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Monitoring Pyrethroid Insecticide Resistance in Major Malaria Vector *Anopheles culicifacies*: Comparison of Molecular Tools and Conventional Susceptibility Test

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ABSTRACT

Background: *Anopheles culicifacies* is a main malaria vector in southeastern part of Iran, bordring Afghanistan and Pakistan. So far, resistance to DDT, dieldrin, malathion and partial tolerance to pyrethroids has been reported in *An. stephensi*, but nothing confirmed on resistance status of *An. culicifacies* in Iran. **Methods:** In current study, along with WHO routine susceptibility test with DDT (4%), dieldrin (0.4%), malathion (5%), permethrin (0.25%), lambadacyhalothrin (0.1%), and deltamethrin 0.025, we cloned and sequenced segment VI of domain II (SII6) in voltage-gated sodium channel (*vgsc*) gene of *An. culicifacies* specimens collected in Sistan and Baluchistan province (Iran). **Results:** A 221-bp amplified fragment showed 91% and 93% similarity with exon I and exon II of *An. gambiae*. The size of intron II in *An. culicifacies* is 62 bp, while in *An. gambiae* is 57 bp. The major difference within *An. culicifacies* specimens and also with *An. gambiae* is in position 29 of exon I, which led to substitution of Leu to His amino acid. **Conclusion:** This data will act as first report on partial sequence of *vgsc* gene and its polymorphism in *An. culicifacies*. A Leu to His amino acid substitution detected upstream the formerly known knockdown resistance **(***kdr)* mutation site could be an indication for other possible mutations related to insecticide resistance. However, the result of WHO susceptibility test carried out in Baluchistan of Iran revealed a level of tolerance to DDT and dieldrin, but almost complete susceptibility to pyrethroids in *An. culicifacies*. We postulate that the molecular diagnostic tool developed for detection and identification of *kdr*-related mutations in *An. culicifacies*, could be useful in monitoring insecticide resistance in Iran and neighbouring countries such as Pakistan and Afghanistan. A phylogenetic tree also constructed based on the sequence of exon I and II, which readily separated *An. culicifacies* populations from *An. stephensi*, *An. fluviatilis* and *An. gambiae*. *Iran. Biomed. J. 11 (3): 169-176, 2007*

Keywords: An. culicifacies, An. gambiae, insecticide resistance, knockdown, Voltage-gated sodium channel (*vgsc*)

INTRODUCTION

Application of Insecticides remains the primary control tool in the majority of vector and pest control programs throughout the world [1]. Most resistance mechanisms can be devided into two groups: metabolic (alterations in the levels of activities of detoxification proteins), and target site (mutation in the receptor genes). Alone or in combination, these mechanisms confer resistance, sometimes at an extremely high level, to all of the available classes of insecticides [2].

Pyrethroids are a large group of highly insecticidal compounds with relatively low mammalian toxicity and low persistence in the environment [3, 4]. The primary site of action of DDT and pyrethroids is voltage-dependent sodium channel [4, 5]. One important resistance mechanism is reduced target-site sensitivity to pyrethroids in the insect nervous system, which is also known as knockdown resistance (*kdr*) [3, 6] and its involvement in modification of voltage-dependent sodium channels

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was first implicated by electrophysiological studies [7]. Sodium channel mutations play a significant role in sodium channel insensitivity to pyrethroids [8].

The *Anopheles gambiae* sodium channel was physically mapped to chromosome 2L, division 20 C [9]. This resistance results from a single point mutation in a gene that encodes a sodium channel [10, 11] and gives the characteristic *kdr* phenotype [12]. Pyrethroid resistance in *An. gambiae* was first reported in West Africa [13]. This resistance was probably initially selected by the intensive use of DDT and more recently pyrethroid for cotton crop protection [14]. However, a second substitution (Leu-ser) has been found in East African *An. gambiae* [9]. On the other hand, both mutations have been reported in *An. sacharovi* from Turkey [15].

The *kdr* is associated with a Leucin to Phenylalanin/Leucin to Serine mutation in segment 6 of domain II [6, 9, 16], and *super-kdr* resistance in house fly with an additional methionine to threonine mutation in the linker region between S4 and S5 of domain II [16, 17]. Voltage-gated sodium channel (*vgsc*) gene has been studied in other pyrethroid resistant insects and arthropods including horn flies (*Haematobia irritans*) [18] with Leu/Phe, *Heliothes virescens* [19, 20] with the Leu/His mutation, cockroaches [3, 6, 8], *Pluella xylostella* and *An. stephensi* laboratory strains [21]. This region and its mutations also have been used as genetic markers for detection of molecular forms and incipient speciation within *An. gambiae* s.s. [22-24].

In Iran, malaria is still the most important parasitic and vector-borne disease. Iran initiated malaria eradication campaign in 1951 and changed to malaria control in 1985 as a result of constraints and challenges. This made its almost all regions free of the disease, with the exception of the thinly populaed southeastern tropical part of Kerman, Sistan and Bluchistan and Hormozgan provinces. Vector control activities in this region is mainly based on residual spraying of human and animal shelters, focal application of larvicides, biological control and insecticide treated net distribution. *An. culicifacies* is the main malaria vector in southeast of Iran and neighboring countries. So far, resistance to DDT in 1963, BHC/cyclodiens in 1964, malathion in 1976, chlorphoxim, fenitrothion, phoxim, and iodofenphos in 1980, carbamates in 1986 and partial tolerance to pyrethroids has been reported in *An. stephensi*, but the only case of resistance in *An. culicifacies* has been related to DDT in 1976 reported from southeast corner of Iran.

Molecular techniques have recently allowed us to start and dissect most of basic mechanisms underlying resistance to commonly used insecticides at the DNA level. With regards to the major role of *kdr* mutation in resistant individuals of main malaria vectors, we studied the structure of sodium channel, especially exon I, II and intron II of *An. culicifacies*, which on the basis of previous studies in *An. gambiae*, should contain the possible *kdr* mutations [9, 10]. This goal has been achieved by carrying out the susceptibility test on field-collected specimens of *An. culicifacies*, followed by cloning, designing new primers, amplification of *kdr* mutation-containing region, sequencing and finally its comparison with molecular evidence of *kdr* mutation in *An. gambiae*.

MATERIALS AND METHODS

Adult *An. culicifacies* specimens collected in a period of two months before, and up to two months after application of insecticides from Saravan, Iranshahr, Nikshahr, Khash and Chabahar in Sistan and Bluchistan province of Iran. WHO susceptibility test carried out with DDT (4%), dieldrin (0.4%), malathion (5%), permethrin (0.25%), lambadacyhalothrin (ICON) (0.1%), deltamethrin (0.025) on *An. culicifacies* specimens based on recommended procedure [25] and percent of death was recoreded after 24 hours recovery. The mortalities were corrected using Abbott's formula [26].

DNA was extracted from individual mosquitoes using slight modification of the techniques described by Collins *et al*. [27] and Xu *et al.* [28]. The genomic region containing the partial sequence of exon I and exon II of *vgsc* gene was PCR amplified in *An. culicifacies* specimens: Saravan (10), Iranshahr (7), Nikshahr (9), Chabahar (8) and khash (5) using primers dinF and dinR [29].

Forward primer (dinF):
5-TGGATTGAATCAATGTGGGATTG-3

Reverse primer (din R):
5-TGCCGTTGGTTGCAGACAAGG-3

DNA genomic (10-50 ng), one unit of Taq polymerase (CinnaGen, Tehran, Iran) and 0.5 mM of each primer were used in a 25 µL total PCR volume. Amplification was performed for 35 cycle at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final extraction step at 72°C for 5 min.

Table 1. Results of insecticide susceptibility tests on *An. culicifacies.* Number of individuals (in parentheses) and percentage of death in specimens of *An. culicifacies* from Sistan and Baluchistan after one hour exposure to DDT (4%), dieldrin (0.4%), malathion (5%), permethrin (0.25%), lambadacyhalothrin (0.1%) and deltamethrin (0.025).

No and % death / Anopheles	DDT (4%)	dieldrin (0.4%)	malathion (5%)	permethrin (0.25%)	lambadacyhalothrin (0.1%,)	Deltamethrin (0.025)
An. Culicifacies (S and B)	(291) 95%	(219) 97%	(288) 99%	(230) 99.5%	(222) 100%	(202) 100%

Target fragments purified from seized gels followed by cloning in T-vector (pTZ57R/T) and $Top10_F$ *E. coli* cells. Selected clones were used for purification of plasmids [30]. In order to optimize the specific amplification of *vgsc* sequence in *An. culicifacies*, we designed a 24 base reverse primer (Foru-R) based on position 31-54 in the sequence of 67GF strain of *An. culicifacies*, which its sequence is as follows:

Foru-R: 5-AAGGATGAAGAACCGAAATTGGAC-3

All PCR reactions were performed in a total volume of 25 µl. Each mixture contained 1× PCR buffer, 1.5 mM of $Mgcl_2$, 0.5 mM of each primer, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 1 unit of Taq polymerase (CinnaGen, Tehran, Iran) and sterile double-distilled water to 25 µL. The PCR reaction condition was 1 min at 94°C, 1 min at 58°C and 1 min at 72°C for 35 cycle with a final extension step at 72°C for 5 min. Amplified fragments were analized by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

Sequenceed data have been analyzed by using Genruner (version 3.05, 1994, Hastings Software Inc.), ClaustalW [31] and MegAlign (version 3.0.3.c 1993-95, DNA star Inc.) and other programs available in NCBI site (National Center for Biotechnology Information).

RESULTS

WHO routine susceptibility test carried out on specimens of *An. culicifacies* collected from Sistan and Baluchistan province of Iran revealed a level of tolerance to DDT and dieldrin, but almost complete susceptibility to pyrethroids (Table 1). These specimens were subjected to PCR amplification of exon I, intron II and partial sequence of exon II, by using specific primers to *vgsc* sequence in *An. gambia* (Fig. 1A). The size of amplified band was 221 bp in all specimens. This band was excised from the gel, purified and cloned in T-vector (CinnaGen, Tehran, Iran). Selected clones were used for purification of plasmids (Fig. 1B), followed by PCR amplification of a 221 bp fragment, which confirmed the right size of insert (Fig. 1C).

Purified plasmids with inserts have been sequenced on both sides by M_{13} forward and reverse primers. Similar to *An. gambiae*, this sequence is Adenine-Thymine-rich in *An. culicifacies* as well. Blast analysis of two sequences showed a 91% similarity in exon I and 93% in exon II between *An. gambiae* and Iranian *An. culicifacies*. However, intron I revealed a significant diversity between two species on both size and nucleic acid compositions. The size of intron I in *An. gambiae* and *An. culicifacies* is 57 bp and 62 bp, while the total size of amplified fragment in two species is 216 bp and 221 bp, respectively. Therefore, the difference in total size of amplified fragment by using primers: dinF and dinR is due to the variation in intron I sequence. A comparison of exon I and II revealed the differences in nucleotide sequence between two species (Table 2), which did not change the amino acid sequence except in position 29 of exone I, where, a transversion-type point mutation caused a substitution in second base of Lue, resulted to amino acid change to His. Nucleotide and amino acid sequences of this specimen (67GF) is available with accession number AY342398 in GenBank, European Molecular Biology Laboratory and DNA Data Bank of Japan.

Based on sequence data from 67GF, a reverse primer (Foru-R) has been designed. The amplified product in different specimens of *An. culicifacies* was a specific sharp band of 205 bp (Fig. 1D). Analysis of exon I and II revealed that all changed bases are similar in different specimens of *An. culicifacies*, except in position 29 of 67GF strain, which resulted in polymorphism within this species as substitution of Lue to His amino acid (Table 3 A and B). However, size polymorphism due to insertion or deletion has not been detected in exon I and II. Comparison of exon I and II between specimens of *An. culicifacies* and *An. gambiae*, also did not show any difference in amino acid composition (Table 3).

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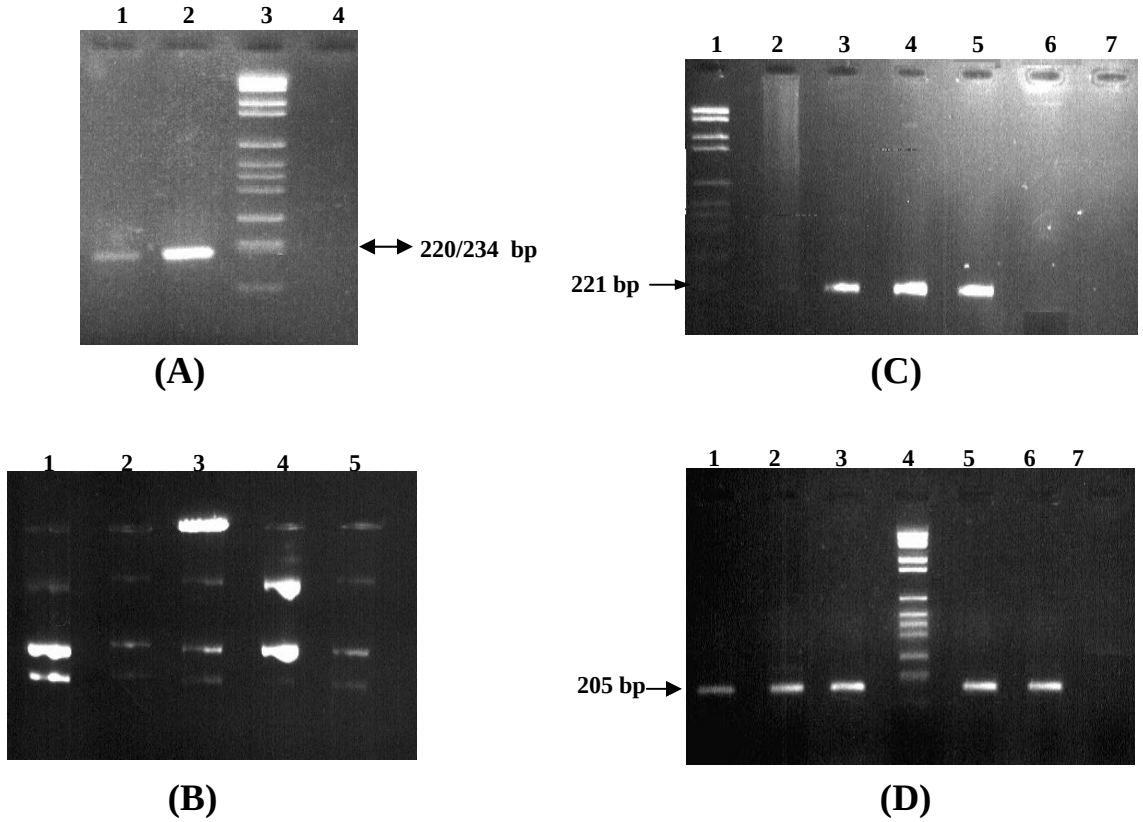

Fig. 1. PCR and cloning analysis of *vgsc* gene (*kdr* region) in *An. culicifacies* (sections A-D). **(A)**, Electrophoresis analysis of *vgsc* -PCR products *in An. culicifacies* (lane 1) and *An. gambiae* (lane 2) by using primers dinF and dinR primers. The size of amplified fragment in *An. culicifacies* is 221 bp. Lanes 3 and 4 are molecular weight marker (VI Rhoch) and negative control; **(B)**, Electrophoresis analysis of extracted plasmid containing the 221bp insert (lanes 2-5) and without insert (lane 1); **(C)**, PCR amplification of clones (lanes 2-5) by primers dinF and dinR. This amplification confirmed the right size of insert (221 bp), which latter has been used for sequencing. Lane 1 is molecular weight marker and negative control is in lane 7; **(D)**, Electrophoresis analysis of PCR products amplified by dinF and Foru-R primers in different specimens of An. culicifacies: 13, 83, 103 (lanes 1-3) and 216, 264 (lanes 5-6). The size of frgament is 205 bp in all individuals. Lanes 4 and 7 are molecular weight marker and negative control, respectively.

Table 2. Nucleotides and amino acid variation in exon I and II of *vgsc* gene of *An. culicifacies* and *An. gambiae* [32].

Exon	Position	Nucleic Acid	Codon	Amino Acid
I	*29	T to A	CTT to CAT	L to H
	33	C to A	GTC to GTA	V
	36	T to C	GGT to GGC	G
	43	A to G	GTA to GTG	V
	46	C to G	TCC to TCG	S
	61	T to C	TTG to CTG	L
	66	C to T	GCC to GCT	A
	69	T to A	ACT to ACA	T
II	3	G to T	GTG to GTT	V
	21	C to T	GCC to GCT	A
	33	A to C	TCA to TCC	S
	39	T to C	TTT to TTC	F
	45	A to T	TCA to TCT	S

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Table 3. Total number and percentage of nucleotides in *vgsc*. **(A)**, *An. culicifacies* and **(B)**, *An. gambiae*.

(A)

		Size (bp)	T	A	C	G	% (A + T)	% (C + G)
Exon	I	90	25	12	22	31	41.11	58.89
	II	69	10	19	10	30	42.03	57.97
Intron	I	43.55	56.45	12	15	19	16	62
Total		%41.63	%57.92	47	45	51	77	221

(B)

		Size (bp)	T	A	C	G	% (A + T)	% (C + G)
Exon	I	38.89	61.11	23	12	21	34	90
	II	42.03	57.97	11	18	12	28	69
Intron	I	33.33	66.67	11	8	19	19	57
Total		%38.43	%61.57	45	38	52	81	216

Blast analysis based on similarity/distance indices revealed that the specimens of *An. culicifacies* are almost identical in amino acid level (considering a single gap), while their similarity with *An. gambiae* is about 92%.

Nucleotide and amino acid sequences related to specimens of *An. culicifacies* (13GF, 83GF, 103GF, 216GF, 264GF, 284Kh) submitted to GenBank and could be reached through the following accession numbers: AY422494, AY342399, AY342400, AY422492, AY422493, AY840370. Accession numbers of proteins are: AAV73776, AAR32092, AAR32094, AAR32093, AAR23796.

Clustal W. (1.83) multiple *vgsc* exon I and II sequence alignment of seven *An. culicifacies* specimens (cul.67GF, cul.83GF, cul.103GF, cul.216GF, cul.264GF, cul.13GF, cul.206N), along with *An. fluviatilis*, *An. stephensi* and *An. gambiae* (Fig. 2) followed by phylogenetic tree construction revealed that all populations of *An. culicifacies* stand in a single clade. The next closer taxa to this clade are *An. fluviatilis*, and *An. stephensi*, while *An. gambiae* appeareed as an out group taxon (Fig. 3).

DISCUSSION

In Culicidae mosquitoes, mutations related to *kdr* has been reported in *vgsc* gene of *An. gambiae* from Africa [9-12, 29], *An. sacharovi* from Turkey [15], laboratory strains of *An. stephensi* [21] and *Culex pipiens* [11]. In current study, *An. culicifacies* specimens were collected from Baluchistan (the main malariuos areas of Iran), bordering Afghanistan and Pakistan, where the indoor space spraying of human and animal shelters for control of adult mosquitoes and application of larvicides in breeding sites are under practice.

Adult *An. culicifacies* specimens collected in a period of two months before, and up to two months after application of insecticides have shown no

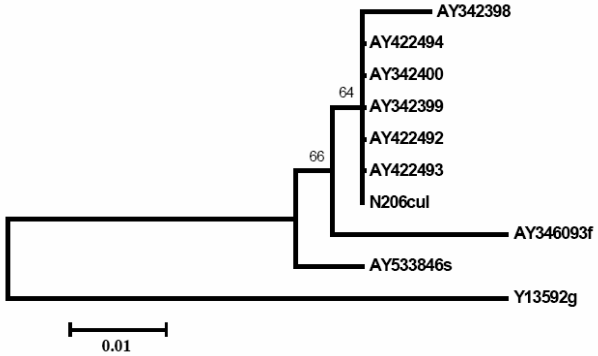

Fig. 3. Phylogenetic tree constructed based on A) exon I and II sequence of *vgsc* gene in *An. culicifacies* (AY342398, AY422494, AY342400, AY342399, AY422492, AY422493, N206cul, *An. stephensi* (AY533846s), *An. fluviatilis* (AY346093f) and *An. gambiae* (Y13592g).

Table 4. Number of individuals (n) and percentage of death in specimens of *An. culicifaciesi* after exposure to DDT (4%), dieldrin (0.4%), malathion (5%), permethrin (0.25%), lambadacyhalothrin (0.1%), deltamethrin (0.025) [32].

No and % death / Anopheles	DDT (4%)	dieldrin (0.4%)	malathion (5%)	permethrin (0.25%)	lambadacyhalothrin (0.1%,)	Deltamethrin (0.025)
An. Culicifacies (S and B)	(125) 99.1%	-	(102) 100%	(92) 100%	(85) 100%	(107) 100%

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67GF                TGGATTGAATCAATGTGGGATTGTATGCATGTAGGCGATGTGTCGTGCATACCATTTTTC 60
206N                TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
13GF                TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
264GF               TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
216GF               TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
103GF               TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
83GF                TGGATTGAATCAATGTGGGATTGTATGCTTGTAGGCGATGTGTCGTGCATACCATTTTTC 60
flu_AY346093_       TGGATTGAATCAATGTGGGATTGTATGCTTGTGGGCGATGTGTCGTGCATCCCATTTTTC 60
Ste                 TGGATTGAATCAATGTGGGATTGTATGCTTGTTGGCGATGTGTCATGCATACCATTTTTC 60
gambiae_Y13592_     TGGATTGAATCAATGTGGGATTGTATGCTTGTCGGTGATGTATCCTGCATACCATTTTTC 60
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67GF                CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
206N                CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
13GF                CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
264GF               CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
216GF               CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
103GF               CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
83GF                CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
flu_AY346093_       CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTCAACCTTTTCTTAGCTTTGCTTTT 120
Ste                 CTGGCTACAGTAGTGATAGGAAATTTAGTCGGTTCTTAACCTTTTCTTAGCTTTGCTTTT 120
gambiae_Y13592_     TTGGCCACTGTAGTGATAGGAAATTTAGTCG-TGCTTAACCTTTTCTTAGCCTTGCTTTT 119
                     **** ** ********************** * ** ************** ********

67GF                GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
206N                GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
13GF                GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
264GF               GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
216GF               GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
103GF               GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
83GF                GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
flu_AY346093_       GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
Ste                 GTCCAATTTCGGTTCTTCATCCTTGTCTGCACCAACGGCAG 161
gambiae_Y13592_     GTCAAATTTTGGTTCATCATCCTTGTCTGCACCAACGGCAG 160
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Fig. 2. CLUSTAL W (1.83) multiple sequence alignment based on exon I and II sequence of *vgsc* gene in *An. culicifacies* (cul. 67 GF, cul. 83 GF, cul. 103 GF, cul. 216 GF, cul. 264 GF, cul. 13 GF, cul. 206N), *An. fluviatilis* (fluviatilis), *An. stephensi* (stephensi) and *An. gambiae* (gambiae_Y13592).

resistance to any of examined insecticides. A similar study in Baluchistan of Iran on resistance status of *An. culicifaciesi* after exposure to DDT (4%), dieldrin (0.4%), malathion (5%), permethrin (0.25%), ICON 0.1%, deltamethrin 0.025, revealed that this species is almost completely susceptible to these insecticides (Table 4) [32]. With respect to these two studies and other related reports by Iranian Center for Diseases Management and Control, it could be concluded that based on WHO routine susceptibility test, *An. culicifacies* in main malarious areas of Iran is not resistant to any insecticides previously used or are currently under application.

In second part of this study, those specimens subjected to DNA extraction were followed by PCR, cloning and sequencing of segment VI of domain II in *vgsc* gene. The outcoming results, for the first time, provided a practical view as nucleotide sequence composition of *kdr* in field populations of a major malaria vector in Oriental region. It also resulted in detection of a transversion-type point mutation polymorphism in position 29 of exon I in *An. culicifacies* specimens, which caused amino acid substitution Lue/His in up-stream of common *kdr* mutation identified in other mosquitoes and insects.

Although needs further investigation to find out the possible role of this amino acid alteration in exon I, but this intra-specific variation and the other main difference in intron sequence between *An. gambiae* and *An. culicifacies* will shed the light on the importance of generating regional genomic data for understanding the mechanisms of insecticide resistance and also to address the speciation process in sibling species of malaria vectors. The prerequisite for developing a simplified methods for detection of resistance in malaria vectors, as reported by Lynd *et al.* [33], is to characterize those target regions in the genome of other main malaria vectors worldwide.

The primer we designed based on the sequence of *vgsc* gene in *An. culicifacies* has been applied for detection of *kdr*-related mutation in other main malaria vectors in the region including *An. stephensi*, *An. fluviatilis*, *An. dthali*, *An. pulcherrimus* and *An. superpictus*. Furthermore, it has been shown that this molecular tool is useful for designing a test, which could be developed for monitoring insecticides against *An. culicifacies* and *An. stephensi* specimens from neighboring countries of Afghanistan and Pakistan (Djadid *et al.*,

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unpunlished data). Surely, this molecular data along with the result of WHO routine susceptibility test and ecological evidence will provide a better understanding for the managers of malaria control program in the states of WHO/EMRO, in order to select the most appropriate tools against anopheline vectors which are mostly common in south-west Asia, Indian subcontinent, and Far East.

On the other hand, a phylogenetic tree constructed based on the sequence of exon I and II, readily separated *An .culicifacies* populations from *An. stephensi*, *An. fluviatilis* and *An. gambiae*. The same phylogram has also been achieved by using intron I sequence of these species, revealing the systematic value of the *vgsc* gene sequences in defining the evolutionary relationships among these species, especially intron sequence which is completely species-specific.

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