BMC Research Notes

RESEARCH NOTE Open Access

Molecular detection of novel Glutamate-gated chloride channel mutations in field collected human head lice (Phthiraptera: Pediculidae) from Iran

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Abstract

Objective Recently, insecticides such as ivermectin, which targets glutamate-gated chloride channel (GluCl) channels in the nervous system of invertebrates, have gained attention for the treatment of head lice. However, resistance to this insecticide threatens the effectiveness of head louse control programs.

Results Molecular bioinformatics sequence analysis revealed that the most common mutations were R37K and E50K with a frequency of 85.71%, followed by D93N (64.28%), M101R (35.71%), and R100Q (28.57%). These mutations are reported here for the first time. The identification of these novel mutations in head louse populations raises concerns about the potential emergence of ivermectin resistance. Further research is needed to explore the functional implications of these mutations and their impact on the effectiveness of insecticide treatments.

Keywords Head louse, Iran, Ivermectin, Glutamate-gated chloride channel, GluCl gene

Introduction

The head louse, *Pediculus humanus capitis* De Geer, is a blood-sucking insect that feeds exclusively on human blood [1]. Head lice are a common public health problem in both developed and developing countries, particularly in poor communities and among s schoolchildren [2]. According to a 2017 survey, the incidence of head louse infection in Iran was about 500,000 out of 80 million people (625 cases per 100,000 population), and the economic burden of head lice in the country was estimated to be $5,790,143 [3].

Red and itchy skin followed by sores on the scalp are major complications of head louse infestations. The head louse is among the most common causes of scalp pyoderma in developed countries [4, 5]. Severe infestations of head lice sometimes lead to the formation of scaly crusts under which lice tend to congregate [6]. Head lice are carriers of *Staphylococcus aureus* and *Streptococcus pyogenes*, both of which can cause skin infections [7]. The DNA of several pathogenic bacteria, such as *Bartonella quintana*, *Borrelia recurrentis*, and *Yersinia pestis*, has been identified in head lice, which can lead to purulent eczema in cases infected with head lice [8–10]. The psychological side effects of head louse infestation stem from its health aspects and have negative effects such as embarrassment and social sanctions. These effects are

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more severe among children and may result in expulsion or suspension from schools [11, 12].

Over the past 80 years, the control of pediculosis has largely relied on the availability of natural and synthetic insecticides, ranging from DDT (introduced in 1943) to pyrethroids (introduced in 1992) [12]. In Iran, the extensive use of pyrethroid insecticides has resulted in the emergence of resistant head louse populations, confirmed through molecular testing [13–15]. Today, insecticides such as avermectin have gained a lot of attention for the treatment of head lice [16, 17]. To date, ivermectin has improve the health of hundreds of millions of people in many developing societies and has been included in the World Health Organization's list of essential medicines due to its broad-spectrum activities [18].

Ivermectins affect the glutamate-gated chloride channels of head lice, and since these channels do not exist in vertebrates, the use of this insecticide in humans has no side effects [19]. In invertebrates, ivermectin binds to glutamate-gated chloride channels (GluCls), leading to a reduction in motor activity, paralysis, and death [20].

The major threats to the sustained use of ivermectin is the emergence of resistance around the world. Various studies from different geographical regions on diverse arthropods have reported mutations such as P299S [21], G323D and G326E [22, 23], A309V [24], G314D and G326E [25] mutations to abamectin and ivermectin insecticides resistance. In 2018, resistance to ivermectin was reported in head louse populations in Senegal due to T136C, C752T, and A815G mutations [26]. To date A309V, G323D, and G326E mutations are associated with target site resistance to abamectin. This study, conducted for the first time in Iran and the region, aimed to investigate GluCl gene sequences in head lice to detect possible mutations. The results of the current study can provide valuable insights into evaluating the appropriateness of using ivermectin insecticide in head louse control programs and be useful for decision-making in head louse control programs, monitoring, and resistance management.

Materials and methods

Sample collection

Head louse samples were collected from families seeking treatment in health centers and from school students between 2017 and 2021.

Before lice sampling, the objective of the study and the method of louse collection were explained to interested individuals, volunteers, and the parents or legal guardians of the younger individuals. Afterwards, written informed consents (in Persian) were obtained from these individuals. Prior to applying permethrin shampoo, a pre-treatment sampling protocol was established to collect and identify head lice (*Pediculus humanus capitis*) from the referrals. A total of three head lice were collected from these individuals. The collection of samples was performed by trained workers from different districts in seven provinces of Iran (Fig. 1). The study's sampling protocol was approved by the Ethics Committee of the Urmia University of Medical Sciences (UMSU), Urmia, Iran (ethical code: IR.UMSU.UC.1401.410), and all participants gave their informed consent. In health centers, head louse treatment with permethrin shampoos was provided free of charge. Details of the infested individuals who provided the samples are not presented due to ethical considerations. The specimens were transferred to the Medical Entomology Laboratory, School of Public Health (SPH), UMSU, and kept in 70% ethanol until use in molecular experiments.

Genomic DNA extraction and PCR amplification

DNA was extracted from each louse using the YTA Genomic DNA Mini Kit (Yekta Tajhiz Azma, Tehran, Iran) following the manufacturer's protocol [13, 14]. The sequence to be amplified was 1722 bp, but due to amplification difficulties, the head louse GluCl gene was amplified in three fragments using three pairs of primers based on Amanzougaghene et al. (2018). The amplified fragment includes exon II to exon VII of the head louse GluCl gene excluding the first exon. The details of the primers used are presented in Table 1.

All PCR reactions were conducted in a 25-µl volume of Master Mix (Yekta Tajhiz Azma). Each reaction contained 1 µl of genomic DNA, 1 µl of each primer (6079 F, 6686R, and 6574 F, and 287R), 12.5 µl of Master Mix, and 9.5 µl of ddH_2O. PCR conditions were started with a hot start at 95 °C for two minutes, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 49.5 °C for 75 s, and extension at 72 °C for 75 s, with an additional 7 min of extension time in the last cycle.

All PCR conditions for amplifying the final fragments of the GluCl gene with 7157 F and 7800R primers were the same, except for annealing (at 48 °C for 60 s) and extension (at 72 °C for 60 s). Fourteen samples were randomly selected from different locations subjected to sequencing in both directions with the primers used for amplification.

Bioinformatics and statistical analysis

Chromatograms and obtained sequences were assembled and analyzed using bioinformatics software such as the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/), Chromas version 2.31 (http://www.technelysium.com.au/chromas.html), and Clustal Omega [27]. The final nucleotide and amino acid sequences were aligned using the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software [28]. DS235786.1 and NW_002987791.1 sequences submitted

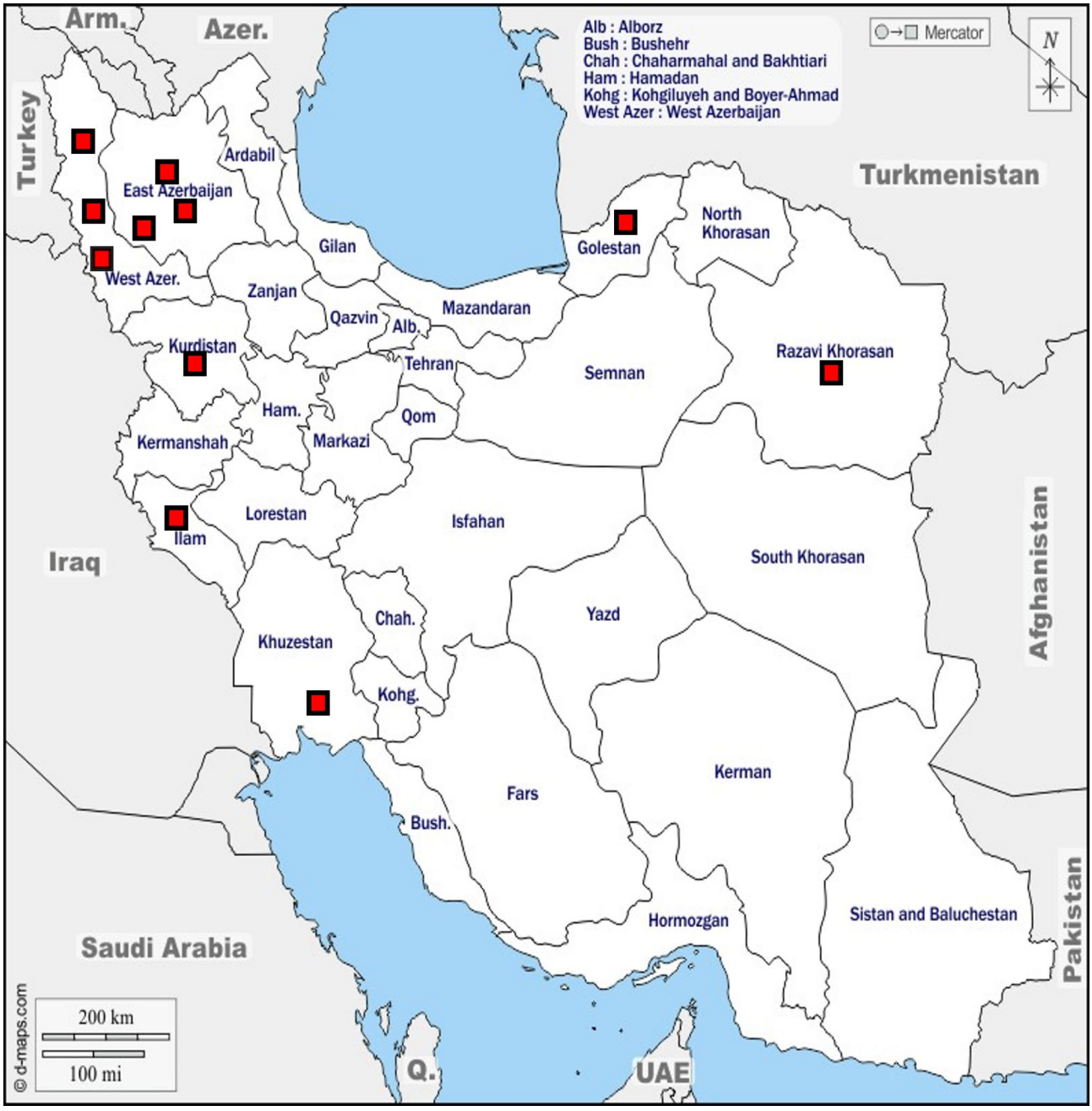

Fig. 1 Locations of collected head louse samples in Iran

Table 1 The details of primers used for amplification and sequencing of head lice GluCl fragment

Purpose	Primer name	Primer sequences
GluCl	6079F	CAATTAATTCGACGGATTCAG
	6686R	CCATCCCTACGACCATCAAATT
	6574F	GCTCACTTCGAATGGCCAGTTG
	7287R	CTAACAAAGCTCCAAATACGAAC
	7157F	AGTGACAACATTACTCACAA
	7800R	GATTGATTTACCAACGACGGC

from the USA and France, respectively, were used as reference genes in the analysis of the samples.

Results

A total of 61 head lice were collected from nine geographic regions in seven provinces of Iran (Fig. 1). Among these 61 samples, 27 were randomly selected for DNA extraction and PCR amplification. Eight specimens from West Azerbaijan Province and one specimen from each of the six other provinces were selected for sequencing in both directions.

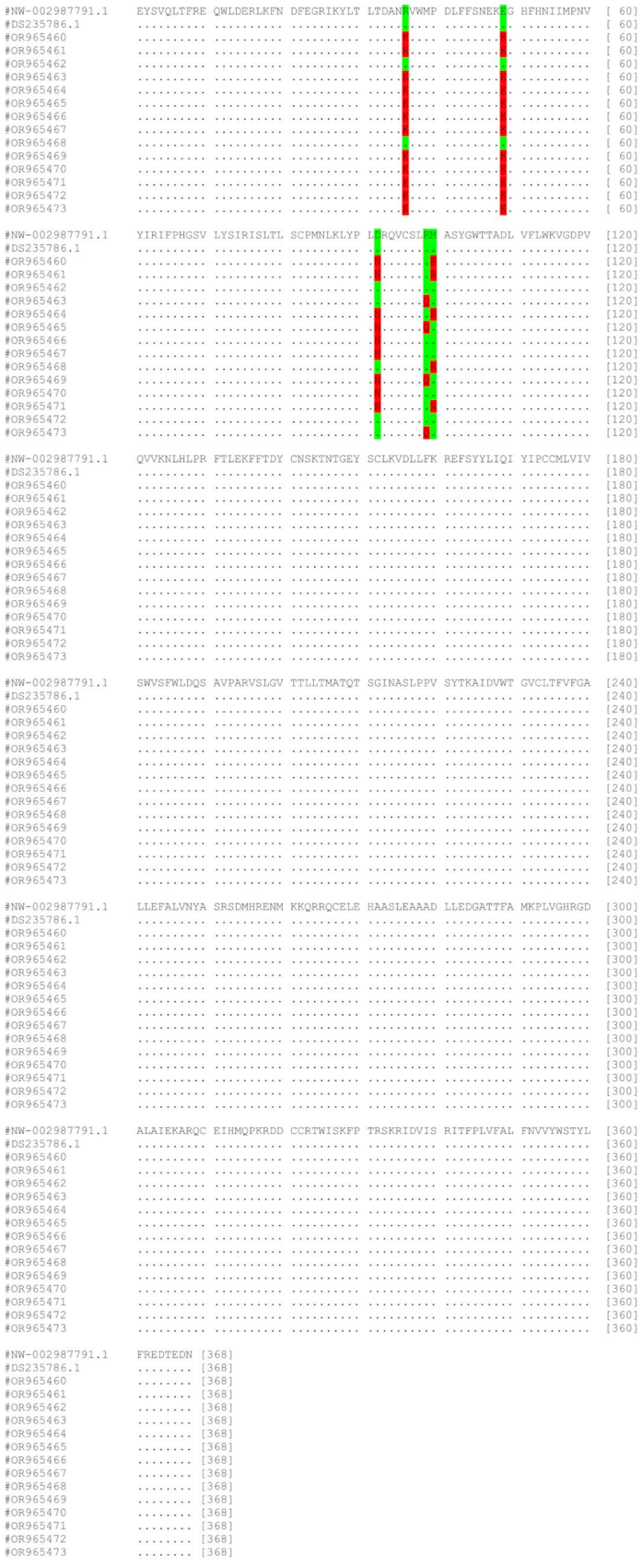

Fig. 2 (See legend on next page.)

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Fig. 2 Multiple alignments of the GluCl channel amino acid sequence of head lice samples collected from Iran. The parts marked in red indicate amino acid substitutions. NW-002987791 and DS235786, represent accessing numbers of reference wild samples. OR965460-OR965467, OR965468, OR965470 OR965471, OR965472, and OR965473 are accessing numbers of subjected samples from West Azerbaijan, East Azerbaijan, Ilam, Khuzestan, Razavi Khorasan, Kurdistan, and Golestan provinces

The first exon of the GluCl fragment in the study by Amanzougaghene et al., which includes 3 bp, was not sequenced in the present study. The GluCl fragment in sequenced samples contained six exons and six introns. Five GluCl sequences of head louse from different countries were submitted to the GenBank with the accession numbers DS235786.1 (USA), NW_002987791.1 (USA), XM_002429761.1 (USA), MT321070.1 (France) and MT321071.1 (France). Sequence similarity within mRNA sequences of GenBank were between 97.21 and 100%, whereas it was 100-99.55% within mRNA sequences of the current study. The 0.45% difference within Iranian sequences was due to six nucleotide mutations at positions 239, 277, 487, 509, 512, and 1050 nucleotides. Approximately 83.33% of the mutations were transition and 16.66% were transversion.

Multiple sequence alignment comparison between the GenBank and Iranian sequences in the exon region showed 99.19–99.73% similarity. There were nine nucleotide mismatches at positions 239, 277, 339, 487, 509, 512, 1050, 1460, and 1496 nucleotides (0.81%), with 77.77% transitions and 22.22% transversions. Multiple sequence alignment revealed a nine-nucleotide size variation between our sequences and GenBank-submitted sequences. This difference was due to the presence of three TAA repeats in the third and fifth introns (Fig. 1. Supp) in 89.78% of the Iranian sequences.

Sequence alignments at the amino acid level among five GenBank sequences showed 97.21–100% similarity. The 0.27% difference was due to the substitution of leucine with serine (L to S) at amino acid position 204 in comparisons with the GenBank sequence (ID: MT321071.1). Amino acid sequence similarity within GluCl sequences of Iranian head lice was 99.18–100%. The 0.82% difference was due to five amino acid substitutions at positions 37, 50, 93, 100, and 101. Amino acid sequence comparisons among Iranian and GenBank sequences revealed 98.64–99.73% similarity. Five non-synonymous amino acid substitutions at five positions caused a 1.36% difference (Fig. 2). These non-synonymous mutations resulted in the substitutions of arginine with lysine (R37K), glutamic acid with lysine (E50K), aspartic acid with asparagine (D93N), arginine with glutamine (R100Q), and methionine with arginine (M101R). The most frequent amino acid substitutions were R37K and E50K (85.71%), followed by D93N (64.28%), M101R (35.71%), and R100Q (28.57%).

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Discussion

The head louse infestation remains a global challenge despite significant efforts and expenditures [29]. Effective head louse control programs needs up-to-date information on the sensitivity and resistance of this insect to commonly used insecticides [30]. Studying insecticide resistance at the molecular level provides valuable insights into the mechanisms underlying resistance and helps develop more effective control strategies [31]. Recently, various studies have aimed to understand the genetic basis of resistance to pyrethroid and avermectin insecticides in head lice [13–15, 26, 32–35]. In Iran, focus has mostly been on the *kdr* resistance in head louse populations collected from different regions and provinces [13–15, 32]. We analyzed the GluCl gene sequence for the first time in head lice from West Azerbaijan and six other provinces of Iran. This study provides crucial insights into the genetic basis of insecticide resistance in head lice (*Pediculus humanus capitis*) in Iran.

We identified five novel non-synonymous mutations (R37K, E50K, D93N, R100Q, and M101R) in the GluCl gene, all located in the N-terminal extracellular domain of the GluCl channel (Fig. 3). A recent study in Senegal identified five mutations (S46P, N143D, T236A, A251V, and H272R) in the GluCl gene sequence of head lice [26]. Of these, S46P, A251V, and H272R were located in the N-terminal extracellular domain, TM3 domain, and M3-M4 connection strand of the GluCl channel, respectively (Fig. 3) [26]. Additionally, Njue et al. (2004) reported several mutations (E114G, V235A, and L256F) in the N-terminal extracellular domain of the GluCla3 channel, as well as V60A and R101H in the same domain of the GluClb channel in Cooperia oncophora, an intestinal worm species related to cattle [36]. Their electrophysiological studies demonstrated that the L256F mutation in the GluCla3 channel led to a threefold decrease in sensitivity to ivermectin [36]. Most of the reported mutations, similar to those identified in the current study, were located in the N-terminal extracellular domain of GluCl channels. The frequency of the S46P mutation in the Iranian head louse population was 28%, whereas it was 85.71% for R37K and E50K, which were 25 and 11 nucleotides away from the S46P mutation, respectively. Based on these findings, we hypothesize that the identified mutations contribute to ivermectin resistance in head lice. The close proximity of R37K and E50K to the previously reported S46P mutation suggests a possible combined effect in resistance mechanisms. Additionally, the high similarity between Iranian and GenBank

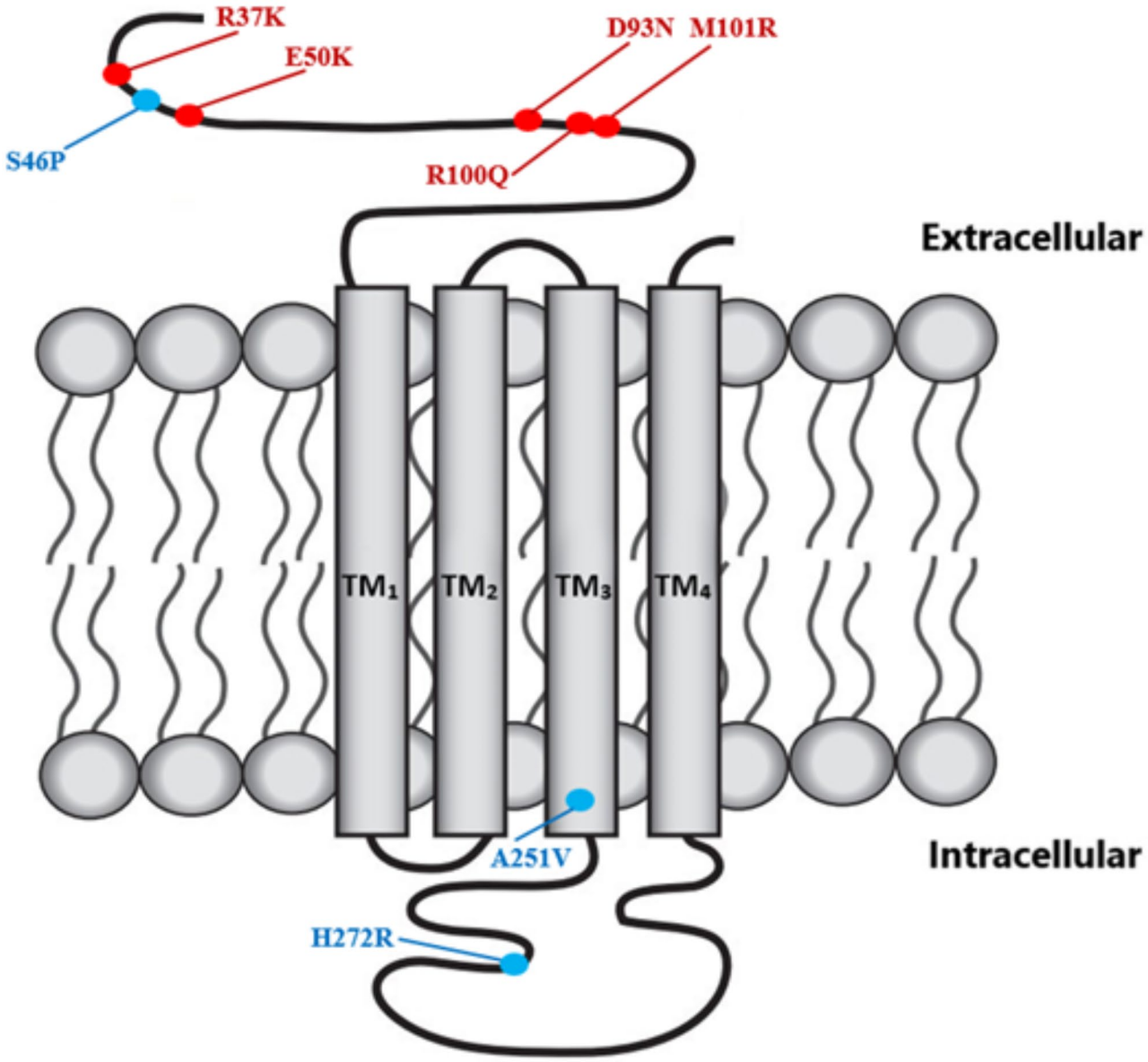

Fig. 3 Glutamate chloride channel subunit of head louse. The red dots indicate the location of nonsynonymous mutations identified in the head lice samples of the present study, the blue dots indicate the location of the mutations of the study by Amanzougaghene and his colleagues in 2018. R = Arginine, K = Lysine, E = Glutamic acid, D = Aspartic acid, N = Asparagine, Q = Glutamine, M = Methionine, S = Serine, Proline = P, Alanine = A, Valine = V, Histidine = H

sequences, coupled with the identified mutations, indicates a conserved yet adaptable genetic structure in response to insecticide pressure. However, more studies are needed to provide more evidence connecting these mutations to ivermectin resistance.

Insects can develop cross-resistance to multiple insecticides, complicating pest management strategies. Cross-resistance, where resistance to one insecticide confers resistance to others with similar modes of action, limits its control options [37, 38]. For example, mutations in the GluCl gene can cause cross-resistance with other insecticides, including Milbemycins, Emamectin benzoate, Fipronil, and Spinosad [39–42]. Recent reports have indicated cross-resistance between milbemycin and avermectin insecticides [43, 44]. Studies show a direct association between abamectin and milbemectin resistance in European two-spotted tick populations, suggesting that abamectin metabolic resistance mechanisms, such as cytochrome P450, are widespread among Tetranychus urticae populations in Europe [39, 45]. Cytochrome P450 enzyme activity is a major mechanism in the resistance of insects to various insecticides, including

avermectins [45–49]. Although the resistance mechanisms to ivermectin in lice are not yet fully understood, there is a known relationship between cytochrome P450 enzyme activity and body louse resistance to ivermectin [50]. Desensitization of target sites is the most common resistance mechanism in insects [40]. Knock-down resistance in head louse populations in several Iranian provinces [13–15, 32] is due to target site desensitization [40]. The correlation between mutations in the GluCl gene and reduced sensitivity of GluCl channels to avermectin insecticides suggests that desensitization may play a role. However, none of the five new mutations identified in this study have been definitively linked to ivermectin resistance. Functional studies, protein structure analysis, or molecular docking studies are needed to confirm this association. These mutations may reflect natural variation among different populations from various geographic regions rather than a specific connection to ivermectin resistance. Without phenotypic experiments and functional studies, it is not possible to determine whether this mechanism is the primary contributor to this reduced sensitivity.

Future research should aim to validate the functional impact of these mutations on resistance phenotypes through bioassays with ivermectin, protein structure analysis, and molecular docking studies. Techniques such as CRISPR/Cas9 and voltage clamp electrophysiology (e.g., expression of mutant GluCl in Xenopus oocytes followed by measuring ivermectin's agonistic activity) can provide deeper insights into the mechanisms of resistance. Additionally, exploring the prevalence of these mutations in other regions and their correlation with resistance levels will enhance our understanding of resistance spread and evolution.

Smart utilization of insecticides and comprehensive resistance management strategies are essential to combat the spread of insecticide resistance. The identification of novel mutations in the GluCl gene raises concerns about the potential emergence of ivermectin resistance. Future experiments should explore these potential links to develop effective control measures. By staying updated with research findings and implementing appropriate measures, we can better address the challenge of insecticide resistance in head louse populations.

Limitations

Our study, while comprehensive, has limitations. The sample size was relatively small, with only 61 head lice collected and 27 sequenced. A larger sample size could provide a more robust dataset and enhance the generalizability of our findings. Additionally, our focus was solely on the GluCl gene, potentially overlooking other resistance mechanisms. Future studies should include other genes and pathways involved in resistance to provide a holistic view.

Acknowledgements

The authors express their gratitude to the Health and Biomedical Informatics Research Center, Urmia University of Medical Sciences, for the financially supporting of this project (No. IR.UMSU.UC.1401.410).

Author contributions

Conceived and designed the experiments: SG. Performed the experiments: VM and SG. Analyzed the data: SG and VM. Contributed reagents/materials/analysis tools: VM and SG. Wrote the paper: VM, and SG. All authors have read and approved the final manuscript.

Funding

The present research has received a financial support from the "Urmia University of Medical Sciences, Urmia, Iran" (Code: IR.UMSU.UC.1401.410).

Data availability

The nucleotide and related amino acid sequences obtained from the current study were submitted to GenBank, the European Molecular Biology Laboratory (EMBL), and DNA Data Bank of Japan (DDBJ) databases and accessible with accession numbers OR965460-OR965473.

Declarations

Ethics approval and consent to participate

The study was granted approval by the Ethics Committee of the Research Department of the School of Public Health, Urmia University of Medical Sciences, with permit number: IR.UMSU.REC.1401.410. Participants signed a written informed consent form before participating in the study, in accordance with the guidelines of the Declaration of Helsinki. Informed consent to participate was obtained from the parents or legal guardians of the minors.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 16 September 2024 / Accepted: 5 March 2025
Published online: 18 March 2025

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