTTGTTGCGCTGGACAAAGCG 420
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Saravan      GAACATCCTCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTGCCATACTACGAGGAA 477
Chabahar     GAACATCCTCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTGCCATACTACGAGGAA 477
Nikshahr     GAACATCCTCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTGCCATACTACGAGGAA 477
Kazeroon1    GAACATCCCCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTGCCATACTACGAGGAA 480
Kazeroon2    GAACATCCCCGGATCTACGGGTGGATCGATCGTCTGAAGCAGCTGCCATACTACGAGGAA 480
             ******** ****************************************************

Saravan      GCGAACGGTGGC 489
Chabahar     GCGAACGGTGGC 489
Nikshahr     GCGAACGGTGGC 489
Kazeroon1    GCGAACGGTGGC 492
Kazeroon2    GCGAACGGTGGC 492
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Nucleotide variation was observed within the Kazeroon and Nikshahr populations in three positions of exon 1 (21, 105, and198). However in intron region, this variation was detected in three corresponding positions (8, 10, and 33) of Kazeroon specimens in compare to Nikshahr population. Moreover, whilst comparing the Kazeroon population with other populations of the Sistan and Baluchistan (Saravan and Chabahar) province, nucleotide differences at one and three positions in exon I (105) and exon II (21, 90, 198) and four positions in intron I (8, 16, 33, 72) of Kazeroon specimens were revealed. Analysis of amino acid sequences in different specimens showed that the nucleotide substitutions in the various strains did not lead to any change in the composition of amino acid sequences. Thus, the resistance status observed after the application of the WHO susceptibility test could be related to over expression of the *GST* gene. Comparisons of nucleotide variations and amino acid replacements between specimens of the control area and areas under insecticide treatment are shown in Table 2. All of the nucleotide variations occur in the third position and therefore based on the wobble hypothesis, do not cause a change in the amino acid codon.

DISCUSSION

Glutathione transferases (GSTs) play a central role in the detoxification of xenobiotics such as insecticides and elevated *GST* expression is an important mechanism of insecticide resistance. The observation that the genomic location of a cluster of GST genes coincides with a region of the genome containing a major locus conferring DDT resistance was the main aim of this study which analyzed and compared *GSTe2* sequences between susceptible and resistant populations of *An. stephensi*, one of the most important malaria vectors present in the Middle East and Indian subcontinent. This has now led to identification of mutations in both exons and intron I of the *GSTe2* gene in various strains. However, it has been observed that none of these nucleotide substitutions results in any amino acid change, eventhough the WHO susceptibility tests confirm DDT resistance in *An. stephensi* specimens collected from the Sistan and Baluchistan province. Furthermore, in a comparative study, Dinparast *et al*. (2006) have shown that the *GSTe2* coding sequences of *An. stephensi, An. culicifacies*, and *An. fluviatilis* show 82 to 86% similarity at the nucleic acid levels with that of *An. gambiae*. In their study, species-specific differences have also been detected in intron I of the *GSTe2* gene, probably useful as a molecular marker for species-specific identification.

The data from this investigation will be used in future studies to express the protein of this gene in different populations consisting of the three major malaria vector species, *An. stephensi*, *An. culicifacies*, *An. fluviatilis*, in Iran and neighboring countries. This may help in understabding the mechanism underlying the resistance of these important but neglected malaria vectors to DDT and pyrethroids, as have already been shown in *An. gambiae* and *Aedes aegypti* (Lumjuan *et al*., 2005). In addition, eventual characterization of recombinant *GSTe2* may shed light on other features of GST, such as glutathione peroxidase activity, which has been detected in *Ae. aegypti*, but not in *An. gambiae*.

In conclusion, it can be assumed that the out coming results of this study may serve as first report and

Table 2. Comparison of Nucleotide variations and amino acid replacements in coding sequence of the GSTe2 gene, between *An. stephensi* specimens.

			Nucleotide variations		Codon variations		Amino acid replacements	
	Number	Position	Sistan & Baluchistan	Kazeroon	Sistan & Baluchistan	Kazeroon	Sistan & Baluchistan	Kazeroon
Exon I	1	105	C	G	GCC	GCG	A	A
Exon II	1	21	A	C	GGA	GGC	G	G
	2	90	A*/G**	A	CTA*/CTG**	CTA	L	L
	3	198	T	C	CCT	CCC	P	P

Samples were coiiected from Kazeroon district in Fars province and Saravan, Chabahar, Nikshahr districts in Sistan and Baluchistan province. Nucleotides and amino acid codons which are related to Nikshahr district are marked by * and those from other districts of Sistan and Baluchistan (Saravan and Chabahar) specimens marked by **.

baseline data regarding the structure of the *GSTe2* gene, including exon I, exon II and intron I, in susceptible and resistance field specimens of *An. stephensi.* Meanwhile the integration of these data into the malaria control programs will still remain a challenge in Iran and neighboring countries, especially Afghanistan and Pakistan.

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