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Allele Specific Polymerase Chain Reaction (AS-PCR) Assay to Detect Knockdown and Acetylcholinesterase Mutations in *Anopheles superpictus*

Anopheles superpictus'ta Knockdown ve Asetilkolinesteraz Mutasyonlarının Belirlenmesinde Allel Spesifik Polimeraz Zincir Reaksiyonunun (AS-PCR) Kullanılması

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ABSTRACT

Objective: The study aims to determine the presence of L1014F, L1014S, L1014C alleles, which are responsible for knockdown resistance and *Ace-1* G119S alleles, which are responsible for acetylcholinesterase insensitivity in *Anopheles superpictus*, the secondary vector of malaria in Turkey.

Methods: In this study, 60 *Anopheles superpictus* adult females were collected from Aydın, Denizli, and Muğla provinces. Then, allele-specific primers for *kdr* L1014F, L1014S, and L1014C alleles, and the *Ace-1* G119S allele were designed. The presence of these alleles was screened in three *Anopheles superpictus* populations by allele-specific polymerase chain reaction.

Results: Although L1014S allele frequency was too low in Aydın, Muğla, and Denizli populations, neither *kdr* L1014F and L1014C nor *Ace-1* G119S mutations were found in any population.

Conclusion: In this study, *kdr* L1014S mutation was detected for the first time in the Aegean *Anopheles superpictus* populations.

Keywords: *Anopheles superpictus*, knockdown, acetylcholinesterase, allele-specific primer

ÖZ

Amaç: Bu çalışmanın amacı, Türkiye'de sıtmanın ikincil vektörü olan *Anopheles superpictus*'ta knockdown direncinden sorumlu olan L1014F, L1014S, L1014C allellerinin ve asetilkolinesteraz duyarsızlığından sorumlu olan *Ace-1* G119S allellerinin varlığını tespit etmektir.

Yöntemler: Aydın, Denizli ve Muğla illerinden 20'şer adet *Anopheles superpictus* ergin dişileri toplanmıştır. Daha sonra knockdown direncinden sorumlu olan L1014F, L1014S ve L1014C allellerine ve *Ace-1* G119S alleline özgü primerler tasarlanarak allele özgü polimeraz zincir reaksiyonu ile bu üç *Anopheles superpictus* popülasyonunda bu allellerin varlığı taranmıştır.

Bulgular: Aydın, Muğla ve Denizli popülasyonlarında çok düşük oranda L1014S mutasyonuna rastlanmakla birlikte, popülasyonların hiçbirinde ne *kdr* L1014F ve L1014C; ne de *Ace-1* G119S mutasyonuna rastlanmıştır.

Sonuç: Bu çalışmanın sonucu olarak, ilk kez *Anopheles superpictus* Ege bölgesi popülasyonlarında *kdr* L1014S mutasyonu tespit edilmiştir.

Anahtar Kelimeler: *Anopheles superpictus*, knockdown, asetilkolinesteraz, allele özgü primer

INTRODUCTION

Mosquitoes impact on over half of the worlds' population through the transmission of harmful diseases such as malaria, dengue, encephalitis and filariasis (1). Of these, *Anopheles* mosquitoes are responsible for the transmission of malaria all over the world. A total of 241 million malaria cases were reported globally and 627,000 people died due to malaria in 2020 (2).

Turkey had a devastating malaria history between the beginning of the First World War (1914-1918) and

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WHO's Global Malaria Eradication Programme established in 1957 in Turkey (3). Success was maintained until about 1978s and then case numbers increased in the following years (4). Although large-scale malaria control operations were reintroduced after 1978, case numbers increased dramatically as a result of intensive DDT use and insecticide resistance in mosquito populations (5). In the following years, malaria transmission increased rapidly in the mid-1990s (6). A significant decrease in malaria cases have been succeeded in Turkey after Tashkent declaration in 2005. Finally, significant progress has been performed toward national malaria elimination by the effort of the Turkish Ministry of Health (7).

The primary vector of malaria in Turkey is *Anopheles sacharovi* while *Anopheles superpictus*, *Anopheles maculipennis* and *Anopheles subalpinus* are known as secondary malaria vectors (5). However, due to exophilic and zoophilic tendencies, the epidemiological role of *An. superpictus* in malaria transmission varies (8).

Pyrethroids (PYs) are synthetic insecticides with high activity. They act on the voltage sensitive sodium channel (VSSC) (9). When PYs are attached to VSSC, they disrupt the opening and closing kinetics of VSSC. The poisoned insect loses control of its nervous system and shows trembling behaviour which is known as the knockdown effect (10). PYs have been widely used as an effective adult and larval mosquito control (11). However, extensive PYs use has led strong selection pressure and resulted in resistance problems in different mosquito populations all around the world. Knockdown resistance (*kdr*) mutations, a target site mutation, occurs in the *vssc* gene and cause reduced susceptibilty to PYs (12). So far, various *kdr* mutations have been reported from different *Anopheles* species as well as other mosquitoes (13). Molecular assays are useful tools in order to monitor the allele frequencies of target site mutations. Of these, polymerase chain reaction (PCR) based approaches are low-cost and practical ways to detect resistance alleles in mosquito populations (14).

Another target site mutation is known as *Ace-1* (G199S) mutation developed against organophosphates and carbamates. G119S mutation causes the conversion of the aminoacid glycine, which encodes the active site of the enzyme, to serine and causes acetylcholinesterase insensitivity (12). To date, the G119S mutation has been reported from different Anopheles species including *Anopheles albimanus* and *Anopheles gambiae* (15).

This study aims to design reliable allele-specific primers (ASP) to use in allele-specific PCR (AS-PCR) to detect *kdr* L1014F, L1014S, L1014C and *Ace-1 G119S* alleles in *An. superpictus* populations. Designed ASP were applied in AS-PCR to analyse *kdr* and *Ace-1* mutation frequencies in *An. superpictus* populations collected from Aydın, Muğla and Denizli provinces of Turkey. This is the first study to determine *kdr* and *Ace-1* mutation frequencies in Turkish *An. superpictus* populations.

METHODS

Allele Specific Primer Design

The ASP for detection of the wild type (Leucin-1014L) or mutated (Phenylalanine-1014F; Cystein-1014C; Serine 1014S) kdr allele in position 1014 of the *vssc* gene was designed through Primer3plus software (16). A 243 base pair (bp) long partial *vssc* gene sequence of *An. superpictus*, uploaded to GenBank by Djadid et al. (17), was obtained from GeneBank (Accession number: AH014535.2). Species specific forward (SupkdrF-5'- GGATTGAATCAATGTGGGATTGT-3') and reverse (SupkdrR-5'-AAGGATGAAGAACCGAAATTGGAC) primers were designed based on the partial sequence of *vssc* gene of *An. superpictus* obtained from GeneBank. Similarly, a 24 bp long each allele specific primers for leucine (SupkdrL_1-5'-GCTACAGTAGTGATAGGAAATTTA-3', phenylalanine (SupkdrF-5'- GCTACAGTAGTGATAGGAAATTTT-3', cysteine (SupkdrC-5'- GCTACAGTAGTGATAGGAAATTGT-3' and serine (SupkdrSer-5'- GCTACAGTAGTGATAGGAAATTCA-3' alleles were designed through Primer3Plus software.

Primers Ex3AgF:5'- GATCGTGGACACCGTGTTCG-3′ and Ex3AgR:5'-AGGATGGCCCGCTGGAACAG-3′ were used to amplify approximately 500 bp partial sequence of *Ace-1* gene (18). Similarly, AS (SupAceS-5'- GCTACAGTAGTGATAGGAAATTTA-3' and (SupAceR-5'- GCTACAGTAGTGATAGGAAATTCA-3' primers were designed to amplify approximately 400 bp glycine and serine alleles at positions 119 of the *Ace-1* gene.

Sample Collection and Study Area

Adult specimen collection was performed in 3 different locations from Aydın, Muğla and Denizli provinces of the Aegean region of Turkey. Sampling was performed between May-September 2018. Adult females were captured from the resting sites through aspirator. Collected samples were transferred in cages to vector insect laboratory of Aydın Adnan Menderes University. Morphological identifications have been performed through an identification key guided by Becker et al. (11). Adult mosquitoes were stored in a -20 °C until genomic DNA have been isolated. A total of 60 genomic DNA was extraction was performed from whole body of individual female mosquitoes using Invitrogen genomic DNA extraction kit (TermoFisher Scientific, USA). The PCR assay was applied to genotype a total of 60 *An. superpictus* specimens collected from 3 sites from Aydın, Muğla and Denizli.

Detection of Alleles Using AS-PCR

Each AS-PCR for detection of *kdr* alleles was performed in a final 25 µL volume. The reaction consisted of: 1× PCR Buffer, 1.5 U DNA polymerase, 2.5 mM dNTP, 10 µM each of primers and 1.5 µL of DNA (~20-50 ng). Reaction conditions were consisted of first denaturation at 95 °C, 5 min; 40 cycles of denaturation at 95 °C, 30 s; annealing at 59 °C, 1 min; extension at 72 °C, 1 min and the finally 72 °C, 5 min. PCR products were run on 2% agarose gel for 75 min at 70 V in 1× tris borate ethylenediamine tetra acetic acid buffer and visualised under UV light. Approximately ~250 bp long fragment for control band and ~190 bp fragment for each allele was visualized if the sample had that allele (Figure 1).

Ace-1 alleles were detected by AS-PCR which was carried out in a final volume of 25 µL containing 1.5 µL 2.5 mM dNTP, 2 µL buffer, 0.2 µL 10 mM from each primer, 0.25 µL (5 U/mL) Taq DNA polymerase and 1 µL DNA. Thermal cycle programme was consisted of a first denaturation at 95 °C, 10 min; 30 cycles of denaturation at 94 °C, 30 s; annealing 56 °C, 30 s; extension 72 °C, 30 s and finally 72 °C, 10 min. PCR products were run on 1% agarose gel and visualized under ultraviole light (Figure 2).

In order to validate the efficacy and reliability of AS-PCR, obtained PCR products were sequenced through a 3730xl capillary system automatic sequencer. Resistant (Serine-1014S) and sensitive alleles (Leucine-1014L) were deposited in GenBank (accession numbers: OP503892-OP503893) (Figure 3).

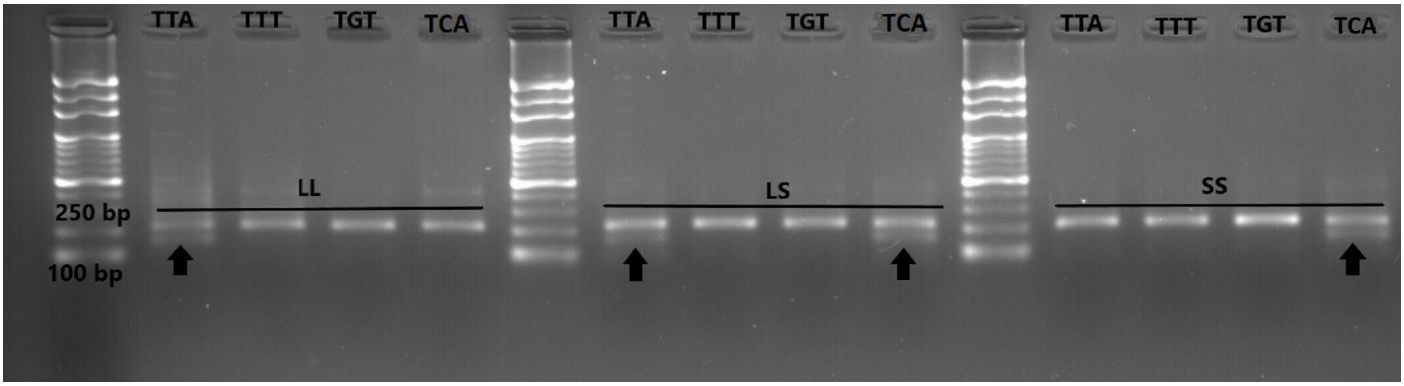

Figure 1. Agarose gel image of *vssc* gene region amplified with allele-specific primers (LL is encoded by the aminoacid homozygote Leucine (TTA/TTA); LS is encoded by Leucine and Serine (TTA/TCA); SS is encoded by homozygote Serine (TCA/TCA)

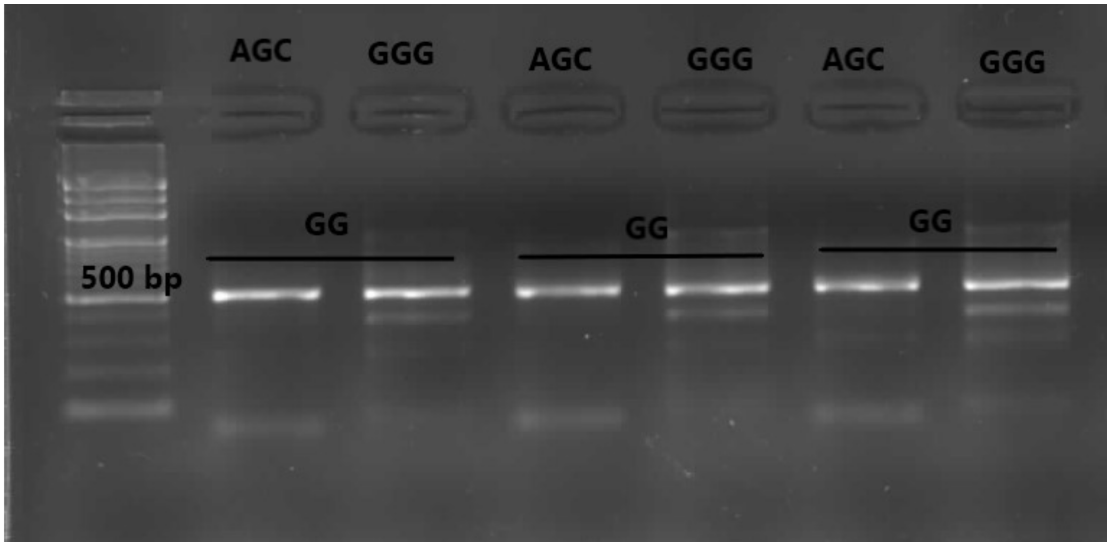

Figure 2. Agarose gel image of *Ace-1* gene region amplified with allele-specific primers (Glycine is encoded by GGG; Serin is encoded by AGC)

Statistical Analysis

Genotyping and kdr allele frequencies and Hardy-Weinberg expectations of each *An. superpictus* populations have been tested via exact probability test via POPGENE (19).

RESULTS

Although all L1014F, L1014C and L1014S alleles were screened in *An. superpictus* populations, only L1014S allele was found in the study area. L allele frequency was 0.8750 in the Aydın and Muğla population while S allele frequency was 0.1250 in these populations. In total, 80% and 85% of the individuals had homozygous L1014L in Aydın and Muğla populations while 15% of the populations had heterozygous L1014L/L1014S in the populations, respectively. L allele frequency was 0.9750 in the Denizli population while S alle frequency was 0.0250. Only 5% of the population had heterozygous L1014L/L1014S while 95% of them had homozygous L1014L. All of the populations were in the Hardy Weinberg Equilibrium ($p>0.05$) calculated based on Fisher's Exact test. The *kdr* genotype, leucine and serine allele frequencies and exact test results of each *An. superpictus* population are given in Table 1.

No *Ace-1* (serine) mutation was found either in any of the *An. superpictus* populations. All specimen had wild type *Ace-1* (Glycine) allele encoded by GGG (Figure 2).

Table 1. Genotypes, allele frequencies and Fisher's Exact test results of *vssc* gene region of *Anopheles superpictus* populations (L/L: homozygous susceptible (Leucin); S/S: homozygous resistant (Serin); LS: heterozygous resistant (Leucin/Serin)

Locality	No	Genotype			Allele frequency		p	SE	F_{IS}
		LL	LS	SS	L	S			
Aydın	20	16	3	1	0.8750	0.1250	0.2472	0.0022	0.3372
Denizli	20	19	1	-	0.9750	0.0250	-	-	-0.000
Muğla	20	17	3	-	0.8750	0.1250	1.00	0.000	-0.1176

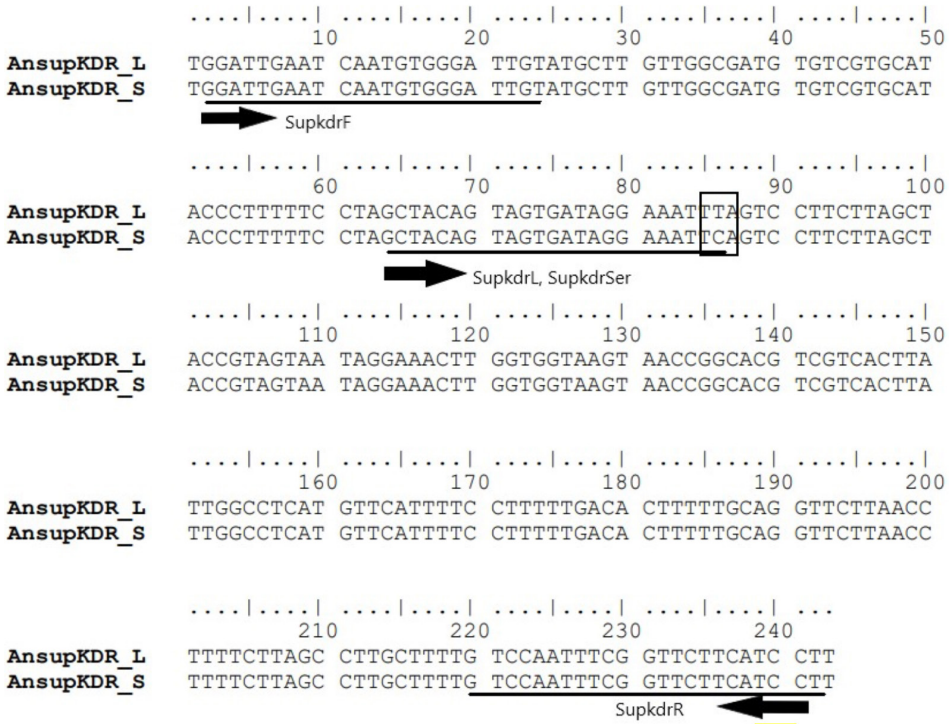

Figure 3. A partial sequence alignment of voltage-sensitive sodium channel (*vssc*) gene region in *Anopheles superpictus*. SupkdrF and SupkdrR indicates forward and reverse primers, respectively. SupkdrL and SupkdrSer indicates leucine and serine alleles

DISCUSSION

A key finding of this research is the identification of the L1014S allele in *An. superpictus* populations collected from Aydın, Muğla and Denizli. This result is a proof of the presence of this allele in *An. superpictus* populations from western Turkey. Since sample size of collected specimen is not wide enough, further studies with larger specimen numbers are needed to understand the exact frequency of resistance alleles in *An. superpictus* populations. The presence of the L1014S alleles was first described in *Anopheles sacharovi* (20). It was also reported in *An. sacharovi* populations collected from the Mediterranean and Aegean populations in Turkey (21). This is the first record of presence of the *kdr* alleles in *An. superpictus*. However, several *kdr* alleles (L1014F, L1014C, L1014W) have been detected in different mosquito populations all over the World. For instance, L1014S alleles have been reported in *Anopheles gambiae* populations in several countries including Burkina Faso (22) and Cameroon (23). The presence of this allele was reported in *Anopheles arabiensis* populations collected from Uganda (24) and Ethiopia (25). All L1014F, L10104F, L1014C and L1014W mutations have been reported in Chinese *Anopheles sinensis* populations (26).

Another important finding of the study is the lack of *Ace-1* mutation (G119S) in *An. superpictus* populations collected from the study area. The serine allele was not found in any of the samples while ASP created a band profile on agarose gel with only the GGG-encoded glycine allele. Similarly, G119S alleles have not been detected in Turkish *An. sacharovi* (20) and Iranian *An. stephensi* populations (27). However, several researchers reported the presence of G119S alleles in different *An. gambiae* populations in Mali (28), Nigeria (29) and *An. arabiensis* populations in Senegal (30).

Although this study provides useful evidence for the presence of L1014S allele in *An. superpictus* populations in the Aegean region of Turkey, it does not allow to evaluate the frequency of the alleles and meaningful population genetic analysis due to limited sample size. Further investigation with larger specimen numbers is required to determine the geographical distribution of this allele in this geographic area. The other limitation of the study is the lack of bioassay tests to test insecticide susceptibility status of the species. Further studies should aim to detect mortality rates against different kind of insecticide with recommended insecticide dose through the susceptibility tests of World Health Organisation. It should be taken into consider that molecular analysis should be supported with bioassays to understand resistance levels and underlying mechanisms.

CONCLUSION

This study proposed a useful and practical allele specific primer to genotype L1014F, L1014C, L1014S *kdr* mutation in *An. superpictus*, a secondary malaria vector species in Turkey. The use of AS-PCR to monitor *kdr* mutation in *An. superpictus* populations might facilitate the detection of the mutation when it is in initial stage before the resistance alleles spread wider. Additionally, results also proved that *Ace-1* G119S mutation associated with acetylcholinesterase insensitivity is not detected in western Turkish *An. superpictus* populations. Regular detection of allele frequencies in vector mosquito species is of great importance to manage insecticide resistance policies at the local levels. This study is a preliminary study that proposes AS primer sequences rather than giving clear information about the *kdr* allele frequencies in Aegean region populations. Clear information about the *kdr* allele frequencies of the populations and the resistance status might be gathered by increasing the specimen numbers in future studies.

*Ethics

Ethics Committee Approval: This article does not contain any studies with animals performed by any of the authors.

Informed Consent: This article does not contain any studies with animals performed by any of the authors.

Peer-review: Externally peer-reviewed.

*** Authorship Contributions**

Concept: S.İ.Y., F.M.Ş., Design: S.İ.Y., F.M.Ş., Data Collection or Processing: S.İ.Y., Analysis or Interpretation: S.İ.Y., Literature Search: S.İ.Y., Writing: S.İ.Y., F.M.Ş.

Conflict of Interest: No conflict of interest was declared by the authors.

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