## Original Article

# Ace-1 Target Site Status and Metabolic Detoxification Associated with Bendiocarb Resistance in the Field Populations of Main Malaria Vector, *Anopheles stephensi* in Iran

**Abdollah Badzohre[1], *Mohammad Ali Oshaghi[1], Ahmad Ali Enayati[2], Seyed Hassan Moosa-Kazemi[1], Seyed Hassan Nikookar[3], Fahimeh Talebzadeh[1], Nazanin Naseri-Karimi[1], Ahmad Ali Hanafi-Bojd[1], *Hassan Vatandoost[1,4]**

[1]Department of Vector Biology and Control of Diseases, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Medical Entomology and Vector Control, School of Public Health and Health Sciences Research Center, Mazandaran University of Medical Sciences, Sari, Iran.
[3]Department of Medical Entomology and Vector Control, Health Sciences Research Center, Addiction Institute, School of Public Health, Mazandaran University of Medical Sciences, Sari, Iran
[4]Department of Chemical Pollutants and Pesticides, Institute of Environmental Research, Tehran University of Medical Sciences, Tehran, Iran

***Corresponding authors:** Dr Hassan Vatandoost, E-mail: hvatandoost1@yahoo.com, vatando@tums.ac.ir, Dr Mohammad Ali Oshaghi, E-mail: moshaghi@sina.tums.ac.ir

**(Received 05 Mar 2023; accepted 20 May 2023)**

## Abstract

**Background:** *Anopheles stephensi* is the main vector of malaria in Iran. This study aimed to determine the susceptibility of *An. stephensi* from the south of Iran to bendiocarb and to investigate biochemical and molecular resistance mechanisms in this species.

**Methods:** Wild *An. stephensi* were collected from Hormozgan Province and reared to the adult stage. The susceptibility test was conducted according to the WHO protocols using bendiocarb impregnated papers supplied by WHO. Also, field *An. Stephensi* specimens were collected from south of Kerman and Sistan and Baluchistan Provinces. To determine the G119S mutation in the acetylcholinesterase (Ace1) gene, PCR-RFLP using AluI restriction enzyme and PCR direct-sequencing were performed for the three field populations and compared with the available GenBank data. Also, biochemical assays were performed to measure alpha and beta esterases, insensitive acetylcholinesterase, and oxidases in the strains.

**Results:** The bioassay tests showed that the *An. stephensi* field strain was resistant to bendiocarb (mortality rate 89%). Ace1 gene analysis revealed no G119S in the three field populations. Blast search of sequences revealed 98–99% identity with the Ace1 gene from Pakistan and India respectively. Also, the results of biochemical tests revealed the high activity of non-sensitive acetylcholinesterase, alpha and beta-esterase in the resistant strain compared to the susceptible strain. No G119S was detected in this study additionally the enhanced enzyme activity of esterases and acetylcholinesterase suggesting that resistance was metabolic.

**Conclusion:** The use of alternative malaria control methods and the implementation of resistance management strategies are suggested in the study area.

**Keywords:** *Anopheles stephensi*; Malaria; Insecticide resistance mechanism; Bendiocarb; Iran

## Introduction

Malaria is the main vector-borne disease (VBD) with approximately 241 million cases and 627000 deaths reported around the globe, during 2020 (1). In Iran, seven anopheline mosquito species have been reported to be malaria vectors and the Persian Gulf border regions are considered endemic foci of malaria (2). *Anopheles stephensi* is dominant and active as



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

a primary vector in these areas. Vector control programs including indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs), have been implemented in the region and indigenous malaria-positive cases have been declining in recent years (3).

Over the past decade, the widespread use of pyrethroids (PY) insecticides in the health and agricultural sectors has led to increased resistance in malaria vectors (4). Pyrethroids are the only group of insecticides applied for net treatment and three groups organochlorine (OC), organophosphate (OP) and carbamate (C) are useful for IRS (5). Development of insecticide resistance, mostly against PYs, in several disease vectors, is a crucial problem arisen by IRS and LLIN interventions (6).

The World Health Organization (WHO) recommends the use of insecticide classes with different modes of action to limit resistance against insecticides in mosquito vectors (7). Carbamate insecticides are a good choice for replacement or rotational use with PYs insecticides in residual spraying due to their different modes of action and are recommended in control programs against malaria, yellow fever, dengue, and onchocerciasis in other countries (8).

Resistance in malaria vectors and other important vectors in medical entomology to various groups of insecticides has been reported in most parts of the world including eastern Mediterranean region (9). Populations of *An. culicifacies* s.l., *An. superpictus* s.l. and *An. subpictus* s.l. have shown resistance to second-generation PYs in Afghanistan (10). In Sudan, *An. arabiensis*, the main malaria vector, has shown resistance to all four main groups of insecticides (PY, OP, C and OC) (11). In China, there have been records of *Culex* and *Aedes* resistance against PYs and OPs (12) (5). *Anopheles gambiae* s.l. (13, 14), *Ae. aegypti* (15) and *Ae. albopictus* (16) show different levels of resistance to insecticide groups in their distribution areas. Resistance of *An. stephensi* to different insecticides classes has been reported in the Horn of Africa and Asia (4, 9, 17, 18).

Development and spread of resistance in mosquito vector populations occurring from prolonged and excessive use of insecticides is a major public health concern, hindering the efficiency of vector control interventions (19). Resistance in mosquitoes can occur in three main ways: metabolic resistance, target-site resistance, and penetration resistance. Malaria vectors resistance to insecticides is mainly due to allelic changes and metabolic resistance (8).

Monitoring and evaluating resistance to insecticides will help identify the threat and how resistance develops in vectors. Three different methods including bioassay tests, biochemical assay, and molecular tests can be used to identify vector resistance and each method will provide different information (8).

Due to the fact that the molecular bases of insecticide resistance mechanisms are very different and these mechanisms must be well defined for each insecticide. Evaluation strategies (Efficient monitoring strategies) can be implemented and finally manage the resistance of vectors to diverse insecticide types (7).

Acetylcholinesterase (AchE) has a role in the transmission of neural messages by hydrolysis of the neurotransmitter acetylcholine and is a common target for two groups of insecticides C and OP (20). Insect paralysis and death occur by interference with the transmission of neural messages in the synaptic space by blocking AchE transmission and causing intoxication signs and respiratory failure (21).

Substitution at locus 119 of the Ace-1 gene from Glycine to Serine plays a key role in creating cross-resistance to OP and C insecticides in various vectors such as *An. gambiae* (6, 22, 23), *An. coluzzi* (24) *An. albimanus* (25) in malarious regions. The present study was designed in order to identify the sensitivity level of *An. stephensi* to bendiocarb (2, 2-Dimethyl-2H-1, 3-benzodioxol-4-yl methylcarbamate) insecticide, and the analysis of biochemical and molecular mechanisms involved in resistance.



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

## Materials and Methods

### Study area

Samples were collected in Iran from three provinces in the Persian Gulf Region including Bandar-Abbas (Hormozgan) (27.3169°N, 56.3131°E) for rearing, bioassay tests and selection process, Kerman (58.41524°E, 27.1777 °N) and Sistan and Baluchistan (25.3062°N, 60.6939°E) for molecular and biochemical assay only. This region is localized in subtropical zone.

### Larval sampling and rearing procedure

Larval samples were collected from November to December 2020 from Bandar-Abbas breeding places such as water containers, freshwater pools, stream margins, discarded tires, plastic containers, cisterns and barrels using the dipping method according to WHO guidelines and were reared to adults in the TUMS insectary maintained at 28±2 °C, 12:12 h L:D photoperiod and 70±10% relative humidity. Larval identification of the mosquitoes was conducted using morphological identification keys (26). The pupae were sorted and transferred with pipettes from the plastic trays to glass cups with small amounts of water inside a cage and were provided with a 10% sugar solution for rearing adults who emerged after one day in the cage.

### Susceptibility test for *Anopheles stephensi*

Insecticide susceptibility tests were carried out following the WHO insecticide susceptibility test procedure. A total of 500 non-blood fed adult female *An. stephensi* (2–3 day-old) from Bandar-Abbas were exposed to insecticide impregnated papers supplied by WHO with discriminating concentration of bendiocarb. Batches of 25 mosquitoes in four replicates were exposed to insecticide impregnated papers for 3.45, 7.5, 15, 30 and 60 min in WHO test tubes. After one hour of exposure the number of knocked-down were recorded. A control in two replicates (50 female *An. stephensi*) was used for each test time, exposed to untreated papers. Mosquitoes were then transferred into holding tubes with papers and were supplied with 10% sucrose solution for 24 hours. At the end of the recovery period, the number of dead mosquitoes was recorded for each exposure time. Knockdown calculations were performed according to WHO protocol (27) using Prism software. These steps were done for the Beech strain as susceptible to insecticides that obtain from TUMS insectary.

When the mortality rate in the control was between 5–20%, it was corrected using Abbott's formula. According to the WHO criteria: 98–100% mortality indicates susceptibility, 90–97% mortality indicates resistance candidate (more investigation is needed) and less than 90 % mortality suggests resistance (27).

### Selection process

The samples which presented the lowest mortality rates to bendiocarb were preceded for selection pressure. This strain was selected for 7 generations by exposing adults to bendiocarb which produced 50–70% mortality (2). Semi gravid or gravid female mosquito samples from each generation were left to egg-lay and mature and adult F1-F7 generations were collected. The selection was continued as long as a homogenous resistant population with resistance ratio 7 fold was attained. In each generation, the mortality rate and $LT_{50}$ were calculated in serial times and regression lines were plotted in each generation using Microsoft Excel. All alive and dead specimens were kept individually in Eppendorf tubes and freezed in -20 ºC for further biochemical and molecular assays.

### Biochemical analysis

In total 545 *An. stephensi* samples were used for biochemical (α-esterase, β-esterase), MFO and AchE enzyme analyses. Randomly each 3-to-5-day-old female from the populations: Beech strain, F7 generation and resistant individual adult that survivors from the bendiocarb bioassay were selected and used for this purpose. Individuals were homogenized in 250



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

μL distilled water on ice and the homogenates were centrifuged at 3000 g for 20 min at 4 °C. Enzyme levels were measured as a single adult female using the WHO's field and laboratory manual (27). Absorption levels based on the specified wavelengths for each of the enzymes were measured spectrometrically by a microplate reader and the average absorption calculated based on the data of two holes for one insect was considered. Spectrophotometric analysis was passed in 96 well flat-bottom microtiter plates by a Biotek Elx808 microplate reader (Biotek Instruments, USA).

**Total protein assay**

This test measures the total amount of protein in each mosquito. This measurement is important because by using this information we can eliminate the errors caused by the different sizes of insects and the amount of their different enzymes. 300 microliters of Bio-Rad solution diluted in distilled water at a ratio of 1:4 was added to 10 μL of mosquito homogenate solution in two replicates and after 5 minutes their absorbance at 570 nm was measured.

**Non-specific esterase assay (NSE)**

NSE activity was measured by using α-naphthyl acetate (α-NA) and β- naphthyl acetate (β-NA). For α-NA and β-NA assays, a 20 μL of mosquito homogenate was added to the well in duplicate. Following this, 200 μL of α-NA (120 μL of 0.03 M α-NA dissolved in 12 ml of 0.02 M sodium phosphate buffer with pH= 7.2) and 200 μL β-NA (120 μL of 0.03 M β -NA dissolved in 12 ml of 0.02 M sodium phosphate buffer with pH= 7.2) were added to each well. After 30 min of incubation, 50 μL of (0.023 Fast Blue Salt dissolved in 2.25 ml of distilled water along with 5.25 ml of 5% phosphate buffered SDS solution) was added to stop the reaction and the end point absorbance measured at 570 nm. The values in units of n moles α and β napthol/min/mg were calculated against standard curves of α and β naphthol acetate (27).

**Mixed function oxidase (MFO) assay**

MFO activity was determined based on the heme amount using the heme-peroxidase assay (9). 20 μL mosquito homogenate and a 200 μL working solution (0.01 g of 3, 3, 5, 5′ tetrametylbenzidine- TMBZ+5 mL methanol+ 15 mL sodium acetate buffer) were added in duplicate in the plate. Following this, 25 μL of 3% v/v hydrogen peroxide was added. After 120 min. incubation, the absorbance was read at 650 nm and protein values were calculated against a standard curve of cytochrome C protein.

**Acetylcholinesterase (AchE) assay**

For this purpose, twenty-five microliters of homogenates in duplicates were solubilized by adding 145 μl of Triton phosphate buffer (1% Triton X-100 in .1 M phosphate buffer pH 7.8) to each replicate. Ten μL of dithiobisnitrobenzoicacid (DTNB) solution (0.01 M dithiobis-2-nitrobenzoic acid in .1 M phosphate buffer pH 7.0) and 25 μL of the substrate ASCHI (0.01 M acetylthiocholineiodide) were added to one replicate to initiate the reaction. The final solution was replaced by 25 μL of the substrate ASCHI having 0.2% of the inhibitor propoxur (0.1 M) for the second test replicate. As control wells contained 25 ml distilled water, 145 ml Triton buffer, 10 ml DTNB solution and 25 ml ASCHI solution without and with propoxur, were used. The kinetics of the enzyme reaction was monitored continuously at 405 nm for 5 min. The percentage of inhibition of AchE activity by propoxur in the test relative to the uninhibited wells was measured. The assay conditions were set so that individuals without an altered AchE based resistance mechanism had >60% inhibition of the AchE activity.

Four blank replicates were set using the same materials of each assay except for distilled water added instead of the mosquito homogenate.

**Data analyses of Biochemical assays**

The activity of the enzymes was measured for mosquito replicates and used for further analysis. The data then were transformed into



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

the actual enzyme activity values and the total protein content of each sample using a standard equation. Mean enzymatic activities were calculated for populations and compared with sensitive *An. stephensi* laboratory strain by way of Mann Whitney test non-parametric using GraphPad Prism version 9.Enzyme ratios (ER) were calculated by dividing the mean activities of each enzyme in strains with those of the Beech susceptible strain.

**DNA extraction, amplification and PCR-RFLP**

For the molecular assay, *An. stephensi* samples were collected from three southern provinces of the country including Hormozgan, Kerman and Sistan and Baluchestan where malaria is endemic. Sixty live samples from three populations including 20 samples remained alive from bioassay tests that were resistance to bendiocarb 0.1% (Bandar-Abbas strain) and 40 samples from two other regions (20 from each) were randomly selected and their genomic DNA was extracted either using QIAamp DNA tissue kit (Cat. No. 51404) according to instrument guideline or the following protocol: a single mosquito is homogenized in a 1.5 ml Eppendorf tube containing 200 µl of Collins buffer (100 mMTris HCL, pH 8.0, 10 mM EDTA, 1.4 M NaCl, 2%) and incubated at 65 °C for 30 min; then 200 µL of ethanol 70% are added (21). After centrifugation (room temperature, 5 min, 12000 rpm), the supernatant is transferred to a fresh tube, 200 µL of ethanol 70% are added, and the mix is centrifuged again (12000 rpm, 15 min). After discarding the supernatant, the pellet is washed with 90% ethanol, dried and suspended in DNAase Free water.

To detect the G119S mutation in the Ace-1 gene, PCR was conducted on the extracted DNA and followed by digestion of PCR products by AluI restriction enzyme. PCR was carried out in 25 µl volumes containing 1 µL of genomic DNA, 1 µL of each primer Ex3Agdir 5′-GATCGTGGACACCGTGTTCG-3′ and Ex3AGrev 5′-AGGATGGCCCGCTGGAACAG-3′, 12.5 PCR master mix Taq DNA Polymerase 1.5X Ampliqon which generating 520 bp PCR fragment. PCR reaction was performed in the initial denaturation step of 5 min at 94 °C, followed by 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s in 35 cycles. Fifteen µL of the PCR product were digested with 1 unit of AluI restriction enzyme in the final volume of 25 µL and incubated at 37 °C overnight. The restriction products were fractionated on a 2% agarose gel and visualized under UV light. PCR product directly sequenced with the same primers were used in amplification. Sequence alignment, homology, and phylogeny were analyzed by bioinformatics software such as Clustal Omega, Geneious Prime, Blast and TranSeq software.

## Results

**Insecticide susceptibility bioassay**

Susceptibility tests showed that *An. stephensi* Bandar-Abbas strain was resistant to bendiocarb (89% mortality). The resistance ratio (RR) of field population compare to beech strain was 1.47 fold. Only this population was chosen for the insecticide selection process. This process continued for 7 generations to achieve RR of 7-fold compared to the susceptible Beech strain in the population exposed to bendiocarb. The equation of the regression line, $LT_{50}$, and resistance ratio in each generation is shown in Table 1 and Fig. 1.

**Biochemical assay**

Activities of MFO and the contents α- and ß-esterases that were measured for the Beech strain, seventh generation and resistant strain *An. stephensi* populations are shown in Table 2 and Fig. 2. The ratio of MFO in the seventh generation and resistant populations were 0.5 and 0.9 when compared with the susceptible strain. The MFO activity levels of *An. stephensi* were lower in the selected population than the Beech strain and significantly different between resistant and field strain ($P<0.05$) Table 3 and Fig. 2.

The enzyme ratios for esterases against α-naphthyl acetate were 3.7 and 5.2 and against



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

β-naphthyl acetate were 1.3 and 8.1 in the seventh generation and resistant populations respectively compared with the Beech strain. The enzyme ratios for both esterases were higher in the resistant population. Statistical analysis showed that alterations in α- and β-esterase activity was detected and significantly increased in α- and β-esterase activity of resistant *An. stephensi* selected populations compared to the α- and β-esterase activity of susceptible *An. stephensi* in (Table 3 and Fig. 2).

Considering that the threshold of <60% set for AchE insensitive to propoxur, the percentage of indivuals that show this value in each strain in resistant generation, seventh generation and Beech strain was 43%, 34% and 31%, respectively. There were significant differences between the two populations in AchE inhibition compare to susceptible Beech strain (Table 3, 4 and Fig. 3).

**PCR and PCR-RFLP**

A total of 60 individuals (20 samples from each region) were tested for the G119S mutation. Amplification of the Ace-1 gene with the primers resulted in a fragment length of 520 bp (Fig. 4i). The length of AluI restricted fragments showed that none of the samples carried the G119S mutation. The PCR-RFLP with AluI restriction enzyme digested the PCR product into two fragments with the lengths of 350 bp and 150 bp which indicate no G119S mutation (Fig. 4.ii). For the PCR product containing the G119 S mutation, three fragments in the lengths of 100, 150 and 250 bp would appear.

**Blast search and sequence alignment**

The amplified region of the Ace1 gene seems to include two exons that can be translated into amino acids and an intron part between the two exons, 72 bases long. Comparing the sequence of Ace1 gene between the three populations of Sistan and Baluchestan, Kerman, and Bandar-Abbas after removing the unreliable parts and equating their length (492 base pairs) showed that there was no polymorphism in the sequence of this gene between the three populations.

Comparison of 492 bp of Ace1 gene sequence of the three populations of *An. stephensi* with other sequences available in the GenBank showed 99.59% and 98.37% similarity with the acetylcholinesterase gene of an Indian *An. stephensi* with the accession number CP032299, and Pakistan *An. stephensi* (SDA-500 strain) with the accession number (CP032232) respectively. In addition, first and second exon of the sequences obtained from this study had 99% and 98% homology with the transcript (mRNA) sequences of two exon parts of *An. stephensi* variant X5 (accession number XM_036043798). The amino acid sequences of Ace1 gene obtained from this study was 100% similar to the amino acid sequences of the above mentioned *An. stephensi* strains available in the GenBank.

The phylogenic tree inferred from Ace1 sequences obtained in this study in combination with the available sequences in the GenBank including *An. stephensi* strains from Chabahar, Iran (HG380320), India (CP032299) and Pakistan (XM036043798), *An. albimanus*, *An. gambiae* and *An. funestus* which has the bendiocarb resistant G119S mutation showed two main clades one with G119S mutations and one without the mutation.



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

**Table 1.** Bioassay mortality of the Beech strain, Bandar-Abbas field populations (F0-F7) of *Anopheles stephensi*, to bendiocarb 0.1%, Iran, 2021

| Population | Regression line | $LT_{50}$ (s) | $R^2$ | RR | MR (%) | Resistance Status |
|---|---|---|---|---|---|---|
| **Beech** | Y= 2.03X-0.28 | 398 | 0.96 | 1 | 99 | S |
| **Field F0** | Y= 1.71X+0.23 | 588 | 0.88 | 1.47 | 89 | R |
| **F1** | Y= 1.98X-0.74 | 794 | 0.95 | 1.99 | 87 | R |
| **F2** | Y= 1.85X-0.6 | 1044 | 0.94 | 2.62 | 78 | R |
| **F3** | Y= 2.11X-1.52 | 1213 | 0.88 | 3.04 | 75 | R |
| **F4** | Y= 2.1X-1.85 | 1460 | 0.88 | 3.66 | 71 | R |
| **F5** | Y= 1.82X-0.98 | 1911 | 0.95 | 4.8 | 64 | R |
| **F6** | Y= 1.73X-0.82 | 2280 | 0.98 | 5.72 | 61 | R |
| **F7** | Y= 1.94X-1.74 | 2767 | 0.95 | 6.95 | 63 | R |

s: Second, MR: Mortality Rate, RR: Resistance Ratio, S: Susceptible, R: Resistance.

**Table 2.** Descriptive results of the biochemical assays in three strains, Beech, G7 (7th generation) and Resistant of *Anopheles stephensi*, Iran, 2021

| Enzyme | Strain | Beech | G7 | Resistant |
|---|---|---|---|---|
| | **Number of tested mosquitoes** | **90** | **64** | **64** |
| **MFO** | Mean ± SD | 4.219e-005 ± 7.863e-005 | 2.189e-005 ± 1.569e-005 | 4.088e-005 ± 1.166e-005 |
| | Enzyme Ratio | 1 | 0.5 | 0.9 |
| **α-esterase** | Mean ± SD | 2.359e-005 ± 6.425e-005 | 0.0008759 ± 0.0006302 | 0.0001235 ± 0.0001134 |
| | Enzyme Ratio | 1 | 3.7 | 5.2 |
| **β-esterase** | Mean ± SD | 5.918e-005 ± 7.594e-005 | 8.147e-005 ± 7.254e-005 | 0.0004798 ± 0.0002745 |
| | Enzyme Ratio | 1 | 1.3 | 8.1 |

**Table 3.** Mann Whitney non-parametric test on the biochemical assay results between three strains of *Anopheles stephensi*, Iran 2021

| Enzyme Activity | MFO | | ACE | | α-esterase | | β-esterase | |
|---|---|---|---|---|---|---|---|---|
| **Strains** | **P value** | **Significant (P< 0.05)** | **P value** | **Significant (P< 0.05)** | **P value** | **Significant (P< 0.05)** | **P value** | **Significant (P< 0.05)** |
| **Beech and G7** | 0.0007 | Yes *** | 0.012 | No | <0.0001 | Yes **** | 0.0079 | Yes ** |
| **Beech and Resistant** | <0.0001 | Yes **** | 0.0006 | Yes *** | <0.0001 | Yes **** | <0.0001 | Yes **** |
| **G7 and Resistant** | <0.0001 | Yes **** | 0.0279 | Yes * | 0.3 | No | <0.0001 | Yes **** |

**Table 4.** Inhibition of AchE activity by propoxur in the three strains of *Anopheles stephensi*, Iran, 2021

| Strain | Total samples | No. inhibition less than 60% | Percent. inhibition less than 60% | No. inhibition upper than 60% | Percent. inhibition upper than 60% |
|---|---|---|---|---|---|
| **Beech** | 90 | 28 | 31% | 62 | 69% |
| **G7** | 64 | 22 | 34% | 42 | 66% |
| **Resistant** | 64 | 28 | 43% | 36 | 57% |



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

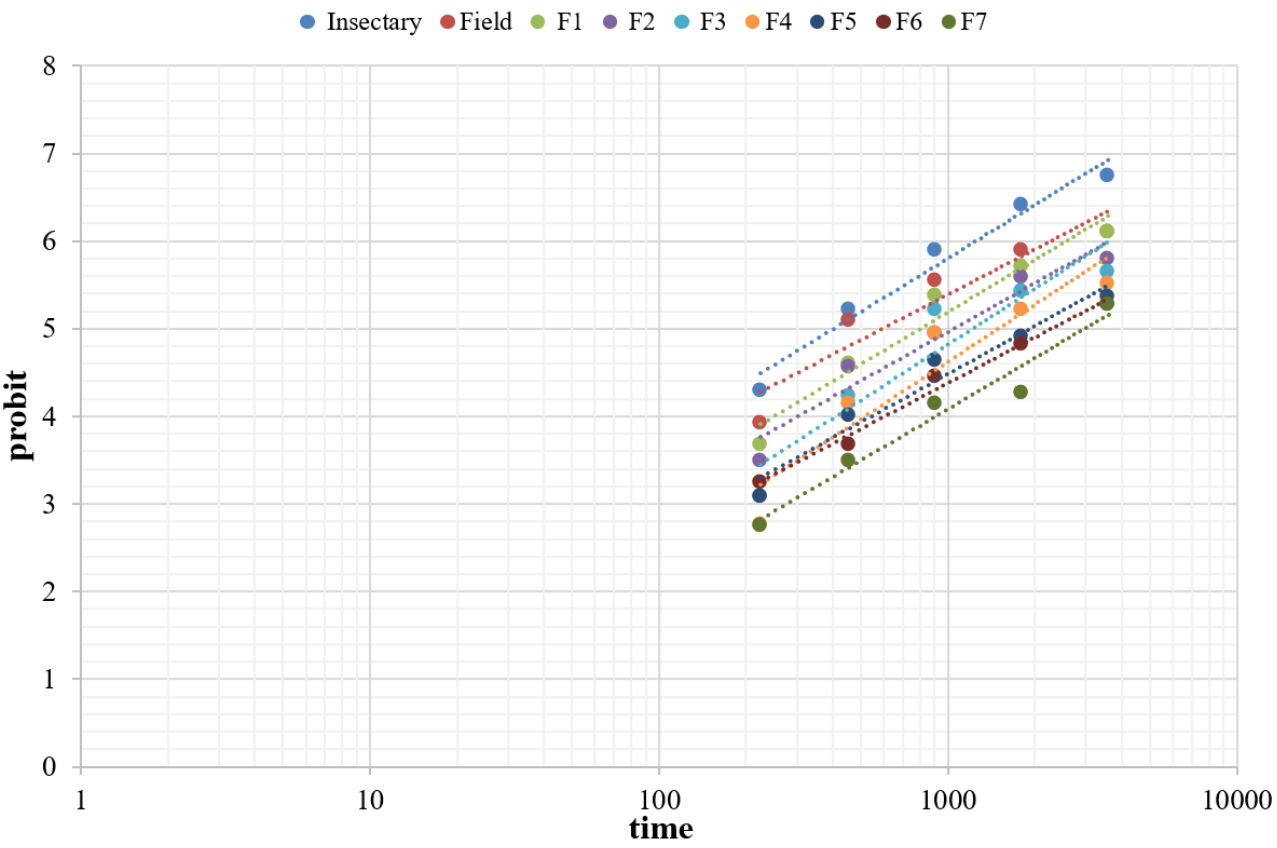


**Fig. 1.** Regression lines of *Anopheles stephensi* populations exposed to bendiocarb 0.1% (time: second), Iran, 2021

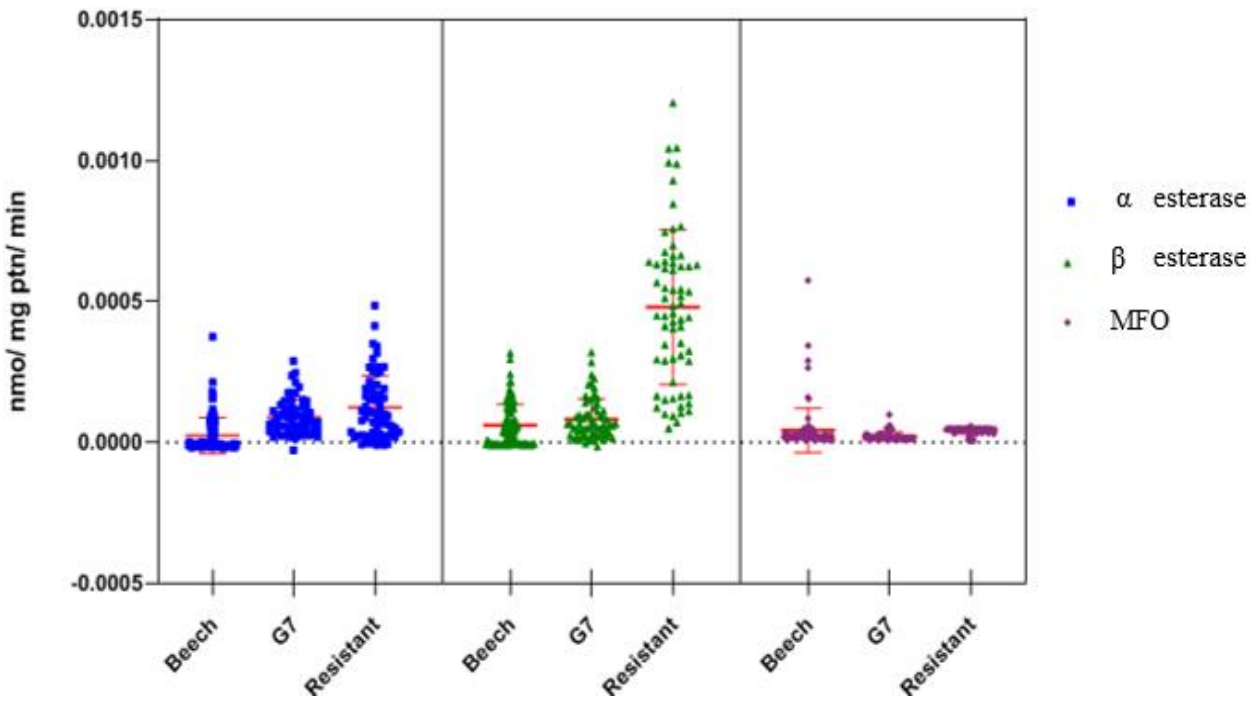


**Fig. 2.** Mean enzyme activities of α and β esterase and Mixed Function Oxidase measured in *Anopheles stephensi* strains from Iran, 2021, (G7: seventh generation)



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

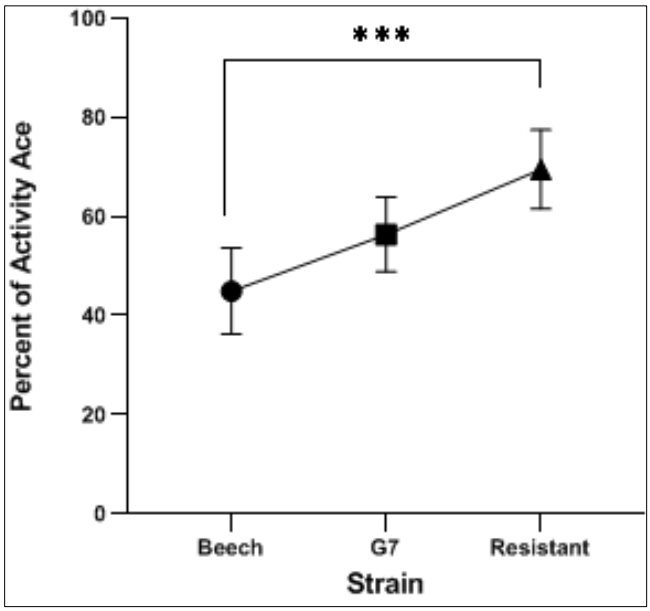


**Fig. 3.** Percent remaining activity Acetylcholinesterase in three strains of *Anopheles stephensi* from Iran, 2021, (G7: seventh generation)

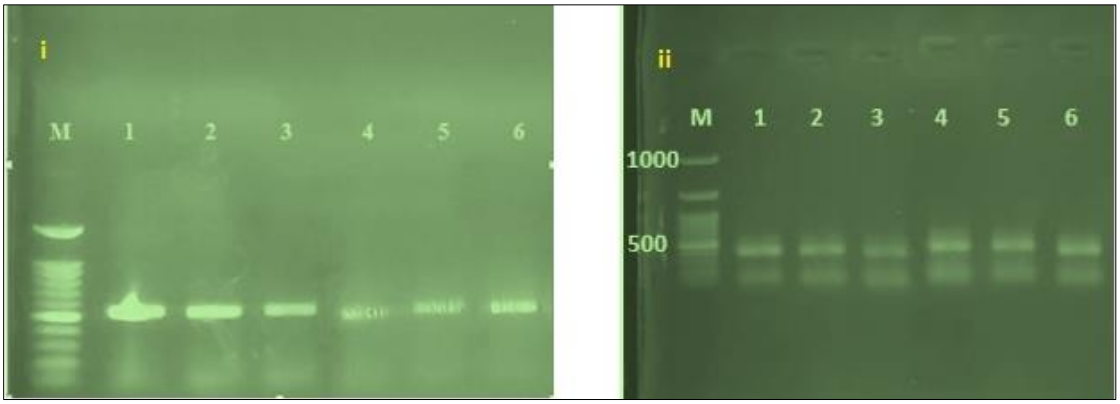


**Fig. 4.** Investigative PCR (i) and PCR-RFLP (ii) to detect G119S mutation in the amplified region of ACE-1 (492 bp) in six strains of *Anopheles stephensi* Iran, 2021. M: 100 bp ladder, 1: bendiocarb-resistant strain, 2: 7$^{th}$ generation of selected strain with bendiocarb, 3: Beech strain, 4: Bandar-Abbas strain, 5: Kerman strain, 6: Sistan and Baluchestan strain

## Discussion

The resistance of *An. stephensi* in southern parts of Iran to bendiocarb raises concerns regarding the control methods of *An. stephensi* based on the use of insecticides in this area, because the use of this insecticide is a recommended alternative to PYs (19, 28). As in the present study, *An. stephensi* was already shown to be resistant to bendiocarb in Hormoodar of Bandar-Abbas, Kerman and Sistan and BaluchIstan in Iran with a mortality rate of 86% (3). However there is an old report of more than 10 years ago that this vector was 100% susceptible to bendiocarb in Hormozgan Province of Iran (29).

Resistance of this species to other insecticides including DDT in Iran (9, 18), Malathion, Deltamethrin and Permethrin in Afghanistan (30–32) has been reported and a similar study has shown resistance to bendiocarb in Somali


http://jad.tums.ac.ir
Published Online: Sep 30, 2023

region (4). In addition to *An. stephensi* in its distribution regions, resistance to bendiocarb has been reported in other Culicidae family such as *An. gambiae* in Benin (22, 33), Nigeria (34, 35) and Tanzania (36), *An. funestus* in Zambia (37, 38) and *An. arabiensis* in Ethiopia (39).

In this study α and β esterase content in the resistant population of *An. stephensi* was increasingly involved in resistance. Values of 1.85 and 2.18 compared to the sensitive strain were recorded for α and β esterase enzymes, respectively. Esterases also confer resistance to OP and cross resistance to pyrethroids (40). The role of esterases in resistance to insecticides in *An. stephensi* from other countries has been reported for example in resistance to pyrethroids and malathion from Dubai and India respectively (30, 41). Evidence of alpha and beta activity has been observed in a population of *An. sacharovi* resistant to OP and carbamates in Turkey compared to the susceptible strain (42).

The finding of this study suggests that altered AchE is present in resistant *An. stephensi*. The mean inhibition rates of AchE in the resistant samples are significantly higher than the Beech strain and confer resistance to bendiocarb because of the activity of this enzyme. The role of AchE in resistance to OP and carbamate insecticides has been already reported in other malaria vectors such as *An. stephensi* in Iran (9, 43), *An. albimanus* in Mexico (44) and *An. maculipennis* in Turkey (45). Our data was parallel to previous investigations conducted supporting the implication of metabolic mechanisms in OP and carbamate resistance such as resistance to malathion and bendiocarb in Afghanistan populations has shown (30).

Two genes Ace-1 and Ace-2 are known to encode the protein acetylcholinesterase in insects, and mutations in them are known to be responsible for resistance in many pests (46). In *Drosophila*, four points mutations (F368Y, G303A, I199V and F115S) (47) have been identified as responsible for insecticide resistance. Also, five mutations (G365Y, F327Y, G262V, G262A and V180L) in houseflies, alone or tuple, cause different levels of resistance (48). The presence of the G119S mutation is also one of the most important mutations in the Ace-1 gene reported in *An. gambiae* (49, 50), *An. coluzzi* (51), *An. arabiensis* (52), *An. albimanus* (53) and *Cx. pipiens* (54) involved in OPs and carbamate insecticide resistance. In *An. funestus*, other mutations in Ace-1, including N485I have been recorded and associated with resistance to bendiocarb in a population originating from Malawi (55). In *Ae. aegypti* two mutations in T506T locus was introduced as a new mutation responsible for resistance to Temephos in Indonesia (56). None of the mentioned mutations have been observed in the examined sequences in this study.

Duplication of Ace-1 in Africa's main malaria vector, *An. gambiae* is a challenge for malaria control (57) and the analysis of three populations in this study did not indicate presence of duplication in Ace-1. The *An. stephensi* resistance to bendiocarb in absence of Ace-1 G119S mutation in this study is like the eastern Ethiopia region (4). No G119S mutation was distinguished in this study suggesting that resistance was metabolic.

## Conclusion

Insecticide resistance in malaria vectors is a global problem, but there are areas where resistance patterns are more serious than others. The selected region in this study was south of the Iran, where malaria cases have been reported every year. In addition, the arrival of many immigrants from malaria-endemic countries causes the disease to rise in the region. The reduction of susceptibility to bendiocarb, which is the insecticide of choice in residual spray, causes concern. Monitoring the status of resistance to this insecticide in malaria vectors is important to control this disease. The finding of bendiocarb resistance in *An. stephensi* emphasizes the need for additional research in



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

other parts of Iran and the Persian Gulf Littoral. The Ministry of Health should implement applicable integrated vector control strategies for vector management which will be crucial in overcoming the spread of resistance.

## Acknowledgements

This work was financially supported by the deputy of research Tehran University of Medical Sciences (TUMS), with the project No. 99-43609, for fulfilment of a PhD degree in Medical Entomology and Vector Control from Department of Medical Entomology and Vector Control, School of Public Health, TUMS. The authors are very grateful to Mr Fekri, Mr Askani and Mr Saberi from Hormozgan University of Medical Sciences, Iranshahr University of Medical Sciences and Kerman University of Medical Sciences respectively.

## Ethical considerations

This research was approved by TUMS ethics committee with code IR.TUMS. SPH.REC. 1398.185.

## Conflict of interest statement

The authors declare there is no conflict of interests.

## References

1. Assogba BS, Djogbénou LS, Milesi P, Berthomieu A, Perez J, Ayala D, Chandre F, Makoutodé M, Labbé P, Weill M (2015) An ace-1 gene duplication resorbs the fitness cost associated with resistance in *Anopheles gambiae*, the main malaria mosquito. Sci Rep. 5: 14529.
2. Soltani A, Vatandoost H, Oshaghi MA, Maleki-Ravasan N, Enayati AA, Asgarian F (2015) Resistance mechanisms of *Anopheles stephensi* (Diptera: Culicidae) to temephos. J Arthropod Borne Dis. 9(1): 71–83.
3. Abbasi M, Hanafi-Bojd AA, Yaghoobi-Ershadi MR, Vatandoost H, Oshaghi MA, Hazratian T, Sedaghat MM, Fekri S, Safari R, Mojahedi AR, Salari Y (2019) Resistance status of main malaria vector, *Anopheles stephensi* Liston (Diptera: Culicidae) to insecticides in a malaria Endemic Area, Southern Iran. Asian Pac J Tro Med. 12(1): 43–48.
4. Yared S, Gebressielasie A, Damodaran L, Bonnell V, Lopez K, Janies D, Carter TE (2020) Insecticide resistance in *Anopheles stephensi* in Somali Region, eastern Ethiopia. Malar J. 19(1): 180.
5. Fang Y, Shi WQ, Wu JT, Li YY, Xue JB, Zhang Y (2019) Resistance to pyrethroid and organophosphate insecticides, and the geographical distribution and polymorphisms of target-site mutations in voltage-gated sodium channel and acetylcholinesterase 1 genes in *Anopheles sinensis* populations in Shanghai, China. Parasit Vectors. 12(1): 396.
6. Ahoua Alou LP, Koffi AA, Adja MA, Tia E, Kouassi PK, Koné M, Chandre F (2010) Distribution of ace-1 R and resistance to carbamates and organophosphates in *Anopheles gambiae ss* populations from Côte d'Ivoire. Malar J. 9(1): 167.
7. Antonio-Nkondjio C, Poupardin R, Tene BF, Kopya E, Costantini C, Awono-Ambene P, Wondji CS (2016) Investigation of mechanisms of bendiocarb resistance in *Anopheles gambiae* populations from the city of Yaoundé, Cameroon. Malar J. 15 (1): 424.
8. Hemingway J (2000) The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. Insect Biochem Mol Biol. 30(11): 1009–1015.
9. Enayati A, Hanafi-Bojd AA, Sedaghat MM, Zaim M, Hemingway J (2020) Evolution of insecticide resistance and its mecha-



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

nisms in *Anopheles stephensi* in the WHO Eastern Mediterranean Region. Malar J. 19(1): 258.
10. Ahmad M, Buhler C, Pignatelli P, Ranson H, Nahzat SM, Naseem M, Sabawoon MF, Siddiqi AM, Vink M (2016) Status of insecticide resistance in high-risk malaria provinces in Afghanistan. Malar J. 15: 98.
11. Casimiro S, Coleman M, Hemingway J, Sharp B (2006) Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. J Med Entomol. 43(2): 276–282.
12. Wu ZM, Chu HL, Wang G, Zhu XJ, Guo XX, Zhang YM, Xing D, Yan T, Zhao MH, Dong YD, Li CX (2016) Multiple-insecticide resistance and classic gene mutations to Japanese encephalitis vector *Culex tritaeniorhynchus* from China. J Am Mosq Control Assoc. 32(2): 144–151.
13. Munywoki DN, Kokwaro ED, Mwangangi JM, Muturi EJ, Mbogo CM (2021) Insecticide resistance status in *Anopheles gambiae* (s.l) in coastal Kenya. Parasit Vectors. 14(1): 207.
14. Opondo KO, Jawara M, Cham S, Jatta E, Jarju L, Camara M, Sanneh F, Gaye PM, Jadama L, Ceesay S, Njie E (2019) Status of insecticide resistance in *Anopheles gambiae* (s.l) of The Gambia. Parasit Vectors. 12(1): 278.
15. Vontas J, Kioulos E, Pavlidi N, Morou E, Della Torre A, Ranson H (2012) Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic Biochem Physiol. 104(2): 126–131.
16. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, Raghavendra K, Pinto J, Corbel V, David JP, Weetman D (2017) Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. PLoS Negl Trop Dis. 11(7): e0005625.
17. Safi NH, Ahmadi AA, Nahzat S, Ziapour SP, Nikookar SH, Fazeli-Dinan M, Enayati A, Hemingway J (2017) Evidence of metabolic mechanisms playing a role in multiple insecticides resistance in *Anopheles stephensi* populations from Afghanistan. Malar J. 16(1): 100.
18. Vatandoost H, Hanafi-Bojd AA (2012) Indication of pyrethroid resistance in the main malaria vector, *Anopheles stephensi* from Iran. Asian Pac J Trop Med. 5(9): 722–726.
19. World Health Organization (2012) Global plan for insecticide resistance management in malaria vectors. World Health Organization, Geneva.
20. Djogbenou L, Labbe P, Chandre F, Pasteur N, Weill M (2009) Ace-1 duplication in *Anopheles gambiae*: a challenge for malaria control. Malar J. 8: 70.
21. Weetman D, Mitchell SN, Wilding CS, Birks DP, Yawson AE, Essandoh J, Mawejje HD, Djogbenou LS, Steen K, Rippon EJ, Clarkson CS (2015) Contemporary evolution of resistance at the major insecticide target site gene Ace-1 by mutation and copy number variation in the malaria mosquito *Anopheles gambiae*. Mol Ecol. 24(11): 2656–2672.
22. Aïkpon R, Agossa F, Ossè R, Oussou O, Aïzoun N, Oké-Agbo F, Akogbéto M (2013) Bendiocarb resistance in *Anopheles gambiae* s.l. populations from Atacora department in Benin, West Africa: a threat for malaria vector control. Parasit Vectors. 6: 192.
23. Dabiré KR, Diabaté A, Namontougou M, Djogbenou L, Kengne P, Simard F, Bass C, Baldet T (2009) Distribution of insensitive acetylcholinesterase (ace-1R) in *Anopheles gambiae* s.l populations from Burkina Faso (West Africa). Trop Med Int Health. 14(4): 396–403.
24. Essandoh J, Yawson AE, Weetman D (2013) Acetylcholinesterase (Ace-1) target site mutation 119S is strongly diagnostic of carbamate and organophosphate resistance in *Anopheles gambiae ss* and *Anopheles coluzzii* across southern Ghana. Malar J.



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

12(1): 404–414.
25. Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M (2004) The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol. 13(1): 1–7.
26. Azari-Hamidian Sh, Harbach RE (2009) Keys to the adult females and fourth-instar larvae of the mosquitoes of Iran (Diptera: Culicidae). Zootaxa. 2078: 1–33.
27. World Health Organization (2016) Monitoring and managing insecticide resistance in *Aedes* mosquito populations: interim guidance for entomologists, Geneva. Available at: https://www.who.int/publications/i/item/WHO-ZIKV-VC-16.1
28. Ossè R, Aikpon R, Padonou GG, Oussou O, Yadouléton A, Akogbéto M (2012) Evaluation of the efficacy of bendiocarb in indoor residual spraying against pyrethroid resistant malaria vectors in Benin: results of the third campaign. Parasit Vectors. 5: 163.
29. Azizi K, Soltani A, Poodat A, Khodadadi M, Yaran M, Hasanvand B (2011) Susceptibility of *Anopheles stephensi* against five current chemical insecticides. Hormozgan Med J. 14(4): 305–311.
30. Safi NH, Ahmadi AA, Nahzat S, Warusavithana S, Safi N, Valadan R, Shemshadian A, Sharifi M, Enayati A, Hemingway J (2019) Status of insecticide resistance and its biochemical and molecular mechanisms in *Anopheles stephensi* (Diptera: Culicidae) from Afghanistan. Malar J. 18 (1): 249.
31. Vatandoost H, Hanafi-Bojd AA, Nikpoor F, Raeisi A, Abai MR, Zaim M (2022) Situation of insecticide resistance in malaria vectors in the World Health Organization of Eastern Mediterranean region 1990–2020. Toxicol Res (Camb). 11(1): 1–21.
32. Susanna D, Pratiwi D (2021) Current status of insecticide resistance in malaria vectors in the Asian countries: a systematic review. F1000Res. 10: 200.
33. Kpanou CD, Sagbohan HW, Padonou GG, Ossè R, Salako AS, Fassinou AJ, Sovi A, Yovogan B, Adoha C, Sominahouin A, Ahogni I (2021) Susceptibility of *Anopheles gambiae sensu lato* to different insecticide classes and mechanisms involved in the South-North transect of Benin. J Entomol. 11(2): 25–37.
34. Olatunbosun-Oduola A, Abba E, Adelaja O, Taiwo-Ande A, Poloma-Yoriyo K, Samson-Awolola T (2019) Widespread report of multiple insecticide resistance in *Anopheles gambiae sl* mosquitoes in eight communities in southern Gombe, North-Eastern Nigeria. J Arthropod Borne Dis. 13(1): 50–61.
35. Wahedi J, Ande A, Oduola A, Obembe A (2021) Bendiocarb resistance and, kdr associated deltamethrin and DDT resistance in *Anopheles gambiae* s.l populations from North Eastern Adamawa State, Nigeria. Ceyl J Sci. 50(1): 63–73.
36. Matiya DJ, Philbert AB, Kidima W, Matowo JJ (2019) Dynamics and monitoring of insecticide resistance in malaria vectors across mainland Tanzania from 1997 to 2017: a systematic review. Malar J. 18(1): 102.
37. Choi KS, Christian R, Nardini L, Wood OR, Agubuzo E, Muleba M, Munyati S, Makuwaza A, Koekemoer LL, Brooke BD, Hunt RH (2014) Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe. Parasit Vectors. 7: 464.
38. Chanda J, Saili K, Phiri F, Stevenson JC, Mwenda M, Chishimba S, Mulube C, Mambwe B, Lungu C, Earle D, Bennett A (2020) Pyrethroid and carbamate resistance in *Anopheles funestus* Giles along Lake Kariba in southern Zambia. Am J Trop Med Hyg. 103(2): 90–97.
39. Mekuriaw W, Yewhalaw D, Dugassa S,

Taffese H, Bashaye S, Nigatu W, Massebo F (2020) Distribution and trends of insecticide resistance in malaria vectors in Ethiopia (1986-2017): a review. Ethiop J Pub Heal Nut. 3(Special): 51–61.
40. Gorouhi MA, Oshaghi MA, Vatandoost H, Enayati AA, Raeisi A, Abai MR, Salim-Abadie Y, Hanafi-Bojd AA, Paksa A, Nikpoor F (2018) Biochemical basis of cyfluthrin and DDT resistance in *Anopheles stephensi* (Diptera: Culicidae) in malarious area of Iran. J Arthropod Borne Dis. 12(3): 310–320.
41. Singh OP, Dykes CL, Lather M, Agrawal OP, Adak T (2011) Knockdown resistance (kdr)-like mutations in the voltage-gated sodium channel of a malaria vector *Anopheles stephensi* and PCR assays for their detection. Malar J. 10(1): 59.
42. Yavaşoglu Sİ, Yaylagül EÖ, Akıner MM, Ülger C, Çağlar SS, Şimşek FM (2019) Current insecticide resistance status in *Anopheles sacharovi* and *Anopheles superpictus* populations in former malaria endemic areas of Turkey. Acta Trop. 193: 148–157.
43. Nejati J, Moosa-Kazemi SH, Oshaghi MA, Badzohre A, Pirmohammadi M, Saeidi Z, Naseri-Karimi N, Parkhideh SZ, Vatandoost H (2021) Insecticide Resistance Status of Malaria Vectors in a Malarious Area, Southeast of Iran. J Arthropod Borne Dis. 15(3): 278–286.
44. Dzul FA, Patricia Penilla R, Rodríguez AD (2007) Susceptibility and inseticide resistance mechanisms in *Anopheles albimanus* from the southern Yucatan Peninsula, Mexico. Salud Publica Mex. 49(4): 302–311.
45. Akiner MM (2014) Malathion and propoxur resistance in Turkish populations of the *Anopheles maculipennis* Meigen (Diptera: Culicidae) and relation to the insensitive acetylcholinesterase. Turkiye Parazitol Derg. 38(2): 111–115.
46. Fournier D (2005) Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. Chem Biol Interact. 157: 257–261.
47. Mutero A, Pralavorio M, Bride JM, Fournier D (1994) Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. Proc Natl Acad Sci U S A. 91(13): 5922–5926.
48. Guo D, Luo J, Zhou Y, Xiao H, He K, Yin C, Xu J, Li F (2017) ACE: an efficient and sensitive tool to detect insecticide resistance-associated mutations in insect acetylcholinesterase from RNA-Seq data. BMC bioinformatics. 18(1): 330.
49. Binyang AJ, Elanga-Ndille E, Tene-Fossog B, Ndo C, Nouage L, Assatse T, Fotso-Toguem Y, Tabue R, Zeukeng F, Nguiffo DN, Etang J (2022) Distribution of acetylcholinesterase (Ace-1R) target-site G 119S mutation and resistance to carbamates and organophosphates in *Anopheles gambiae sensu lato* populations from Cameroon. Parasit Vectors. 15(1): 53.
50. Keïta M, Kané F, Thiero O, Traoré B, Zeukeng F, Sodio AB, Traoré SF, Djouaka R, Doumbia S, Sogoba N (2020) Acetylcholinesterase (ace-1R) target site mutation G119S and resistance to carbamates in *Anopheles gambiae* (*sensu lato*) populations from Mali. Parasit Vectors. 13(1): 283.
51. Chabi J, Baidoo PK, Datsomor AK, Okyere D, Ablorde A, Iddrisu A, Wilson MD, Dadzie SK, Jamet HP, Diclaro JW (2016) Insecticide susceptibility of natural populations of *Anopheles coluzzii* and *Anopheles gambiae* (*sensu stricto*) from Okyereko irrigation site, Ghana, West Africa. Parasit Vectors. 9: 182.
52. Sy O, Sarr P, Assogba B, Ndiaye M, Dia A, Ndiaye A (2021) Detection of Kdr and Ace-1 mutations in wild populations of *Anopheles arabiensis* and *An. melas* in a residual malaria transmission area of Senegal. Pestic Biochem Physiol. 173: 104783.
53. Liebman KA, Pinto J, Valle J, Palomino M,



http://jad.tums.ac.ir
Published Online: Sep 30, 2023

Vizcaino L, Brogdon W, Lenhart A (2015) Novel mutations on the ace-1 gene of the malaria vector *Anopheles albimanus* provide evidence for balancing selection in an area of high insecticide resistance in Peru. Malar J. 14: 74.

54. Tmimi F-Z, Faraj C, Bkhache M, Mounaji K, Failloux AB, Sarih Mh (2018) Insecticide resistance and target site mutations (G119S ace-1 and L1014F kdr) of *Culex pipiens* in Morocco. Parasit Vectors. 11(1): 51.
55. Ibrahim SS, Ndula M, Riveron JM, Irving H, Wondji CS (2016) The P450 CYP 6Z1 confers carbamate/pyrethroid cross-resistance in a major African malaria vector beside a novel carbamate-insensitive N485I acetylcholinesterase-1 mutation. Mol Ecol. 25(14): 3436–3452.
56. Hasmiwati H, Rusjdi SR, Nofita E (2018) Detection of Ace-1 gene with insecticides resistance in *Aedes aegypti* populations from DHF-endemic areas in Padang, Indonesia. J Biolo Divers. 19(1): 31–36.
57. Elanga-Ndille E, Nouage L, Ndo C, Binyang A, Assatse T, Nguiffo-Nguete D, Djonabaye D, Irving H, Tene-Fossog B, Wondji CS (2019) The G119S acetylcholinesterase (Ace-1) target site mutation confers carbamate resistance in the major malaria vector *Anopheles gambiae* from Cameroon: a challenge for the coming IRS Implementation. Genes. 10(10): 790.



http://jad.tums.ac.ir
Published Online: Sep 30, 2023