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Resistance profile *of Aedes aegypti* (L.), towards commonly used insecticides in Jeddah Governorate, Kingdom of Saudi Arabia

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Abstract
Dengue fever is prevalent in western Saudi Arabia since 1990. Dengue fever vector Aedes mosquito is mostly control by different chemical insecticides Aedes vector developed resistance to different insecticides as reported from different parts of the world. The effectiveness of vector control is reduced. Little information is available in the study area about the insect resistance to Aedes mosquitoes which are vector for Dengue fever virus. Present research work was taken to study the presence and degree of resistance to commonly used insecticides in Saudi Arabia to control the vectors and to understand the mechanism of physiological resistance to adult mosquitoes *Aedes aegypti*.
WHO recommended tube assay method were applied to observe mortality and to investigate the insecticide resistance in Aedes mosquitoes. WHO resistance bioassay of mosquitoes with Deltamethrin (0.05%), Malathion (5%), Bendiocarb (1.0%) and Permethrin (0.75%), was conducted. To detect the target site mutation Polymerase chain reaction (PCR) technique was used.
Present study indicated that the exposure of adult Aedes female mosquito to the diagnostic doses Permethrin (0.75%), Deltamethrin (0.05%), Malathion (5%), and Bendiocarb (1.0%) caused 11,49,61.99 and 23% mortality respectively show resistance to these insecticides.
Multiple knocks down resistance (Kdr) Mutations are found in local population of *Aedes aegypti* in Jeddah region associated with Pyrethroid resistance. DNA sequencing for Voltage gated sodium channel (VGSC) was done on *Aedes aegypti* collected from different points of Jeddah for the presence of Kdr mutations. The present study revealed mutations at codon 989, 1016 and 1534.
These findings showed that the *Aedes aegypti* population has developed resistance against Pyrethroid insecticides. There is an urgent need to find better solution for the mosquito control.

Keywords: *Ae. Aegypti*, bendiocarb, deltamethrin, KDR mutations, Malathion, PCR and permethrin,

Introduction
Dengue fever is also an arbovirus disease which is vector borne like Chikungunya, Zika and Yellow fever, spreading all over the world and becoming a public health problem. There have been reports of endemic break of Dengue fever from different parts of the world including Arabian Peninsula. First recorded dengue fever case from Saudi Arabia was reported in 1994 (Fakeeh & Zaki, 2003) [13]. Primary strategy for controlling vectors of different viral diseases was spraying chemical insecticides. The field controlling authorities relied heavily on using organophosphates and pyrethroids insecticides to control disease vectors resulting into insecticide resistances. The control program received a set back and new chemicals are being investigated. The use of pyrethroid was reported from different parts of Saudi Arabia including Jazan, Al Quoz, Jeddah, Makkah and Madina resulting the development of mosquito resistance (Al Ghamdi & Mahyoob, 2022) [5].
The survival of insects with insecticide resistance is an evolutionary phenomenon. The altered metabolic pathways, target site insensitivity, and reduced insecticide penetration are some of the physiological mechanisms involved in the resistance. Other factors for such resistance in mosquitoes are behavioral physiological changes, genetics and ecology (Zhu *et al*., 2016) [40].

Due to the short life of mosquitoes there are rapid changes in genetic makeup over multiple generations. Lui (2015) [22] suggested that mosquito can quickly develop resistance to chemical insecticides due to their repeated used against them.
The resistance to Aedes mosquitoes to different chemical insecticides was first reported in 1949 (Brown, 1986) [7] when Aedes species in Florida showed resistance to DDT. Recently many chemicals including Cyclodiene, Organophosphates, Carbamates, Pyrethroids and IGRs (insect growth regulators). (Karunaratne *et al*., 2018) [19] are found resistant to mosquitoes.
Insecticides are documented to show resistance in mosquitoes
It is essential to understand the different factors involved in resistance to develop effective mosquito control strategies and to reduce mosquito borne diseases.
Knockdown resistance (kdr) is a completely recessive trait which is commonly used for the detection of Voltage-gated channel (VGSC) gene mutations and target site resistance in *Aedes aegypti* to Pyrethroids (Chang and Huang, 2012) [9]. Gene mutations at codons 1016 and 1534 occur in Ae. aegypti at the pyrethroid receptor sites in Domains II (S6) and III (S6) of the protein molecules (Du, *et al*., 2013) [11] of Voltage-sensitive sodium channel (VSSC). S989P is a third mutation which does not reduce the sensitivity of the sodium channel (Du, *et al*., 2013) [11], but confers some additive pyrethroid resistance in the recessive homozygous state in combination with 1016G (Wuliandari J *et al*., 2015) [38]. Gene mutations involved in Pyrethroid resistance and (VSSC) have been identified in Ae. aegypti from the Kingdom of Saudi Arabia (Dafalla *et al*., 2019; Alqahtani *et al*. 2022 [10, 4].

Materials and Methods

Jeddah Governate, being a gateway to Makkah, located in the middle of the Red Sea coast and is the major urban center of the western part of the country. Being a coastal city Jeddah suffers high humidity.

Sample collection

Black Hole Light Traps were used to collect adult mosquitoes in various habitats of the city. Main sites for collection of mosquito larvae were: water storage tanks and drums, air conditioners and disposed small cans and car tires. Samples of ten water dips were taken from water storage tanks and drums by using a scoop hand of 350 ml in volume following Service (1980) to collect *A. aegypti* Larvae.
The collected samples were brought to the Entomological Exploration Department laboratory for counting and identification of different species. The identified mosquito specimens were sorted to males and females.

Rearing of mosquitoes

The larvae were then reared until emergence into complete insect fed with a special nutritional environment for mosquitoes, until they reach the virgin stage. The virgins were then collected daily using a plastic pipette and placed in plastic boxes with water inside a wire cage till hatching. Three days after the complete emergence of the insects; females were given a blood meal from a pigeon, so that they can lay eggs. Also, males and females were fed with a 10% sugar solution. The resulting groups of eggs were collected to repeat the breeding process (Pritam and Moore, 1985) [27].

Sensitivity of the Mosquitoes to the Pesticide: The resistance pattern against the four pesticides Bendiocarb (0.1%), Permethrin (0.75%), Deltamethrin (0.05%), and Malathion (5%), Bottle bioassays were conducted to assess the sensitivity of the field mosquito strains to pesticides. One hundred and twenty-five mosquitoes which have been identified, were divided equally among five test tubes: one control and the other four for testing different insecticide concentrations. Mosquitoes were exposed to insecticides for 1 hour, followed by a 24-hour recovery period in insecticide-free tubes. To estimate the sensitivity of field strains, the concentration causing 100% mortality in sensitive strains within one hour was determined. Mortality rates were calculated using the Abbott equation (1925) [1]. This method follows the guidelines outlined by WHO (1975) [37]. All experiments were performed under standardized conditions to determine lethal concentration Lc50 and Lc99. Analysis, which relies on converting death percentages into probability values using SAS (1990).

Statistical Design and Analysis

This study employed a completely randomized design (CRD). The analysis of variance (ANOVA) was used to determine if there were significant differences among the treatment groups. A post-hoc test, the Least Significant Difference (LSD), was applied at a significance level of 0.05 to identify specific treatment groups that differed significantly. Statistical analyses were conducted using SAS software, version 9.3. SAS is a widely used statistical software package capable of efficiently performing ANOVA and LSD calculations.
To account for the natural mortality rate in the control group, the percent mortality data were corrected using Abbott's formula. This correction is particularly valuable when dealing with survival data.

Molecular Study

Ae desaegypti voltage-gated channel (VGSC) gene was amplified using single-step polymerase chain reaction (PCR) with specific primer pairs (Untergasser *et al*., 2012) [36] for detecting Kdr mutations.

DNA Extraction

Mosquitoes were chosen based on their resistance to the selected Pesticides (Permethrin, Bendiocarb, Deltamethrin, and Malathion). Genomic DNA was extracted from 30 females *Ae desaegypti* from different collection points using QIAGEN DNA Extraction Kits following manufacturer instructions.

Measuring DNA Concentration and Purity

DNA concentration and purity were measured using a Nano drop Spectrophotometer (ND1000 Spectrophotometer, Nano drop Technologies, Inc.). The DNA concentration was measured as ng /µl. The purity (indication of the quality) was calculated as the ratio of absorbance reading at wavelength 260 and 280 nm. Also DNA samples were then run in 1%agarose gel, observed under ultraviolet light (UV) to check the quality of the DNA.

Polymerase Chain Reaction (PCR): To amplify (*VGSC*) gene specific primer pairs (Untergasser *et al*., 2012) [36] for detection of Kdr mutations were used. F: 5′ ATC GCT TCC CGG ACA AAG AC 3′ and R:3′GTT GGC GAT GTT CGA CTT GA 5′. The PCR reaction mixture consisted of 25 µl

containing 5 µl template DNA; 50 mMKCl; 10 mMtris-HCl, PH 8.3; 1.5 mM Mgcl2; 200 mMdNTP; 1 U taq polymerase and a primer pairs 2µl each. The reaction was performed in thermocycler machine (BioerPCR molecular) with 5 minutes at 95 °C for initial denaturation, followed by 40 cycles with: 30s at 95 °C for denaturation, 30s at 58 °C for annealing, and 30s at 72 °C for elongation and eventually 72 °C for final extension. Electrophoresis of 5 µl aliquots of the PCR product was run in 1.5% Agarose gels. The use of 1.5% Agarose gels helps to visualize the amplified products, under UV light.

Samples Sequencing

The most successful sharp bands (Twenty) PCR products were selected for sequencing to identify Kdr mutations in the *VGSC* gene responsible for resistance. Sequencing was carried at Macrgen Co. - South Korea).

Sequence analysis: Analysis of the obtained molecular sequence data was performed using a collection of bioinformatics tools, including FinichTv for assembly, BLAST for sequence similarity searches, BioEdit for sequence editing and analysis, ORF Finder for identifying open reading frames of protein, MEGA7 for phylogenetic analysis, and ProjectHope for downstream functional annotations. Banklit software for sequence submission.

Results

Results of targeted mosquito sensitivity measurement

The sensitivity of mosquitoes to insecticides was measured according to the World Health Organization (WHO) standard criteria.

Diagnostic dosages were determined for each tested insecticide, representing the concentration that kills 99% of susceptible mosquitoes within 24 hours; results were shown in table (1).

Table 1: Evaluation of some insecticide efficacy against *Aedesaegypti*in Jeddah

Mosquito insecticide	D.D[a]	Mortality (%) (Mean ± Se) [b]	Susceptibility Status
Permethrin	0.75%	11[d]±0.01	Resistant strain
Deltamethrin	0.05%	49[b]±0.25	Resistant strain
Malathion	5%	61.99[a]±0.05	Resistant strain
Bendiocarb	1.0%	23[c]±0.75	Resistant strain
LSD	5.67		

According to the method, if the death rate ranges from 98-100%, then mosquitoes are sensitive to the insecticides recommended to be used. If the death rate ranges from 85 to 98% then the mosquitoes are regarded as less sensitive. If the death rates are less than 85%, the mosquitoes are regarded as showing resistance. The effectiveness rate of Permethrin repellent was only 11%; The efficacy rate of Bendiocarb was only 23%; Efficacy rate of the used deltamethrin was 49% only and the efficacy rate of the Malathion insecticide was recorded only 61%. (Table-1).

Molecular Results

DNA Quality of *Aedes aegypti*

The mean DNA quantity was 30-120 ng/µl and DNA quality was 1.5-2.3 for a single *Aedes aegypti* sample.

PCR results for *VGSC* gene

Gel images showing the results of amplification of *VGSC* gene using specific primers detecting the Kdr mutations with a ~ 437bp band size were shown in Fig 1.

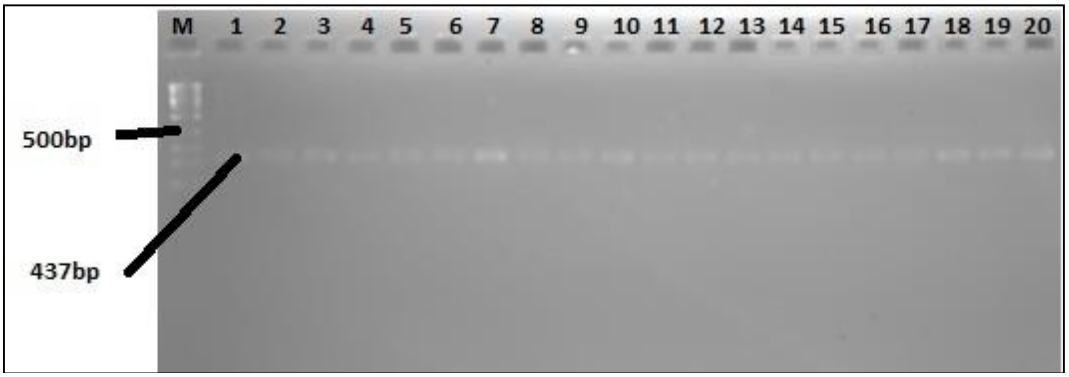

Fig 1: Agrose gel for *VGSC* gene, Lane M, 500bp Molecular ladder, lane 1-20 Positive*VGSC* gene ~ 437bp

Target DNA Sequence Analysis

Analysis of the sequence was conducted using the following software:

1. **FinichTv:** Software for trimming the unclean ends according to the their scores for assembly.
2. **Basic Local Aliment Search Tool (BLAST):** The clean sequences were blasted separately to the NCBI for identification. All sequences were identified as *VGSC* gene for *Ae. aegypti*. Results were illustrated in table (2). The 20 isolates of *Aedes aegypti* were found to be genetically similar to that retrieved from the GenBank with different identities.

Table 2: Comparison of isolates with those retrieved from the GenBank

No.	Accession no.	Identity	Country
1.	LC605642.1	100%	Laos(Asia)
2.	KY626180.1	100%	Saudi Arabia
3.	KJ957885.1	99.75%	Indonesia
4.	MT237429.1	100%	Malaysia
5.	MK005580.1	99.61%	Malaysia
6.	KM677310.1	99.60%	India
7.	KM677318.1	99.60%	India
8.	MT237424.1	100.00%	Malaysia
9.	LC557556.	99.21%	Brazil
10.	MK977840.1	99.21%	USA
11.	MN997335.1	100%	Saudi Arabia

Results of Multiple Sequence Alignment (MSA)

Highly similar sequences retrieved from NCBI were subjected to multiple sequence alignment using BioEdit version 7.0.9.0 software using (ClusalW) to find the homology between the sequences; results are illustrated in Figure 2. The nucleotide sequences were converted to amino acids using the online Open Reading Frame (ORF) finder software. Comparing the protein sequences of the study isolates to the reference sequence from the NCBI was also done using BioEdit version 7.0.9.0 software. Results were shown in Figure 3.

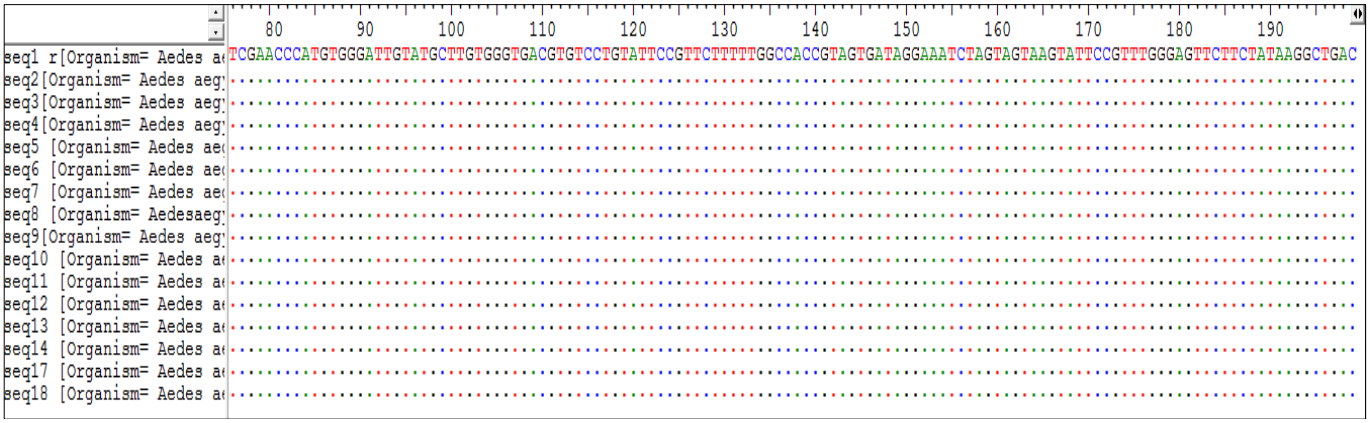

Fig 2: Alignment of 20 isolates using BioEdit (nucleotides) showing 100% identity of the isolates

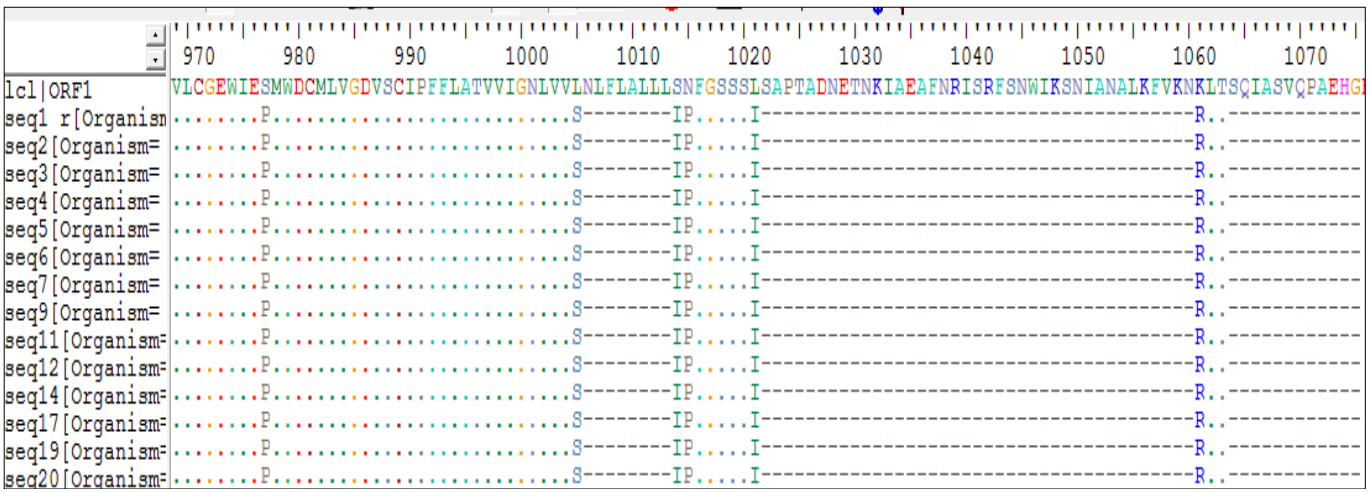

Fig 3: Alignment of 20 isolates using BioEdit (amino acids), showing the differences between the reference sequence and the study isolates (1-20)

MEGA7 for Phylogenetic trees

The multiple sequence alignment of the isolates was done using ClustalW in MEGA7 software. Aligned sequences were analyzed by Neighbor-joining to infer evolutionary relationships among the *Aedes aegypti*. Results were shown in Figure 4.

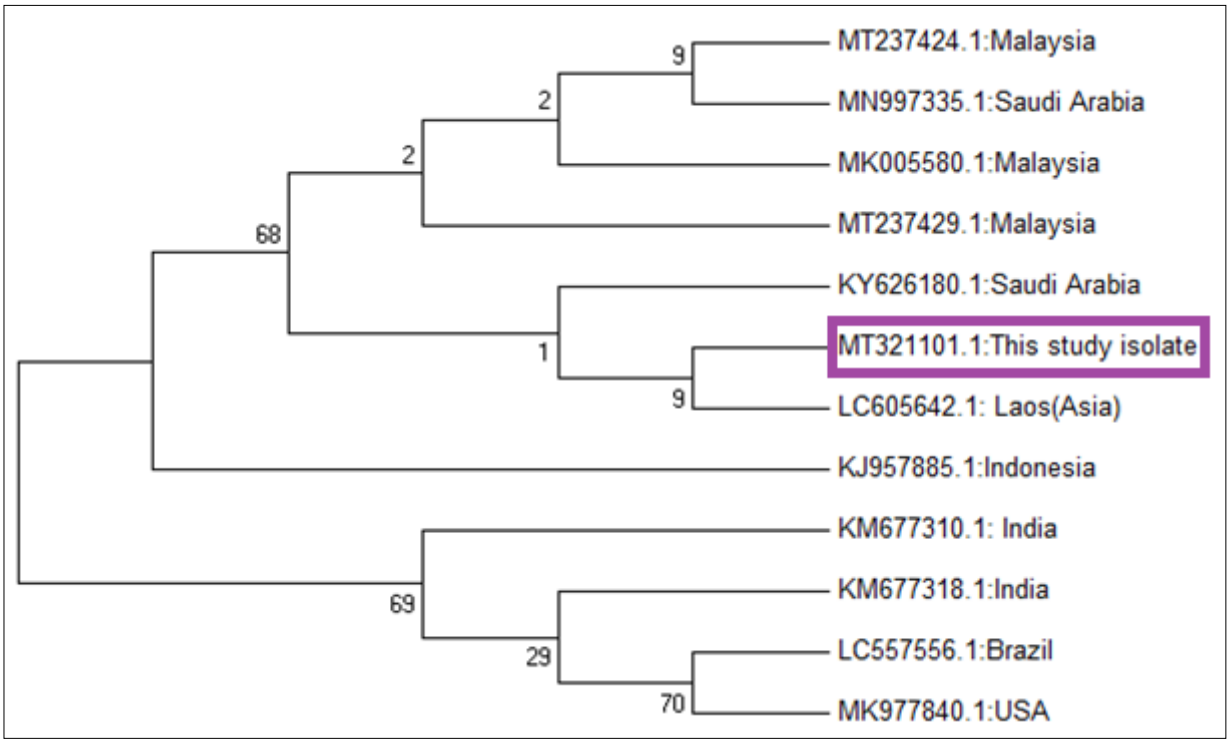

Fig 4: Neighbor-joining tree (with 500 bootstrapping) based on genetic distance analysis of *VGSC* gene sequences showing the genetic relationships of *Aedes aegypti*

Chromatogram curve of *VGSC* gene sequences from several *Aedes aegypti* populations for Detection of the mutation in the *VGSC* gene

Mutation sites in the domain II of *VGSC* gene sequence were checked by MSA. There are four important sites in this sequence, namely bases number 70, 137, 146 and 401. These sites represent codon 989, 1011, 1014, and codon 1016. The base substitution of codon 989 is known by TCC to CCC which likewise changing serine to proline. The substitutional of codon 1016 bases are GTA to GGA which mutates valine to Glycine. Figure 5 (A) and Figure 5 (B) demonstrate the Chromatogram curve of *VGSC* gene sequences from several *Aedes aegypti* populations. A change of base sequence from TCC to CCC corresponding to S989P mutation and the Chromatogram curve of *VGSC* gene sequences from several *Aedes aegypti* populations. A change of base sequence from GTA to GGA corresponding to V1016G mutation respectively.

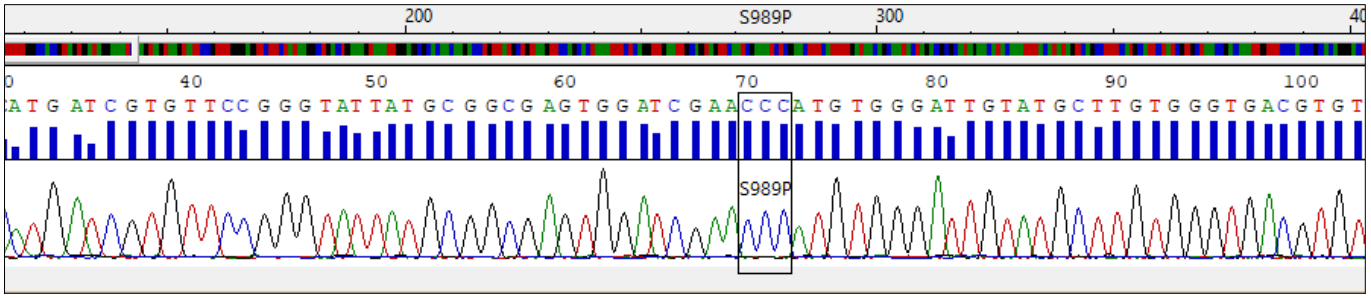

Fig 5(A): Chromatogram curve of *VGSC* gene sequences from several *Aedes aegypti* populations. A change of base sequence from TCC to CCC corresponding to S989P mutation

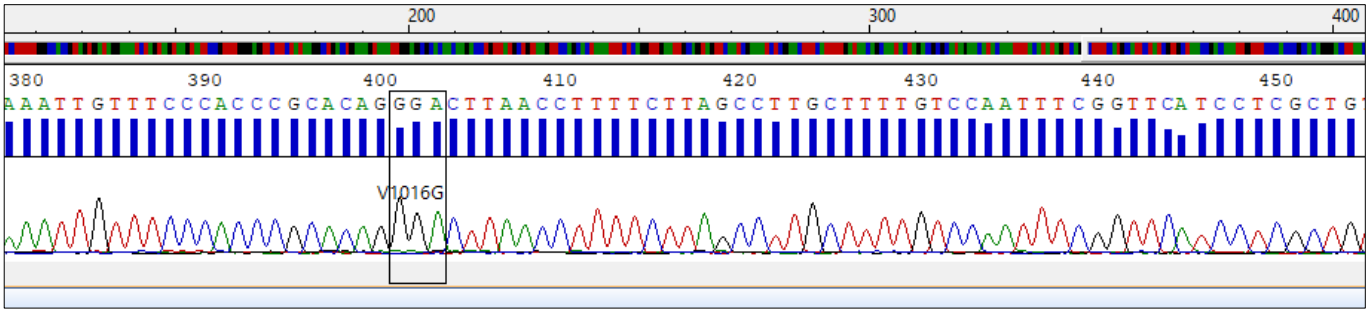

Fig 5(B): Chromatogram curve of *VGSC* gene sequences from several *Aedes aegypti* populations. A change of base sequence from GTA to GGA corresponding to V1016G mutation

Detection of the effect of the mutations on the *VGSC* protein function using ProjectHope software

For confirmation of the results one of the detected mutations (Serine to Proline at position 989) were checked using Project Hope software. The results showed that the mutation was highly effective for the function of the protein demonstrating a strong correlation between the presence of the mutation and the resistance to pyrethroids. The results shown in Figure 6 where Serine is replaced by Proline, there are significant changes in the protein's properties. The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is colored red. The side chain, unique for each amino acid, is colored black.

Fig 6: Serine to Proline at position 989.

The mutant residue is bigger than the wild-type residue. The mutant residue is more hydrophobic than the wild-type residue. The wild-type residue is much conserved, but a few other residue types have been observed at this position too.

Sequence submission to NCBI using Banklit software

One haplotype was submitted to GenBank using Banklit software. The haplotype was designated accession number by GenBank as MT321101.

Discussion

The climatic conditions that prevail in Jeddah city make it an ideal environment for the multiplication and widespread distribution of mosquitoes. Humid conditions, suitable temperatures, building constructions, overflowing sewers, and uncovered domestic water storage tanks constitute favorable foci for breeding of mosquitoes. All these conditions play a very important role in spreading dengue fever in Jeddah (Aburas, 2007; Fakeeh and` Zaki 2001) [2, 12]. Although there is a scarcity of natural fresh water sources, however, the storage methods and development of water resources and urbanization provide breeding sites for mosquitoes which are the potential vectors for many arbo-virus diseases. The poor sewage system in the country, which is in the developing stage, provides an ideal breeding ground for different mosquito species. Manmade water storage tanks and receptacles are the ideal places for the breeding of fresh water species.

Resistance is a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species. Cross-resistance occurs when resistance to one insecticide confers resistance to another insecticide, even where the insect has not been exposed to the latter product. Clearly, because pest insect populations are usually large in size and they breed quickly, there is always a risk that insecticide resistance may evolve, especially when insecticides are misused or over-used. (Subramanyam, and Hagstrum, 2018) [34]. Many of the most dangerous human diseases are transmitted by insect vectors. After decades of repeated insecticide use, all of these vector species have demonstrated the capacity to evolve resistance to insecticides. Insecticide resistance is generally considered to undermine control of vector-transmitted diseases because it increases the number of vectors that survive the insecticide treatment. Disease control failure, however, need not follow from vector control failure. There are different resistance mechanisms such as metabolic resistance or modification in the behavior resistance, but this study deals with resistance by chemical insecticides caused by modification, which modifies the insect molecules and suppress its effects (Hafeez *et al.*, 2021) [15]. Pyrethroid insecticides are supposed to be less harmful to the environment and animals other than insects, but act very fast on the central nervous system of insects like DDT (Zaller, and Zaller, 2020) [39], leading to the paralysis and convulsions with ultimate death of the insect.

In the present study the effectiveness rate of Permethrin repellent was only 11%;the efficacy rate of Bendiocarb was only 23%;efficacy rate of the used deltamethrin was 49% only and the efficacy rate of the Malathion insecticide was recorded only 61%. (Table-1).Based on the results of sensitivity tests for all the insecticides used showed a very high resistance rate for Aedes mosquitoes in Jeddah.

The knockdown resistance phenotype (Kdr) is the result of point mutation in the Voltage gated sodium channel (Vgsc) in the neurons. Pyrethroid are neurotoxin insecticides and they target different molecules in the neurons especially the voltage gated sodium channel (Vgsc) which are closed to block the entry of insecticides inside the neurons (Soderlund, 2020). These mutations in *Vgsc* may help the insects to resist the effect of Pyrethroid insecticides on the nervous system (Niklas *et al.*, 2023) [26].

Aedes aegypti population has high resistance to Bendiocarb and Malathion with mortalities ranging from 0 to 89% while high resistance intensity against both Permethrin and Deltamethrin was recorded by Talipouo *et al*., (2021) [35].

The difference in the response of the fourth larval stage to *A. aegypti* for the tested insecticides may be due to the type of pesticide used and the way it is done, and this conclusion is consistent with many previous studies (Saleh and Aly, 1987; Canyon and Hii, 1999; Nazni *et al*., 2005) [8, 25].

This study confirmed that the differences in the levels of addition or strengthening resulting from mixing the compounds together may be due to the difference in both the action of these compounds and the level of the tested concentrations (Kelada and Shaker, 1988) [21].

Due to mutations, the knockdown effect is inhibited and momentary paralysis followed by complete recovery, this phenotype is called Knockdown resistance (Kdr). Sofia Balaska *et al*., (2020) [33] recorded this Kdr mutations in *Ae. Albopictus* mosquito. First Kdr trait (Leu1014) was identified in *Muscadomestica* (Ingles *et al.*, 1996) [18]. Since then, large number of insects was explored for genomic sequence Kdrs. In *Aedesaegypti* this effect was first studied by Severson *et al.*, in 1997 [31]. Unfortunately. In *Aedesaegypti*Vgsc mutation is coded by CTA in place of Codon TTA (Found in other insects) For *Aedesaegypti* substitute from CTA to TTT or TCA are required for nucleotide substitutions (Martins *et al.*, 2009a; Saavedra-Rodriguez *et al.*, 2007) [23, 28]. In *Aedesaegypti* mutations related to pyrethroids resistance

V1016G, S989P and F1534C were observed (Valine to Alanine or Glycine; Proline; Phenylalanine to Cysteine) were recorded from different parts of the world (Harris *et al*., 2010; Martins *et al*., 2009a and Saavedra-Rodriguez *et al*., 2007). [16, 23, 28] Same mutations were also recorded from Saudi Arabia's Jazan Area (Al Sheikh *et al*., 2016) [6].

This is seen in the high levels of resistance expressed by the mosquitoes that carried the mutations S989P, V1016G. These mutations were found to be common in the *Aedesaegypti*, suggesting their significant role in conferring resistance of the protein due to the fact that each amino acid has its own specific size, charge, and hydrophobicity-value. The results that the Serine is replaced by Proline, there are significant changes in the protein's properties. Proline has a rigid, cyclic structure that introduces kinks in the protein chain, reducing its flexibility. This can disrupt the normal folding and structural integrity of the protein. Additionally, Proline's different size, charge, and hydrophobicity compared to Serine can affect the protein's interactions and stability. As a result, the Serine to Proline mutation at position 989 can lead to a significant alteration in the protein's normal function, potentially impairing its biological activity and interactions due to these structural and physicochemical changes.

During the present study Vgsc mutations were recorded at codons 1016, 989, and 1534 in *Aedesaegypti* form 10 districts of Jeddah. Same results were obtained by Pyrethroids, showing a high resistance in *Aedes aegypti*. Three Kdr substitutions were found to be strongly associated with Permethrin and Deltamethrin resistance. Permethrin resistance was found more than Deltamethrin.V1016G causes insensitivity to Permethrin and Deltamethrin while F1534C causes resistance to Permethrin only. S989P causes very little resistance to Pyrethroids (Hirate *et al*., 2014) [17]. Several studies showed that S989P mutation in the sodium channel when associated with the V1016G mutation can increase the resistance levels to Pyrethrin in *Ae*. *aegypti.*

Voltage gated sodium channel mutations at V1016G, S989P were recorded from all areas studied during this work. These studies suggest that the resistance against Pyrethroids may be due to target mutations in Vgsc genes. The genotype of three codon combinations S989P, V1016G and F1534C are spreading in *Aedes aegypti* population in and around Jeddah. The presence and prevalence of Kdr mutations suggests that the resistance with insecticides is becoming common due to their extensive use (Al Nazawi *et al*., 2017) [3].

Conclusion

This study provides on how insecticide resistance mechanisms can affect the behavior of an *Ae. aegypti*, population from the Saudi Arabia. In Ae.*aegypti* mutations related to Pyrethroid, Organophosphorus and Carbamates resistance V1016G, S989P and F1534C were observed (Valine to Alanine or Glycine; Proline; Phenylalanine to Cysteine).

The evidence generated by this study has advanced understanding of resistance phenotypes, mechanisms and their possible consequences in a little-studied region, and local control programmers should consider adopting vector control strategies far less reliant on conventional insecticides before control failure ocurs.

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