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Molecular Analysis of Targeted Insecticide Resistance Gene Mutations in Field-Caught Mosquitos of Medical Importance From Saudi Arabia

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Abstract

Gene mutations on target sites can be a valuable indicator of the status of insecticide resistance. Jeddah, a global commercial and major port-of-entry city, is bearing the brunt of dengue disease burden in Saudi Arabia. In the current study, six genotypes of three codon combinations (989, 1016, and 1534) were observed on voltage-gated sodium channel (*VGSC*) gene in Jeddah's *Aedes aegypti* population, with PGF/PGC as the dominant one. Two types of introns between exon 20 and 21 on *VGSC* have been identified for the first time in *Ae. aegypti* in Saudi Arabia. Statistical and phylogenetic analyses showed that the intron type was significantly associated with the 1016 allele and may reflect the history of insecticide treatment in different continents. In addition, fixation of the L1014F allele on *VGSC* and G119S on *acetylcholinesterase 1* gene was detected in local *Culex quinquefasciatus* populations, with frequencies of 95.24 and 100%, respectively. To the best of our knowledge, this is the first report of resistant-associated mutations in field-caught *Cx. quinquefasciatus* in Saudi Arabia. The high prevalence of insecticide resistance gene mutations in local primary mosquito vector species highlights the urgent need to carry out comprehensive insecticide resistance surveillance in Saudi Arabia.

Key words: *Aedes aegypti*, *Culex quinquefasciatus*, acetylcholinesterase 1 gene, dengue, knockdown resistance gene

Mosquito-borne diseases, including dengue, malaria, West Nile, Japanese encephalitis, Rift Valley fever (RVF), chikungunya, Zika, and other emerging arboviruses, are prevalent and represent an enormous threat to public health worldwide. Intensive international trade and travel, influenced by global economic integration, has accelerated the spread of mosquito-borne viruses, resulting in occasional disease outbreaks even in previously endemic or disease-free areas. At least four vector-borne viruses, including dengue virus (DENV), Alkhurma haemorrhagic fever virus, RVF virus, and Crimean-Congo haemorrhagic fever virus, have been circulating in Saudi Arabia (Al-Saeed et al. 2017). The most important of them is dengue, with most of the disease burden and severity, which is mainly prevalent in the western and southwestern regions in Jeddah and Makkah of Saudi Arabia (Heilman et al. 2014, Organji et al. 2017, Mohammed et al. 2018). The third dengue-affected city in the country after Jeddah and Makkah is Jezan (Ministry of Health of Saudi Arabia 2019), the most southwestern region of Saudi Arabia on the border with Yemen, which suffered the 2000 RVF outbreak together with Jeddah (Miller et al. 2002). A total of 5,345 confirmed dengue cases were reported in 2018, with

an incidence rate of 16/100,000 population. Most of the cases (92.46%, *n* = 4,942) were in Jeddah and 3.05% (*n* = 163) were in Makkah, according to the Ministry of Health of Saudi Arabia report (Ministry of Health of Saudi Arabia 2019).

Jeddah is the financial and diplomatic center of Saudi Arabia and is the main point-of-entry for the Holy City of Makkah (only 80 km SE of Jeddah) for millions of visitors of perennial religious rituals for pilgrimage (Hajj) and Umrah as well as workers and other visitors. *Aedes aegypti* (Linnaeus, 1762), the major mosquito vector of dengue, chikungunya, and Zika, and *Culex quinquefasciatus* (Say, 1823), a vector of West Nile, RVF and Japanese encephalitis, are the two dominant mosquitoes in Jeddah (Khater et al. 2013, Alikhan et al. 2014). Although there have been no reports of West Nile virus outbreaks in Jeddah, it has been reported in countries neighboring Saudi Arabia, such as Iran and Egypt (Aghaie et al. 2016, Sayedahmed 2016). Since the distribution of *Ae. aegypti* and *Cx. quinquefasciatus* is widespread and both are domestic and endophagic species, there is an ever-present risk of arboviral disease outbreaks. In our previous paper, DENV-2 and four strains of insect-specific flaviviruses, including 1 cell-fusing agent virus and 3 *Phlebotomus*-associated flaviviruses, were detected in pools of *Ae. aegypti*, and 10 strains of *Culex flavivirus* were detected in pools of *Cx. quinquefasciatus* from Jeddah (Fang et al. 2021).

Since no protective vaccines or effective drugs are available for the majority of mosquito-borne diseases, the major strategy for reducing the risk of transmission is the control of disease vector mosquitoes. Vector control has been relying heavily on insecticide applications to reduce larval and adult mosquito populations in their natural habitats as well as personal protection using mosquito repellents (Sayono et al. 2016). Pyrethroids (mainly deltamethrin, cypermethrin, and cyfluthrin) and the organophosphates, pirimiphos-methyl and diazinon, have been the most common insecticides used in different commercial formulations over the last 20 yr in routine mosquito control operations in Saudi Arabia. *Aedes aegypti* populations from Jeddah and Makkah showed high resistance to permethrin, deltamethrin, and bendiocarb and were tolerant to fenitrothion, based on conventional insecticide bioassays performed on adult mosquito samples collected in 2015 (Al-Nazawi et al. 2017). Noteworthy, in a 10-yr period, the mortalities to pyrethroids decreased from 75 to 93% in 2008 to <20% in 2017 in the *Ae. aegypti* population in Makkah (Al-Nazawi et al. 2017). In addition, the *Ae. aegypti* population in Jezan was only sensitive to cyfluthrin, tolerant to deltamethrin, permethrin, and fenitrothion, and resistant to Lambda-cyhalothrin, dichlorodiphenyltrichloroethane (DDT), and bendiocarb (Alsheikh et al. 2016). However, to our knowledge, no relevant record exists on insecticide resistance in field-caught *Cx. quinquefasciatus* from Saudi Arabia.

Selection pressure on insecticide target sites of mosquito vectors, resulting in the occurrence and development of single- or multiple-site mutations in the responsible genes, has been reported and subsequently detected globally in major mosquito vector species belonging to the genera *Anopheles*, *Aedes*, and *Culex* in the last few decades (Sokhna et al. 2013, Scott et al. 2015, Moyes et al. 2017). Increased insecticide target-site insensitivity due to gene point mutations and the increased or elevated activity of insecticide-detoxifying enzymes are two of the major mechanisms that have evolved in mosquito vectors for surviving the lethal effect of insecticides (Brengues et al. 2003, Liu 2015). The voltage-gated sodium channel (*VGSC*) gene is the major target site for pyrethroids and DDT (Hemingway and Ranson 2000), which comprises four homologous domains (DI to DIV), and each domain contains six transmembrane helical segments (S1–6) (de Lera Ruiz and Kraus 2015). The most prevalent insecticide resistance gene mutation is the knockdown resistance (*kdr*), which is linked to single nucleotide polymorphisms on the transmembrane segment 6 of domain II of the *VGSC* (Soderlund and Knipple 2003). The mutation sites on the amino acid 1014 position on *VGSC* IIS for both *Anopheles* and *Culex*, and the 1016 position for *Aedes*, were well confirmed to reduce mosquito sensitivity to pyrethroid, and the organochlorine DDT due to cross-resistance effect (Donnelly et al. 2009; Du et al. 2013, 2016). In particular, several point mutation sites on *VGSC* are speculated to be involved in pyrethroid and DDT resistance in *Ae. aegypti*, including G923V, L982W, S989P, M1011I/G, V1016G, and F1269C in domain II and T1520I, F1552C, F1534C, and D1763Y in domain III of *VGSC* (Chang et al. 2009, Yanola et al. 2010, Kushwah et al. 2015, Sayono et al. 2016). Among them, two point mutations, V1016G and F1534C, that confer resistance to pyrethroids and DDT, are the most widespread (Harris et al. 2010, Dusfour et al. 2015, Li et al. 2015, Du et al. 2016, Kawada et al. 2016). Four non-synonymous (A99S, L1014F/S/C, V1016G, and W1594R) and six synonymous, (L884L, G923G, A1262A, D1266D, P1270P, and G1754G) mutations were found to be linked with the levels of permethrin resistance in *Cx. quinquefasciatus* populations (Komagata et al. 2008, Li et al. 2012, Xu et al. 2012). In addition, the replacement of the amino glycine (Gly, G) with serine (Ser, S) on position 119 of acetylcholinesterase 1 (*ace-1*) was found to be associated with resistance to organophosphate and carbamate insecticide groups (Weill et al. 2003). The G119S mutation was commonly detected in *Anopheles* (Essandoh et al. 2013, Fang et al. 2019) and *Culex* (Bourguet et al. 1998), while seldomly recorded in *Aedes* species (Sayono et al. 2016).

In Saudi Arabia, S989P, V1016G, and F1534C mutations on *VGSC* were reported in *Ae. aegypti* samples collected from Jeddah and Makkah in 2015 (Al-Nazawi et al. 2017). Al-Nazawi et al. (2017) was the first to report insecticide resistance gene mutations in *Ae. aegypti* populations from Saudi Arabia. However, point mutations in the *VGSC* of *Ae. aegypti* populations in Jeddah have not been fully characterized, especially the types of introns between exons 20 and 21, as well as whether the *ace-1* mutations were in Jeddah *Ae. aegypti* populations. In *Cx. quinquefasciatus*, the V1016G mutation was detected in the JPP-B strain, originating from Jeddah and selected by permethrin for 20 consecutive generations (Amin 1989, Komagata et al. 2008). This mutation has not been reported in other populations of the *Cx. pipiens* complex (Scott et al. 2015). To the best of our knowledge, since the first studies performed in the mid-1980s, no study on insecticide resistance gene(s) in *Cx. quinquefasciatus* populations has been reported so far in Saudi Arabia. Thus, currently, there is limited information on insecticide resistance gene mutations in *Cx. quinquefasciatus* against chemical insecticides in Jeddah. Bioassay tests are designed to detect insecticide resistance in vector populations collected from the field or those reared from field larval collections (Centers for Disease Control and Prevention [CDC] 2012). Such tests require large number of live and homogenous population of mosquitoes and are time-consuming compared with molecular methods (Chung et al. 2019). Early detection and characterization of target mutations of insecticide resistance genes, as potentially valuable molecular markers, can provide a reference to estimate the local insecticide resistance level (Saavedra-Rodriguez et al. 2007, Wuliandari et al. 2015, Sayono et al. 2016).

This study aimed to determine the status of insecticide resistance attributed to *kdr* mutations and the phylogeny of the introns between the exons 20 and 21 on *VGSC*, and *ace-1*, in *Ae. aegypti* and *Cx. quinquefasciatus*. The results of our study will be important for the early detection of developing resistance and in guiding integrated

vector management and insecticide application policies for the prevention and control of mosquito-borne diseases in Saudi Arabia.

Materials and Methods

Mosquito Collection

Mosquitoes were collected with Black Hole strap (BioTrap Co., Ltd, Seoul, South Korea) during routine mosquito surveillance in 2016. More details about the collection method and the results of mosquito diversity and seasonal density are were reported by in Fang et al. (2021). A total of 25 *Ae. aegypti* and 24 *Cx. quinquefasciatus* female mosquitoes were analyzed, representing different districts and municipalities of Jeddah Governorate. Collection sites for the detection of target-site mutations on insecticide resistance genes are shown in Fig. 1, and a map was generated using ArcGIS 10.1 ArcMap software (ESRI, Redlands, CA). The species name, geographic information (including the latitude and longitude information), and GenBank accession numbers of each randomly selected samples are shown in Supp Table S1 (online only).

DNA Extraction and Target-Gene Mutation Sequencing

For DNA extraction, individual mosquitoes were homogenized using two 3-mm steel balls in a mixer mill (Jingxin, Shanghai, China) in 250 µl of ATL (Qiagen, Hilden, Germany) and 20 µl of proteinase K (Qiagen, Hilden, Germany). The homogenates were incubated at 56°C overnight in an oscillating thermo-block. They were then centrifuged at 1,000 rpm for 3 min at room temperature. 200 µl supernatant from each homogenate was added to the Roche MagNA Pure 96 sample plate, and placed into to the MagNA Pure 96 System for automated DNA extraction (MagNA Pure 96 DNA and Viral NA Small Volume Kit, Roche, Basel, Switzerland) according to the manufacturer's instructions DNA was eluted in 100 µl of buffer solution.

For *Ae. aegypti*, the primer set IIS5-6(3)F and IIS5-6(3)R (Al-Nazawi et al. 2017) were used to amplify the IIS6 domain of the *VGSC*, spanning the codons 989, 1011, and 1016 and the introns between exons 20 and 21, while the codon 1534 domain IIIS6 was amplified by the primer set AaNa31F and AaNa31R (Harris et al. 2010). The *ace-1* gene region spanning the position 119 was amplified by the primer set Ace-1F and Ace-1R (Hasmiwati et al. 2018). For *Cx. quinquefasciatus*, the *VGSC* region spanning the 1014 codon was amplified by the primer set Cq1 and Cq2 (Sarkar et al. 2009). The fragment containing the *ace-1* mutation was amplified by the primer set Moustdir and Moustrev (Huchard et al. 2006).

The PCR amplification products were separated on 1.5% agarose gel electrophoresis, purified, and sequenced in both directions by Sangon Biotech (Shanghai, China). The peak spectrum (chromatogram) of the sequencing data was determined using BioEdit v.7.2.6 (Hall 1999). The allelic information for certain ambiguous sequences, cloning into pESI-T vector (Yeasen Biotech Inc., China), and nucleotide sequencing were performed for confirmation.

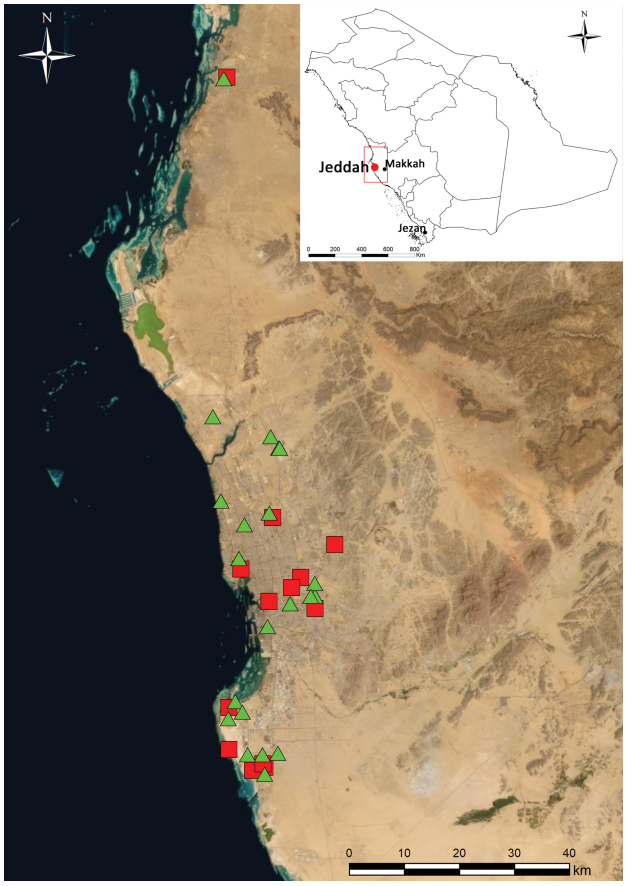

Fig. 1. Map of mosquito collection sites for detection of target-site mutations on insecticide resistance genes in 2016 in Jeddah, Saudi Arabia. Squares represent collection sites of *Aedes aegypti*, triangles represent sites of *Culex quinquefasciatus*.

Phylogenetic Analysis

The obtained *VGSC* IIS6 fragments spanning the introns flanked by exons 20 and 21 were aligned with –136 homologous sequences retrieved from GenBank using ClustalW2 (Larkin et al. 2007) with default settings, which were manually adjusted when was necessary. Based on the Akaike information criterion, the best-fit model for the alignment was determined using Model test 3.7 (Posada and Crandall 1998), in cooperation with PAUP* v.4.0b10 (Wilgenbusch and Swofford 2003). Consequently, maximum likelihood (ML) and Bayesian likelihood trees were constructed using the TVM+ G model for introns between exons 20 and 21 on the *VGSC*. Both the ML and neighbour-joining trees were constructed using MEGA v.7.0 (Kumar et al. 2016), with 1,000 bootstrap replicates. The Bayesian tree was built with MrBayes v3.2.6 (Ronquist et al. 2012) on the CIPRES portal (www.phylo.org/) (Miller et al. 2010), run for 10 million generations, with the first 25% generations discarded as burn-in. The trees were visualized using Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Both intra- and inter-specific divergences of introns spanning between exons 20 and 21 were calculated using the K2P distance model (Kimura 1980) in MEGA v.7.0 .

Statistical Analysis

Pearson's chi-squared test was applied to analyze associations among mutation codons and type of introns between exons 20 and 21 on *VGSC*. The tests were performed in SPSS v.20 (IBM Corp., Armonk, NY).

Results

Mapping of the Target-Site Resistance–Associated Genes in *Ae. aegypti*

The target-site resistance–associated genes were successfully amplified from a total of 20 *Ae. aegypti* and 21 *Cx. quinquefasciatus*.

The sequences originally obtained in this study were deposited in GenBank under the accession numbers MN997310–MN997399. Three non-synonymous *VGSC* mutation alleles, S989P, V1016G, and F1534C, were identified from specimens of wild *Ae. aegypti*. The presence of a silent mutation (TTG to TTA) at position (Leu, L) 982 (14/20) was found. No I1011M or F1552C mutations were identified. In addition, two types of introns between exons 20 and 21, type A (250 bp) and type B (234 bp), were identified.

Six genotypes of the combination of the 989, 1016, and 1534 positions were documented including SVF/PGF, SVF/SVC, SVC/SVC, PGF/PGF, PGF/PGC, and PGC/PGC (Table 1). Among them, PGF/PGC was the dominant genotype, detected in 45% of the examined samples, followed by the SVC/SVC genotype with 25%. Genotypes of SVF/PGF, SVF/SVC, and PGC/PGC were observed less frequently. Allele frequencies are shown in Table 2. The V1016G mutation seems to be more widely distributed at high frequency (67.50%) in Jeddah, than the F1534C mutation (55.00%). Both TTG982TTA (χ^2 = 22.000, df = 2, P < 0.001) and S989P (χ^2 = 44.000, df = 4, P < 0.001) co-occur in the presence of V1016G. All TTG982TTA and S989P coexist with the haplotype harboring the V1016G mutation. In individuals carrying the homozygous F1534C mutation in the IIIS6 region, no other coexisting mutations were observed on 982, 989, and 1016 codons in the IIS6 domain.

The type A intron between exons 20 and 21 was strongly linked to the V1016G mutation (Table 3). The haplotype with the type A intron was in association with V1016G mutation (χ^2 = 22.00, df = 2, P < 0.001) and wild-type F1534 (χ^2 = 11.75, df = 2, P = 0.003). By contrast, the F1534C mutation was strongly linked to the type B intron with all of the SVC/SVC genotype linked to the type B intron too. The allele type on 1016 position was highly correlated with the other two mutations, 989 and 1534, especially the 989 position (χ^2 = 44.00, df = 4, P < 0.001). All homozygous V1016G mutations are linked to homozygous S989P mutation, and V1016G heterozygous mutation is linked to heterozygous S989P mutation, whereas the wild-type of V1016 is linked to the S989. Individuals possessing homozygous wild-type V1016 harbored homozygous (5/6) or heterozygous (1/6) of the F1534C mutation; however, no wild-type homozygous F1534 was observed coexist.

Table 1. Frequency (percentage) of triple-locus *kdr* genotypes, shown as amino acid codon number 989, 1016, and 1534 in the *VSGC* gene of *Aedes aegypti* collected from Jeddah, Saudi Arabia

Genotype	SVF/PGF	SVF/SVC	SVC/SVC	PGF/PGF	PGF/PGC	PGC/PGC
No.	1	1	5	3	9	1
Percentage (%)	5.00	5.00	25.00	15.00	45.00	5.00

Phylogenetic Analysis of Intron Types Identified in the *VSGC* Gene of *Ae. aegypti*

The intron located between exons 20 and 21 on VGSC identified in our study and those retrieved from GenBank were compared and used to calculate the evolution of the kdr mutations. The 158 studied sequences can be clearly divided into two clades with high bootstrap values, one clade contained the sequences possessing type A intron, while the second clade contained those sequences with type B intron (Fig. 2). The intra-group genetic distances of type A and type B introns were 0.001 and 0.049, respectively, whereas the inter-group was 0.355. The two intron types were detected in four continents, Africa, Asia, and South and North America, suggesting that they were distributed paraphyletically. In South America (Brazil, El-Salvador, Guatemala) and North America (California and Hawaii), the ratio of type A to type B intron was 5:1 and 4:0, respectively; while in Asia (China, India, Indonesia, Malaysia, Myanmar, the Philippines, Saudi Arabia, Singapore, Sri Lanka, Thailand, Viet Nam), the ratio was 88:37, and in Africa (Ghana, Kenya, Malawi, Nigeria, Zambia, Zimbabwe), the ratio was 4:17. In addition, for global analysis of the associations among mutation codons and the types of introns between exons 20 and 21 on VGSC, we added the specific 989, 1016, and 1534 allele types of those sequences, available from GenBank. The results of the analysis of the associations between the three codons and the intron types reported, showed that the allele type of the 1016 position was more related to the 989 position (χ^2 = 49.910, df = 4, P < 0.001) than to the 1534 position (χ^2 = 16.297, df = 4, P = 0.003). While the intron polymorphism was more correlated to the 1016 position (χ^2 = 34.333, df = 2, P < 0.001) than the 1534 position (χ^2 = 18.005, df = 2, P < 0.001) (Table 3).

Point Mutations and Polymorphisms on *VGSC* and *ace-1* in *Cx. quinquefasciatus*

The majority of the successfully sequenced partial *VGSC* gene with the *kdr* mutation in *Cx. quinquefasciatus* was the homozygous genotype TTT/TTT (90.48%), except for two of them, which were the heterozygous genotype TTT/TTA.

All the detected *ace-1* at 119 location was the heterozygous genotype GGC/AGC.

Discussion

The application of indoor residual spraying (IRS) and larvicides to reduce and control the population abundance of *Aedes*, *Culex*, and *Anopheles* mosquitoes witnessed a significant decline in the 1970s and 1980s. The persistent mosquito-borne disease foci and endemicity are in the major Saudi cities of Jeddah, Makkah, Madinah, Aseer, and Jezan, and in the southwestern and southern border

Table 2. Frequency (percentage) of point mutations (S989P, V1016G, F1534C) and intron types identified in the *VSGC* gene of *Aedes aegypti* collected from Jeddah, Saudi Arabia

Codon	989		1016		1534		Intron type between exons 20 and 21		
Allele type	S	P	V	G	F	C	Type A	Type B	
No.	13	27	13	27	18	22	28	12	
Percentage (%)	32.50	67.50	32.50	67.50	45.00	55.00	70.00	30.00	
Genotype	SS	SP	PP	VV	VG	GG	FF	FC	CC
No.	6	1	13	6	1	13	4	10	6
Percentage (%)	30.00	5.00	65.00	30.00	5.00	65.00	20.00	50.00	30.00

Table 3. Chi-squared analysis of the linkage between point mutations and type A and B introns located between exons 20 and 21 on *VGSC* reported in *Aedes aegypti* from Jeddah, Saudi Arabia and homologous sequences retrieved from the GenBank from other geographic regions

Point mutations		Geographic regions	χ^2	df	*P*-value
1016	982	Jeddah	22.000	2	0.000
	989	Jeddah	44.000	4	0.000
		Global	49.910	4	0.000
	1534	Jeddah	15.402	4	0.004
		Global	16.297	4	0.003
Intron type between exons 20 and 21	1016	Jeddah	22.000	2	0.000
		Global	34.333	2	0.000
	1534	Jeddah	11.746	2	0.003
		Global	18.005	2	0.000

regions, renewed government efforts have ushered in enhanced pest surveillance coupled with integrated vector control programs, which have shown a significant decline in the total national cases (e.g., dengue) from 2000 till 2019 (Ministry of Health of Saudi Arabia 2020). However, as a side-effect, insecticide resistance have developed in local populations.

In the current study, three non-synonymous point mutations on 989, 1016, and 1534 positions and type A and B introns on *VGSC* were detected in field-caught *Ae. aegypti* mosquito samples in 2016 from Jeddah, Saudi Arabia. No samples that harboured the wild type at the three mutation loci simultaneously were identified. The I1011M/V mutation was absent in Jeddah, even though this substitution of isoleucine on position 1011 is prevalent in South America (Saavedra-Rodriguez et al. 2007, Martins et al. 2009a, b; Siller et al. 2011), and has spread in Thailand and Vietnam at low frequencies (Rajatileka et al. 2008, Bingham et al. 2011).

So far, to our knowledge, Saudi Arabia has the most variation in *kdr* mutations that may be due to long-term usage of pyrethroids in vector control programs in major cities, especially in Jeddah since the first dengue epidemic in 1994. The *kdr* polymorphisms of *Ae. aegypti* populations in Jeddah are different from those reported in other Middle Eastern countries. High prevalence of the F1534C mutation, and low frequencies of I1011M/V and V1016I mutations, with the absence of the 989P allele, were observed in India (Kushwah et al. 2015). In Pakistan, all the studied mosquito samples harboured the homozygous F1534C mutation, whereas no mutation at the 1016 position has been identified (Stenhouse et al. 2013).

The percentage of homozygous V1016G and S989P mutation co-occurrence was high (65.0%) in Jeddah. The V1016G mutation plays a critical role in *kdr* resistance against pyrethroid compounds (Du et al. 2013, 2016; Stenhouse et al. 2013). Linkage association has been observed between the 989 and 1016 loci in *Ae. aegypti* populations of Saudi Arabia (Al-Nazawi et al. 2017), Myanmar (Kawada et al. 2014), and Thailand (Srisawat et al. 2010).

It has been speculated that the S989P may serve as a compensatory mutation to the V1016G, to reduce the fitness cost to the mosquito (Du et al. 2016) and strengthen the response of V1016G to permethrin and deltamethrin (Hirata et al. 2014). Additionally, double homozygous mutations at positions 989 and 1016 combined with either wild-type or heterozygous mutations at the 1534 position were common in Jeddah's population, in agreement with the speculation that the 1016 and 1534 loci were not independent in a linkage disequilibrium analysis (Vera-Maloof et al. 2015). Further, the genotype with the triple mutation haplotype predominated in the tested mosquito samples at 45% (PGC/PGF). Additionally, one individual possessing triple homozygous mutation on 989, 1016, and 1534 loci was found in this study. In field-collected mosquitoes, the triple homozygous mutation was observed at low frequency in the population (Yanola et al. 2011, Stenhouse et al. 2013, Kawada et al. 2014, Dusfour et al. 2015, Marcombe et al. 2019), probably due to the fitness cost exerted on the mosquito by this triple mutation (Brito et al. 2013, Hirata et al. 2014). However, the artificially introduced triple mutation, using the *Xenopus* oocyte expression systems, appeared to confer a higher level of resistance to both permethrin and deltamethrin than those carrying the individual mutations, probably due to the synergistic effect of the combination of mutant alleles (Du et al. 2013, 2016; Hirata et al. 2014). Li et al. (2015) suggested that the F1534C mutation could possibly serve as a compensatory mutation for reducing the fitness cost to the mosquito induced by the V1016G mutation. Thus, whether the homozygous triple mutations on 989, 1016, and 1534 loci lead to insecticide resistance in field mosquitoes is yet to be ascertained.

The intron polymorphism of *VGSC* could serve as a marker to study the evolution of *kdr* mutations (Martins et al. 2009b, Chung et al. 2019). Based on sequence size, the introns located between exons 20 and 21 were classified into type A (250 pb) and type B (234 pb) (Martins et al. 2009b). Analysis of intron sequences obtained in our study and homologous sequences retrieved from GenBank showed that the intron type was significantly associated with 1016 (χ^2 = 34.333, df = 2, *P* = 0.000) and 1534 (χ^2 = 18.005, df = 2, *P* = 0.000) allele type. Further, all individuals possessing the V1016G mutation, either heterozygous or homozygous, harboured the type A intron, but not vice versa. This result was in contrast to the suggestion of Saavedra-Rodriguez et al.'s (2007) that the V1016G mutation was distributed independently in haplotypes possessing either the type A or type B intron. Additionally, the global data analysis showed that the two intron types were distributed evenly in both heterozygous and homozygous F1534C mutations, but the homozygous wild-type F1534 was mainly associated with the type A intron, and not vice versa as well. In Jeddah, six of the examined mosquito individuals were harbouring the homozygous F1534C mutation. Among them, five individuals with the SVC/SVC genotype possessed the type B intron, while the individual carrying the homozygous triple mutation (PGC/PGC) was coupled with the type A intron. In Taiwan Province, China, the F1534C was found to be strictly associated with the type B intron, and the V1016G mutation coexisted with the type A intron (Chung et al. 2019). By contrast, in Africa, the point mutation at the 1534 site was found to be strongly linked with the type A intron in Ghana, but only one heterozygote point mutation V1016I was recorded, except for the F1534C mutations (Kawada et al. 2016). Additionally, V1016I was reported to be located at the haplotype possessing the type A intron, with no 1534 variation observed in Brazil (Martins et al. 2009b, 2013). The mutation at position 1016 was associated with resistance to both type I and II pyrethroids, while the F1534C allele was primarily associated with resistance to type I pyrethroids, and the V1016I mutation did not alter channel sensitivity to pyrethroids in *Xenopus* oocytes (Du et al. 2013, 2016). Based on the evidence above, we speculate that *kdr* mutations are inclined to coexist with the type A intron. When a mutation occurred in either 1016 or 1534 positions, it would be linked to the type A intron. While in case of co-mutation, the type A intron was distributed more frequently with the V1016G/I mutation rather than the F1534C mutation. This is probably due to balancing the fitness cost to the mosquito by increasing the compensatory advantage or linkage disequilibrium (Lynch 2002, Hirata et al. 2014).

Fig. 2. Maximum likelihood phylogeny of introns between exons 20 and 21 of the voltage-gated sodium channel (*VGSC*) gene of *Aedes aegypti*, constructed by 158 sequences under the TVM+ G model. The GenBank accession number, collection country/province, and year of each sequence are noted. The sequences obtained from Saudi Arabia are in italics (red color in online version). The numbers above the branches represent the bootstrap support of the maximum likelihood, neighbour-joining, and Bayesian analyses, respectively, based on 1,000 replicates. The scale bar indicates 0.01 nucleotide substitutions per site. Dark gray and light gray indicate the type A and B of the introns located between exons 20 and 21.

It has been speculated that the type B intron is the ancestor of introns between exons 20 and 21, because *Ae. aegypti* was originally from Africa and the majority of African individuals belong to type B (Gloria-Soria et al. 2016, Kawada et al. 2016). In view of the phylogenetic tree obtained with available global data of the intron sequences, 100, 83.33, and 70.40% individuals from North

American, South American, and Asian countries, respectively, were of type A intron. By contrast, 73.91% of sequences from Africa belonged to the type B intron. According to our analyses above, type A introns commonly coexist with *kdr* mutations, especially the V1016G mutation; while the type B couples with the wild-type or F1534C mutation when the V1016G mutation is absent. This variable pattern of association may reflect the history of insecticide treatment in different continents. The major disease burden of malaria is in Africa and South-East Asia (World Health Organization [WHO] 2019). The mutation on 1534 locus may have arisen in *Aedes* species due to the use of DDT for larviciding and IRS to control malaria since the middle of the last century (Balkew et al. 2010, Harris et al. 2010). Whereas in some South and North American countries, vector control is mainly focused on *Aedes* species (mainly *Ae. aegypti* and *Aedes albopictus*) that are vectors of dengue, chikungunya, yellow fever, and Zika (Weaver et al. 2018). In domestic and endophagic habitats for *Ae. aegypti*, pyrethroid-based insecticides are widely used in household aerosols for public and personal protection, since pyrethroids are highly efficacious against mosquito vectors, and have low mammalian toxicity and a short residual action (WHO 2013). In South Asian countries, the higher frequency of type A intron than that of type B may indicate different selective pressures resulting in different histories of intense and unrestricted insecticide usage. It is likely that the F1534C mutations occurred due to the extensive use of DDT or type I pyrethroids, as both insecticides have the same target site (Harris et al. 2010). Then V1016G has been selected by the subsequent and wide use of type II pyrethroids, such as cypermethrin and deltamethrin (Kawada et al. 2014).

Both the non-synonymous mutation G119S (GGC/AGC) and silent mutation T506T on *ace-1* (Hasmiwati et al. 2018) were not detected in *Ae. aegypti* samples from Jeddah. However, this does not indicate that the *Ae. aegypti* population was completely susceptible to organophosphate and carbamate compounds, as both types of insecticides have the same target site. In bioassay tests, the *Ae. aegypti* population from Jeddah was tolerant to fenitrothion (Al-Nazawi et al. 2017), indicating that other than *kdr* mutations, or more likely, enzymatic mechanisms are involved in conferring resistance in mosquitoes. In Central Java, Indonesia, although the G119S mutation was absent, a degree of resistance to malathion was observed in *Ae. aegypti* (Sayono et al. 2016).

Reports on target-site mutations on *VGSC* and *ace-1* of *Cx. quinquefasciatus* are rare compared to those on *Ae. aegypti*. However, the insecticide resistance level in *Cx. quinquefasciatus* in Jeddah may be even worse as inferred from the fixation of the mutation allele both on *VGSC* and *ace-1*. In the studied mosquito samples in Jeddah, the *kdr* homozygous genotype TTT/TTT occupied 90.48% of the individuals. However, the heterozygous genotype GGC/AGC on *ace-1* was detected in all samples of *Cx. quinquefasciatus*. This is probably related to constant exposure due the extensive use of insecticides for routine spraying campaigns of public health, or for domestic use against mosquito vectors and other insect pests in Jeddah region. Similarly, the homozygous susceptible 1014 genotype was absent in samples collected in 2011 from Zanzibar, East Africa (Jones et al. 2012). In India, most of the studied mosquito samples harboured the heterozygous genotype TTT/TTA, at a frequency of 75% in *Cx. quinquefasciatus* samples (Sarkar et al. 2009).

One major limitation of our study was the small sample size of mosquitoes used for detection of target-site mutations in insecticide resistance genes. However, the primary survey revealed widespread *kdr* in the predominant mosquitoes in Jeddah. Further, bioassay tests on both larvae and adult mosquitoes are required to evaluate the status of insecticide resistance, and the association of *kdr* mutations and insecticide resistance in practice, and the underlying metabolic mechanism responsible for insecticide resistance is worth exploring.

Conclusions

The current study involved a wider geographic region of *Ae. aegypti* vector populations in Jeddah. The results showed that six genotypes of the three codon combinations, 989, 1016, and 1534, were spreading in Jeddah's *Ae. aegypti* population, with the PGF/PGC as the dominant one. Two types of introns between exons 20 and 21 were identified in Saudi Arabia for the first time. Linkage associations have been shown between V1016G with S989P, V1016G with F1534, and V1016G with type A introns. High prevalence of *kdr* mutations implies that the local *Ae. aegypti* mosquitoes are pyrethroid-resistant, and the level of resistance may increase with the extensive and intensive use of pyrethroid, as the active ingredients in household insecticide and spatial repellents. To the best of our knowledge, this is the first report of resistant-associated mutations in field-caught *Cx. quinquefasciatus* in Saudi Arabia. The fixation of L1014F allele on *VGSC* and G119S on *ace-1* was detected in local *Cx. quinquefasciatus* populations, at the frequencies of 95.24% and 100%, respectively, demonstrating that traditional insecticides are likely to be losing efficacy through fogging activities or IRS. In addition, analysis of intron sequences obtained in our study and homologous sequences retrieved from GenBank showed that the intron type was significantly associated with 1016 (P = 0.000) allele type in *Ae. aegypti* and may reflect the history of insecticide treatment in different continents.

The primary analyses showed the widespread distribution of *kdr* mutation both in *Ae. aegypti* and *Cx. quinquefasciatus*. The situation may become even be worse with increasing urbanization, climate change, and sustained *Aedes–Culex* endemicity estimated at a 31–56% prevalence rate, thus threatening the efficiency of vector control against mosquito-borne diseases. It raises the attention that continuous wide range of surveillance of the polymorphism and distribution of insecticide target-site mutations in mosquitoes in this region is highly needed. The mapping of the nation-wide distribution and polymorphism of target-site mutation against insecticide resistance will provide valuable consensus for forecasting the status of insecticide resistance development in mosquito populations of Jeddah and other regions of Saudi Arabia. Additionally, regular monitoring and documenting of the dynamics of target mutation genes and their associations with the insecticide resistance phenotypes is important in the surveillance system for preventing mosquito-borne diseases through long-term and sustainable vector control strategies.

Meanwhile, the above findings suggest that alternative insecticides in areas and the proper use of insect growth regulators or biocontrol approaches for integrated vector control programs should be considered in Jeddah and neighborhoods to mitigate and slow down the spread and impact of insecticide resistance development in disease vector populations. The Saudi government and Jeddah municipality commitment and investment in innovative operational research and development on early detection methods for insecticide resistance, and effective control measures, including vaccines for local and pilgrims' immunization programs. With the establishment of the Public Health Pest Laboratory of Jeddah Governorate, sustainable funding is imperative to provide evidence for coherent and coordinated temporal and spatial epidemiological integrated vector management and pesticide implementation among all stakeholders. Furthermore, strengthening laboratory and epidemiological capacity building

programs for health workers, health professionals, and community engagement including private–public sectors partnerships is important for furthering our ability to control mosquito vectors of dengue, and other emerging threats and epidemics of mosquito-borne diseases.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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