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ORIGINAL ARTICLE

Presence of the knockdown resistance (kdr) mutations in the head lice (*Pediculus humanus capitis*) population in the North of Iran

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Abstract
Head lice infestation continues to be a major health problem worldwide. In order to solve this problem, frequent use of pediculocides has caused growing resistance among head lice population. This study aims to investigate the presence of the knockdown resistance (*kdr*) mutation in the head lice population in northern Iran. Adult head lice were collected from 115 infested individuals referring to the health centers in different parts of Mazandaran province, northern Iran. 38 samples were randomly selected, and Polymerase chain reaction (PCR) was used to amplify a 332-bp and ~ 900-bp fragment of the α-subunit of the voltage-sensitive sodium channel (*VSSC*) gene. According to the analysis of a 332-bp fragment of the *VSSC* gene, the frequency of kdr T917I mutation including homozygous susceptible (SS), heterozygous resistant (RS), and homozygous resistant (RR) were 45.83%, 12.5%, and 41.66%, respectively. The total frequency of the resistance allele was 54.16%. The results of the 900-bp fragment of the *VSSC* gene showed two new mutations in the IIS1-2 extracellular loop (H813P) and IIS2 (S825R) and old well-known kdr mutations (M815I–T917I–L920F). The results of Hardy-Weinberg's exact equilibrium test showed that the frequency of genotypes in the studied areas is different from expectations. Moreover, a positive inbreeding coefficient value (F_{is}>0) was found in studied areas which indicated an excess of homozygotes. Overall, the results showed a high frequency of resistant alleles in the northern region of the country. Therefore, it is necessary to develop appropriate control programs for the treatment of pediculosis.

Keywords *VSSC* gene · Head lice · *Pediculus humanus capitis* · *Kdr* · Iran

Introduction

The head louse, [*Pediculus humanus capitis*], is an obligate, blood-sucking ectoparasite with global distribution (Coates et al. 2020). Pediculosis is an important public health problem affecting individuals of all socio-economic levels (Falagas et al. 2008). Despite improvements in health care and medical education, head lice infestation has dramatically increased recently and threatened community health, particularly among school-age children (Li et al. 2010; Toloza et al. 2009; Hatam-Nahavandi et al. 2020). The prevalence of pediculosis was reported to be 0.47–56.15% in different parts of Iran (Akbari et al. 2022). In addition, the prevalence of head lice infestation varies from 1.65 to 7.9% in the northern part of the country (Moosazadeh et al. 2019; Motevalli Haghi et al. 2014; Modarresi et al. 2013). According to this estimated prevalence, it seems that pediculosis is increasing in the country and globally. Head lice bites cause severe itching and provide the conditions for secondary bacterial infection. The most common clinical signs caused by lice infestation are itching, papular urticaria, skin scraping and cervical/occipital lymphadenopathy. In some rare cases, iron deficiency and anemia also occur (Coates et al. 2020). On the other hand, head lice infestation causes social-behavioural complications such as stigma in individuals (Smith and Goldman 2012). Hence the

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World Health Organization (WHO) has recently paid more attention to the some ectoparasitic skin diseases like head lice as neglected tropical diseases (Casulli 2021). Control measures include removing adult lice and eggs. Therefore, mechanical removal and combined treatment with insecticides are usually recommended (Coates et al. 2020). People routinely treat pediculosis with pyrethroid compounds. The extreme and over-the-counter use of these compounds was observed in most countries (Clark 2010; Fu et al. 2022; Toloza et al. 2014). Currently, the pediculicide drugs available in Iran are permethrin 1%, lindane, and 4% dimethicone which are commercially available in the form of shampoos, topical creams, and lotions (Firooziyan et al. 2017; Mohammadi et al. 2022). By binding to voltage-sensitive sodium channels in the nervous system of head lice, permethrin leads to nerve depolarization and hyperpolarization which causes muscle paralysis and death of the louse (Gellatly et al. 2016). It seems that its overuse has caused failure in the treatment of pediculosis and the emergence of resistance. Of course, insecticide resistance has also been observed in other insects. Molecular markers called knockdown resistance (kdr) have been introduced to identify point mutations (Clark 2009). Three point mutations (amino acid substitutions at M815I, T917I, and L920F) in the α-subunit of the voltage-sensitive sodium channel (*VSSC*) gene have been identified as known kdr markers (Lee et al. 2000). According to Clark's research, the M815I and L920F mutations, when expressed alone, reduce permethrin sensitivity, but when the T917I mutation is present, either in combination with other mutations or alone, it plays an important role in permethrin resistance (Clark 2009).

Since the kdr mutation can be closely related to the lack of response to pyrethroids, basic knowledge of the frequency of genetic resistance markers in the head lice population and the use of alternative insecticides or other treatment methods are necessary actions. Hence, the present work has aimed to investigate the presence and distribution of kdr resistance in head lice population in the northern region of Iran.

Methods

The present study was approved by the Ethics Committee at Babol University of Medical Sciences, Babol, Iran (IR.MUBABOL.REC. 1402.150).

Lice collection was carried out at randomly selected health centers throughout three cities located in the center (Babol) and eastern (Sari and Neka) of Mazandaran Province, the North of Iran, between September 2022 and March 2023. Research staff and medical entomologists screened each participant for lice infestation after receiving written consent. We did not consider individuals who did not want to give written consent or whose visit was for any other scalp disease. Overall, 115 patients were enrolled and thoroughly examined for detection of head lice using a metal comb, then louse specimens (totally 135 head lice samples) were transferred to the vials containing 70% ethanol and kept at − 20 °C for further analysis.

DNA extraction

The samples of head louse were washed twice with sterile distilled water and then left to dry at room temperature. The half of each louse transferred to a sterile 1.5-ml tubes and was crushed with a sterile pestle. Then, the contents of the tube were homogenized with cell lysis solution and proteinase K, and incubated at 55 °C for 3 h. Finally, DNA was extracted using the Insect DNA extraction kit (Viragene, Iran) as described by the manufacturer. The quality and quantity of extracted DNA were assessed using a NanoDrop instrument (Thermo Fisher Scientific Inc., USA) (Candy et al. 2018).

VSSC gene amplification by PCR

38 out of 135 head lice specimens from the studied were randomly selected and subjected to PCR test. The primer pairs for the a-subunit of the voltage-sensitive sodium channel gene including forward 5-AAATCGTGGCCAACGTTAAA-3 and reverse 5-TGAATCCATTCACCGCATAA-3 for amplification of 332-bp fragment selected according to Durand et al. (Durand et al. 2007). The PCR amplification was performed in a total volume of 25 µl including 12.5 µl of 2× Red Master Mix (Ampliqon, Odense, Denmark), 0.5 µl of each primer, 3 µl DNA template, and water, with the thermal program as follows: an initial denaturation step at 95 °C for 5 min; 35 cycles at 95 °C for 45 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 1 min (extension); and a final extension step at 72 °C for 7 min (Boumbanda Koyo et al. 2019). For the amplification of a ~ 900-bp fragment of the *VSSC* gene, specific primers (HLF: 5- ATTTTGCGTTTGGGACTGC-3; HLR: 5- CCATCTGGGAAGTTCTTTATC-3) were selected according to Firooziyan et al. (2017), considering the suggested thermal conditions (Firooziyan et al. 2017). The PCR products were electrophoresed on a 2% agarose gel, and the bands were visualized under UV light.

DNA sequencing and statistical analysis

The PCR products of the *VSSC* gene were purified and subjected to sequencing by Genfanavaran Company (Iran). To compare the sequencing findings to each other and the reference sequence in the GenBank database, BLAST analysis was performed using BioEdit version 7 software (www.

ncbi.nlm.nih.gov). The multiple alignments of sequences were performed using the ClustalW method of MEGA 11 software (Tamura et al. 2011). The nucleotide sequences were translated into amino acid protein sequences. For calculation of the frequency of resistance alleles, the total number of resistant alleles divided by the total number of alleles. The distribution of genotypes was examined for differences from the Hardy-Weinberg equilibrium (HWE) using a Chi-squared test (Black and Krafsur 1985). To evaluate for heterozygous excess or deficiency, the Wright's inbreeding coefficient (F_{is}) was used (Weir and Cockerham 1984).

Results

A total of 115 participants who were infested with head lice were included in the study. For molecular analysis of the 332-bp fragment of the *VSSC* gene, 38 head lice samples were randomly selected and analyzed. Finally, 24 samples were selected and sequenced. The PCR results showed the successful amplification of the 332-bp DNA fragment of the *VSSC* gene in all samples.

The obtained nucleotide and amino acid sequences were aligned and compared with the insecticide-susceptible (SS) reference sequence published in the GenBank database (accession number: AY191156). The results of multiple alignments of the nucleotide and amino acid sequences revealed that 45.83% (11) of samples were homozygote susceptible or wild-type (SS) genotypes with no substitution on three markers of kdr codons. In addition, homozygous resistant (RR) sequences found in 41.66% (10) of samples showed the T917I and L920F point mutations. In three samples (12.5%), only a point mutation in codon L920F was detected, which was identified as a heterozygote sequence (RS) (Fig. 1). The obtained sequences from the present study were deposited in the GenBank database under accession numbers PP156902-PP156915. A chromatogram image for three genotypes sensitive homozygous, resistant homozygous, and resistant heterozygous is shown in the Fig. 2.

In general, the frequency of the kdr T917I mutation was 54.16% in the studied regions. The analysis of the Hardy-Weinberg (H-W) model showed that the frequency distribution of kdr genotypes in the studied regions deviated from the H-W equilibrium. The investigated head lice had a Wright's inbreeding coefficient (F_{is}) higher than zero, indicating an excess of homozygotes (Table 1).

The 900-base pair fragment of the sodium channel gene was amplified in 24 randomly selected samples, but only 10 samples were successfully amplified, and then six sequences were registered in the GenBank database with accession numbers PP187206-PP187215. Multiple alignments of these sequences revealed six single nucleotide mutations in the amplified sequences. All the sequences collected from our study, compared to the Genbank data, showed a close relationship with *Pediculus humanus capitis* with an identification percentage between 99.54% and 100%. The results of the multiple alignment of the amino acid sequence in the haplotypes showed five polymorphic sites in these fragments, at the connecting region of exon I to exon II, nucleotide mutations at positions 95 (A/C) and 102 (G/T) lead to changes in amino acid structure. These mutations lead to H813P and M815I amino acid substitutions. These mutations have been observed in 100% of the sequences. In exon II, a new point mutation has led to the substitution of amino acid S825R, which was observed in 33.3% of the sequences. Moreover, two point mutations in nucleotide positions 584 and 592 have led to the change of amino acids in codons T917I and L920F. These mutations have also been observed in 100% of the sequences. Therefore, sequence analysis has shown two distinct haplotypes in lice. Haplotype I has four amino acid substitutions (H813P, M815I, T917I, and L920F) observed in all sequences, and haplotype II, which, in addition to the above four mutations, has another substitution in amino acid S825R (Fig. 3).

Discussion

Despite the improvement in health in different societies, head lice infestation is still an important health problem in poor and developing countries. According to previous studies, the prevalence of pediculosis is estimated at up to 80% worldwide. It seems that one of the reasons for the increase in the population of lice and its high prevalence is the emergence of resistance to the insecticides used. The degree of resistance varies from one region to another and in different regions of the world. Pyrethroid compounds are the safest and most common pediculicide drugs; however, their frequent use has led to resistance (Lee et al. 2000; Gao et al. 2003). Resistance usually occurs in the form of detoxification, oxidation, esterification, and changes in nerve sodium channels (*VSSC*) called kdr. This is the first study in Iran that carried out the kdr mutation in the population of lice with a plot of 332-base pairs in the northern region of the country. The frequency of resistant allele was 45.83% in the present work. In the study conducted by Ghavami et al. (2023) in the northwestern regions of Iran, the frequency of the resistant allele was 51.68% in the head lice population (Ghavami et al. 2023). Ghahvechi et al. (2021), in their investigation, reported that the frequency of resistant alleles was 25.9% in the head lice population in western and southwestern Iran (Ghahvechi Khaligh et al. 2021). In addition, another study by Firoozian et al. (2017) stated the frequency of the resistant allele to be 66.6% (Firooziyan et

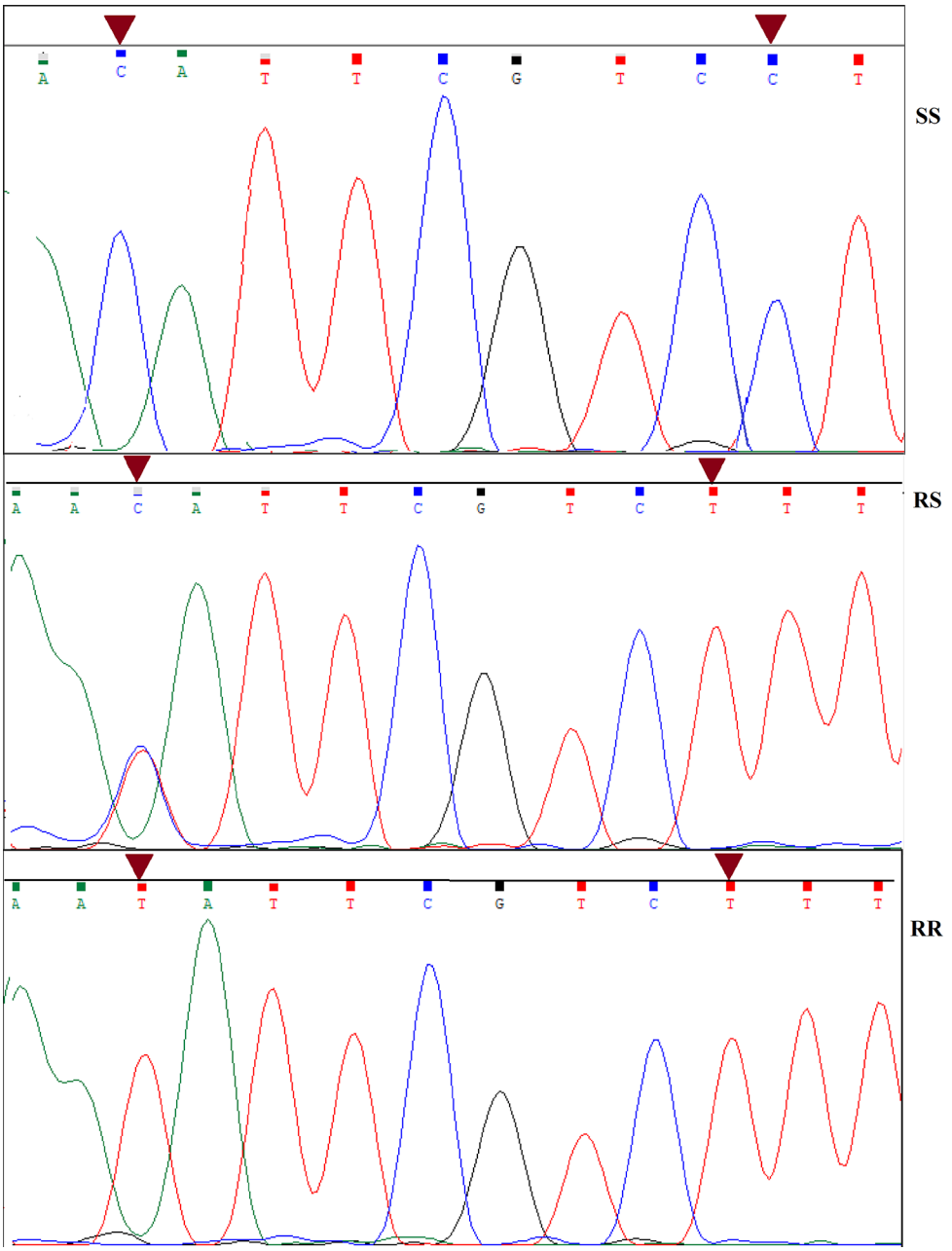

Fig. 1 The chromatogram image for three genotypes sensitive homozygous, resistant homozygous, and resistant heterozygous

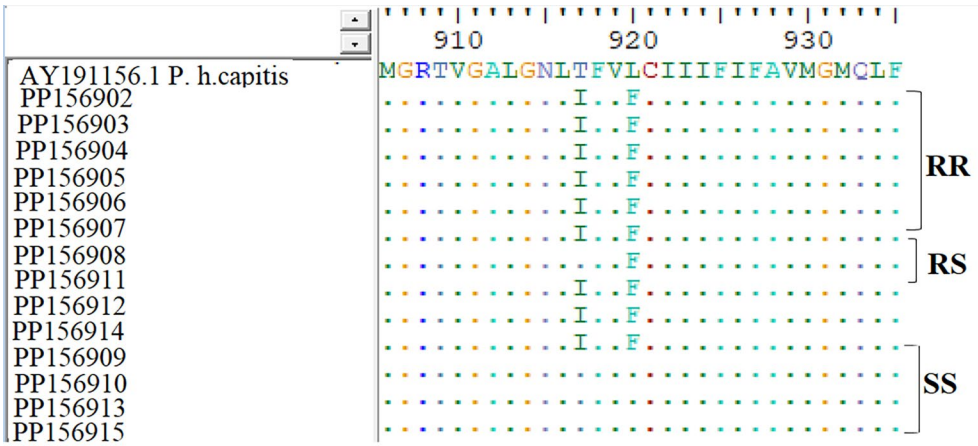

Fig. 2 Alignment of the amino acid sequences of *VSSC* gene 332 bp fragment in the studied head lice with the standard reference. The sequence showed the position of the kdr T917I mutation as well as the position of L920F mutation indicated by a red vertical column and three genotypes as RR, RS and SS

Table 1 Frequency of kdr T917I genotype in human head lice population from northern Iran

Region	No. of louse collected/analyzed/sequenced	Genotype			Frequency of resistance allele (%)	H-Wb(x^2)	F_{is}[c]
		RR	RS	SS			
Babol	100/30/20	8	2	10	50	12.73[a]	0.8[b]
Sari	10/4/2	1	1	0	100	0.22[a]	-0.33[c]
Neka	5/4/2	1	0	1	50	2[a]	1[b]
Total	**115/38/24**	**10(41.66)**	**3(12.5)**	**11(45.83)**	**54.16**	**13.48[a]**	0.75[b]

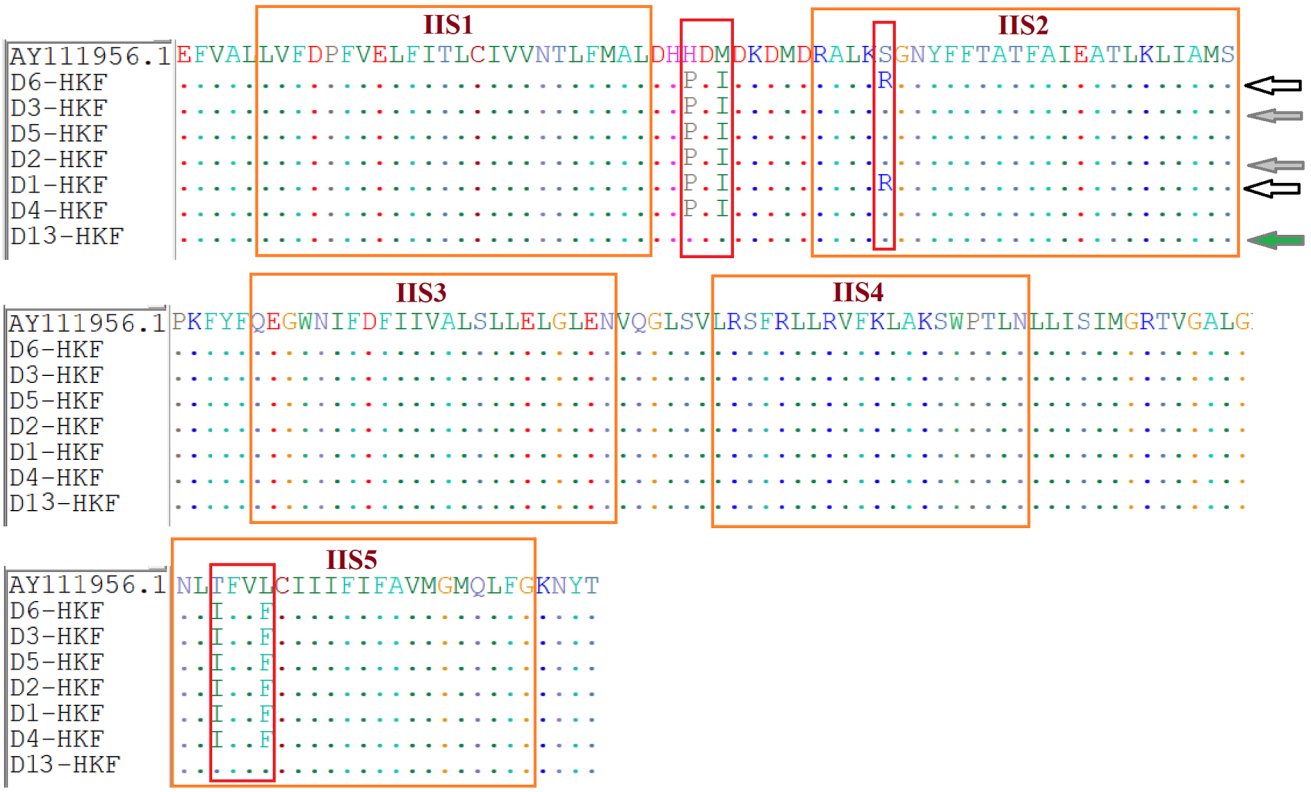

Fig. 3 Alignment of the amino acid sequences of *VSSC* gene 900 bp fragment in the studied head lice with the standard reference. The segments S1–S5 of domains II are showed in orange box. The sequence showed the position of the kdr point mutations in two novel haplotype I (white arrow) and haplotype II (gray arrow) indicated by a red vertical column. The sequence of susceptible genotype (without mutation) recovered from head lice samples are shown in green arrow

al. 2017). The results of all these studies conducted in Iran agree with the present study and indicate a range of resistance between 25 and 50% in the Iranian head lice population. On the other hand, studies conducted in other parts of the world also indicate the high frequency of kdr-resistant alleles in head lice in different geographical regions such as Africa, Asia, South America, the United States, the European Union, and Australia, between 15 and 100% (Fox et al. 2020). The frequency of resistant alleles seems to be more common in countries with widespread use of pyrethroid compounds (Toloza et al. 2014). These results show that the resistance to pyrethroids through this mechanism of neural insensitivity (kdr) is expanding, but it is not geographically uniform. This phenomenon can be related to the selection pressure caused by the frequent use of pediculicide drugs, especially permethrin, and the Iranian head lice population is currently under active selection pressure (Ghavami et al. 2023). In contrast, in the study conducted by Brownell et al. (2020) in Thailand, the prevalence of kdr mutation among the head lice population was about 30%, and the authors suggested it could be related to the low use of pyrethroids in rural areas, more emphasis on traditional medicine, and the use of medicinal plants as the main treatment to eliminate lice infestation (Brownell et al. 2020). Moreover, in the study of Kasai et al. (2009) in Japan, the level of resistance in the head lice population was reported as 8.7%, which is the lowest level of resistance compared to our country and other countries (Kasai et al. 2009).

The investigation on the 900 bp fragment of the *VSSC* gene in the present study has revealed one of the characteristics of kdr resistance in the M815I region in 100% of the samples. In addition, another mutation in the H813P region

was identified in 100% of the samples, which was shown as a new mutation compared to other sequences around the world (Fox et al. 2020). However, in other studies conducted in Iran, H813P mutation in the head lice population has also been observed. In addition, a new substitution in the S825R region, which was detected in 30% of the samples, has been observed for the first time in Iran. This mutation is located in the IIS2 and linker IIS1–S2 near the location of known kdr-related mutations. The analysis of H-W equilibrium revealed that the genotype frequency in the collected head lice population varied across all investigated regions. Moreover, the inbreeding coefficient was positive in the studied regions and overall (Fis > 0), which indicates the presence of homozygous excess. The frequency of heterozygous alleles in the present study, which was determined as RS at the T917I site, was 12.5% (3).It is well documented that the markers M815I and L920F are the first loci mutated against pyrethroids, and only the mutation at the T917I site is considered a heterozygous allele (Toloza et al. 2014). Brownell et al. (2020) also concluded that the presence of excessive homozygotes indicates inbreeding or stabilization of mutant alleles in the lice population, which was in agreement with the results of our study (Brownell et al. 2020). In a recent study performed by Karakuş et al. (2020) in Turkey, the high frequency of the resistant allele (99%) was found among head lice, and the low number of heterozygous alleles (4.4%) (Karakuş et al. 2020). In contrast, a study by Larkin et al. (2020) found that the majority of lice harbored heterozygous kdr mutations, indicating active selective pressure among lice populations in Honduras (Larkin et al. 2020). Similar to the present study, Ghavami et al. (2023) found a significant difference in inbreeding values among mitochondrial groups of lice, and the inbreeding coefficient in lice in clade A was positive and different from the Hardy-Weinberg equilibrium, while the inbreeding coefficient was negative in clade B lice (Ghavami et al. 2023).

Conclusions

This is the first study to determine kdr mutations related to pyrothyroid compounds in head lice from northern region of Iran. The findings of the present study indicate the existence of resistance to permethrin in the head lice populations in the studied area. Therefore, to prevent the increase of resistant alleles, changes in the current control programs, alternative treatment measures, and their improvement in the investigated areas are required. For better understanding of the occurrence of resistance in human lice population, more comprehensive studies in other parts of the country are also suggested in the future.

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Author contributions All authors contributed to data acquisition. Substantial contributions to the conception and design of the study was performed by Tahmineh Gorgani-Firouzjaee, Mohsen Karami and Reza Eslami. Experimental works were performed by Tahmineh Gorgani-Firouzjaee and Seyedeh Maedeh Mirtabar-Darzi. The analysis, interpretation of data was done by Tahmineh Gorgani-Firouzjaee. The first draft of the manuscript and critical revision was written by Tahmineh Gorgani-Firouzjaee. All authors read and approved the final manuscript.

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Data availability Raw data is available upon request to the authors.

Declarations

Competing interests None of the authors have declared a conflict of interest.

Ethical approval The present study was approved by the Ethics Committee at Babol University of Medical Sciences, Babol, Iran (IR.MUBABOL.REC. 1402.150).

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