ORIGINAL ARTICLE

Permethrin-associated *kdr* Mutations through Molecular Analysis of Human Head Lice (Phthiraptera: Pediculidae) Populations in School Children in the South of Iran

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Abstract

Background: Human head lice is one of the most invincible neglected skin diseases. The use of pyrethroid insecticides is a standard method of treating the disease, which leads to lice population resistance in the long run. The main aim of the current survey was to screen the biomarkers of permethrin-associated *kdr* (knockdown resistance) point mutations through molecular analysis of the human head lice populations in primary school children in the south of Iran.

Methods: In an experimental study, Field-collected head lice from infested students were fixed in ethanol, identified using valid taxonomic keys, and processed by PCR for *kdr* mutant studies. Sequencing partial voltage-sensitive sodium channel gene in different head lice populations was subsequently implemented and compared with the permethrin-resistant diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) as the gold standard.

Results: Human head lice appeared to reflect *kdr* point mutations in specimens from the city of Shiraz. At least three amino acid mutations at designated sites of D820E, L840F, and N874G, corresponding to replacements of aspartic acid to glutamic acid, leucine to phenylalanine, and asparagine to glycine, are clear in this representative population, respectively. At the same time, only L840F is reported as a new mutant in this survey.

Conclusion: The ongoing treatment of head lice infested in school children harboring *kdr*-mutated or permethrin-resistant mutants in Shiraz is risky, illogical, and contrary to the one Health initiative of the World Health Organization. Health executives should thus immediately take the indispensable steps to prohibit further procurement of permethrin.

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Introduction

Human lice often called sucking lice (Order: Anoplura), are hemi-metabolic ectoparasitic insects. They feed directly from blood vessels by piercing host skin using their long extruded proboscis (solenophagy), engorging exclusively on blood, the sole source of their diet.[1] Over 530 different lice species have been identified to be associated with placental mammals.[1] Only two genera of these are obligatory ectoparasites of *Homo sapiens,*

Pediculus humanus, and *Pthirus pubis*.[2] The latter crab-like louse lives commonly among pubic hairs of primates (gorillas), while *Pediculus* is found on chimpanzees and New World monkeys.[2-4] It is thus estimated that hematophagous lice coevolved with primates about 25 million years ago, and head lice differentiated within the recent 2 million years of this co-evolutionary life history.[5, 6]

Pediculus humanus has two genetically distinct bodies (*P. humanus corporis*) and head (*P. humanus capitis*) lice ecological types, which are morphologically indistinguishable but have disparate eco-genetic niches.[7, 8] The head louse, which permanently lives and lays eggs (nits) at the base of hair shafts, has three nymphs, and adult male and female head lice feed on human blood about six times a day. Each adult female may lay around 150 eggs during her lifetime, lasting 2-4 weeks.[9]

Head lice infestation or pediculosis capitis due to the presence of *Pediculus humanus capitis* De Geer, 1767, (Phthiraptera; Anoplura: Pediculidae) is a deleterious public health menace in school-aged children in both developing and developed worlds.[10] This infestation, also known as Vagabond's disease, instigates intense scalp irritation and social ostracism , leading to anxiety and school exclusion. Because of the variable morbidity, social stigma, and reduced efficiency associated with the louse burden, the World Health Organization (WHO) has lately added "scabies and other ectoparasitic infestations and leishmaniasis" to the priority list of neglected tropical diseases.[11-14]

Female school children are more susceptible than males (2-12 times higher).[15] The prevalence of this infestation globally varies between 0.7%-59%, depending on several variables such as hygienic status, overcrowding, season, prior pediculicide usage, *etc*.[16] According to epidemiological research around the world, different countries have reported different numbers of pediculosis infestations amoung school children. For example, in Mexico 13.6%,[17] in Jordan 26.6%,[18] in South Africa 15.3%,[19] in Thailand 23.32%,[20] in Nigeria 26.4%,[21] and in the United Kingdom 28.3%.[22]

Researchers conducted epidemiological studies of pediculosis in different regions of Iranand reported the prevalence of head lice in the central region of Iran (Khomein city) to be 11.9%.[23] Also, pediculosis is 7.6% prevalent in girls' primary schools in Qom province.[24] The prevalence of pediculosis (head lice) in Iran shows that the prevalence of total pediculosis varies from 0.47% in Isfahan to 27% in the southeastern region of Iran (Sistan Baluchestan).[25] Student family size, number of students in the classroom, history of head lice infestation in student parents, bathroom sharing shoulder and towel sharing behavior are important risk factors in the prevalence of head lice in the northwest of Iran.[25] Point prevalence is, however, a more accurate indicator.[26] The elimination of *P. humanus capitis* is usually based on the topical application of insecticide formulated products. These usually are available as over-the-counter (OTC) medicines that may have different classes of insecticides, in particular pyrethroid. Large-scale use of these OTC products is inevitable since the infestation is widely spread worldwide. Consequently, incrementing treatment resistance in different head lice populations is widely recorded.[15, 27-30]

It has been a long time that administrators know OTCs have lost their efficacies in Iran. Most of these comprise permethrin, lindane, phenothrin, or Sumithrin.[30] Pyrethroid insecticides, such as permethrin, target the site of voltage-sensitive sodium channels (VSSC) along the nerve axon, causing the prolonged opening of the gates and extensive influx of sodium ions which culminates in continued depolarization and muscle seizure, and finally, death of head lice.[31] Excessive application of permethrin induces single-base point mutations in subunits of the VSSC gene, which leads to operational nerve insensitivity in head lice.[32] In addition, other studies have reported mutations in human genes due to environmental factors.[33, 34] Knockdown resistance (*kdr*) mutation is the most typical resistance mechanism to insecticides through target site insensitivity of the VSSC α-subunit gene,[35] where single-base mutations are used as biomarkers in *kdr.* Molecular resistance mechanisms investigated the determination of this single nucleotide polymorphism (SNP) with genomic DNA extracted from *P. humanus capitis* specimens.[36]

High and rapid diversification into different phylogenetic clades points to the association between humans and head lice dating back to millions of years ago.[37] Researchers have globally differentiated head lice into six clades (A-F) based on their mitochondrial DNA data and grouped them according to their geographical propagation. There is thus an indispensable need to monitor these mutations through geographically-specific genetic biomarkers[16] because of increasing failures to first-line treatment. This failure happened since allele mutations related to pyrethroid resistance differ between regions. This study mainly addressed permethrin-associated *kdr* mutations through molecular analysis of human head lice in school children in the south of Iran.

Methods

Sample Collection

P. humanus capitis specimens were collected from primary schools in four different cities, including Shiraz (n=50; 29°36′36″N, 52°32′33″E),

Zarin-Dasht (n=35; 28°20′N, 54°20′E), Sepidan (n=40; 30°10′N, 52°00′E), and Firuzabad (n=45; 28°50′38″N, 52°34′15″E) (Figure 1) using special plastic detection combs (PDC) as implemented in our previous study.[30] *P. humanus capitis* specimens were examined for their morphological specifications using the stereomicroscope according to a valid diagnostic key for the adult stage.[38] The Directorate of Shiraz University of Medical Sciences (SUMS), School of Health, Ethics Committee, confirmed the head lice collection protocol under the licence number IR.SUMS.REC.1399.542. The parent of each child provided the written informed consent. Collected *P. humanus capitis* specimens were transferred to the laboratory and preserved in 70% ethanol until identification and molecular tests.

Primer Design

The partial genome of Cytochrome c Oxidase subunit 1 (CO1) in P. humanus capitis that other researchers have sequenced, distinguished using the National Center for Biotechnology Information (NCBI). First, the DNA sequences of voltage-sensitive sodium channel genes, including AY191157.1, AY191157.1, MK673335, KX301990.1, KX301989.1, and AB090951.1 aligned using the MEGA6.0 software (Version 6.0). Following analysis, two regions were selected to design gene-specific primers (GSPs).[39] Two forward and reverse primers, including Fcap (5'-ATTTTGCGTATGGGACTGC-3') and Rcap (5'-TCCATCTGGGAAGTTCTTTATCC-3'), were assigned as starter and end primers, respectively, to characterize the partial sequence of the target gene. The expected size amplicon was 903 bp. This study used Gene Runner 0.04, Oligo 0.7, and BLAST (online tool) to design primers on the exon regions.[40]

DNA Extraction

Head lice were grouped into pools of 5-10 specimens transferred to sterile micro-tubes and subjected to DNA extraction and molecular experiments. Each specimen was homogenized using a tissue homogenizer (Thomas Scientific, United States) at 6000 g for 110 s, and DNA extraction was done using the commercial kit (Gene All, South Korea) by following the manufacturer's instructions and then stored at -20 °C.

Polymerase Chain Reaction (PCR) Assay

The PCR assay was performed on all specimens of head lice that were collected from studied areas. The total volume of PCR mixtures was 20 µl and comprised 0.2 µl *Taq* (thermophilus aquaticus) DNA polymerase, 8 µl of 2.5X Master mix, 9.8 µl DNase/RNase-free double distilled water ($2H_2O$), 1 µl of 10 pmol/µl of each primer, and 1µl of DNA template (100-200 ng/µl). All PCR components were bought from Sinaclone Company, Iran. Negative control was included in each trial. PCR program was as follows: 5 min at 94 °C as initial denaturation, and 35 cycles of 30 s each at 95 °C, 56 °C, and 72 °C, with a terminal extension for 10 min at 72 °C.[39] All PCR constituents were subjected to agarose gel electrophoresis in 0.5× Tris-Acetate EDTA buffer, and the amplified fragments were discerned after staining using the DNA safe stain (Sinaclone Co., Iran) with UV light in Gel Documentation systems (Bio-Equip, UK).

DNA Extraction from Agarose Gel

The expected size bands were recovered from the agarose gel using the GF1 gel extraction kit (Vivantis, Malaysia) according to the manufacturer's protocols

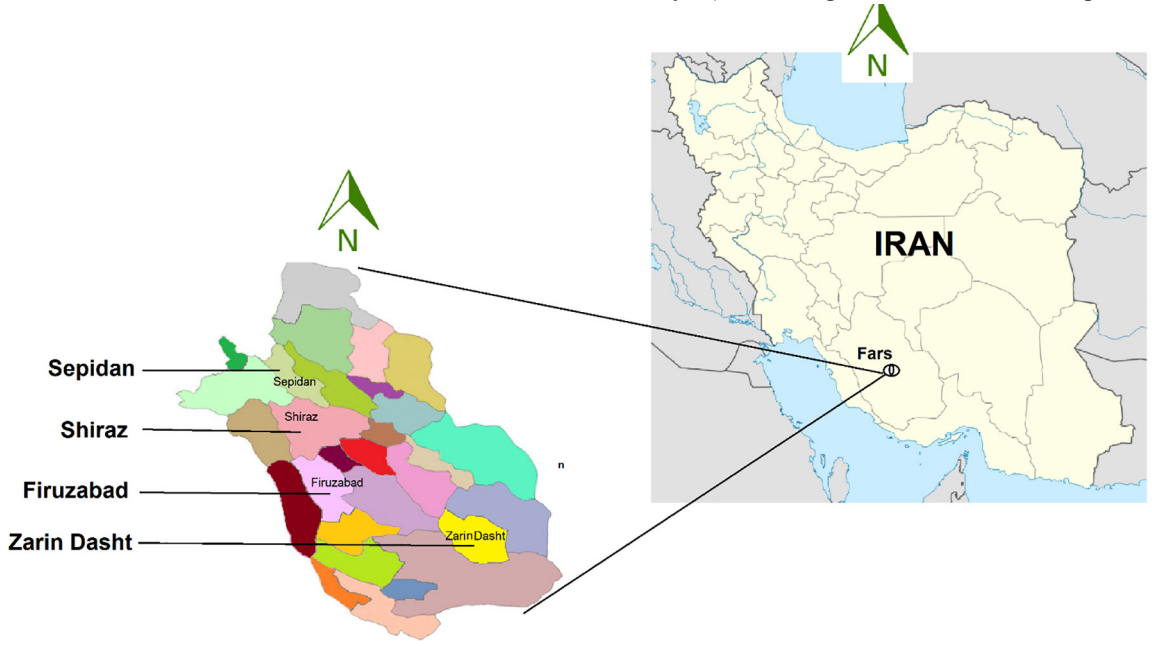

Figure 1: Map of Fars province, Iran, indicating the capital city of Fars province, Shiraz, and three other nearby cities including Sepidan, Firuzabad, and Zarin Dasht.

(Reference is required). Then, sequencing in both directions was conducted based on the forward and reverse GSP primers obtained from Pishgam Company in Iran.

Sequencing

One PCR production sample from each population (n=4) with sizes close to the predicted range (900 bp) was sequenced using the GSPs forward (Fcap) and reverse primers (Rcap), then their analyses were performed by EditMan and SeqMan (Version 7.10, 2006). Finally, the samples were characterized based on the saved sequences in GenBank.

Results

The present study showed that collected head lice species in Shiraz are resistant to permethrin, and a new mutation was observed in amino acid No. 840, in which leucine (L) was replaced by phenylalanine (F). The amplified region on DNA incorporated 903 bp with 4 introns and 5 exons. The forward and reverse primers were designed on these exons. The PCR on DNA revealed an expected band of 903 bp (Figure 2). The amplified genome had its 5 exon regions encoding 130 acids from amino acids 784 to 913 by performing bioinformatics analysis on *kdr* genes of human head lice using MEGA0.6 (Molecular Evolutionary Genetics Analysis version 0.6) (Figure 3).

In the present study, researchers have sequenced a genomic fragment of 903 bp for the *kdr* gene from head lice originating from four cities in Fars province. The result of *kdr* gene sequencing in Shiraz was registered with the accession number MZ688383 in the Genebank. These samples were aligned with permethrin-resistant diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) (designated as DBM-R) as well as permethrin-sensitive human head lice (HL-S) with MEGA0.6 (ClustalW) software and compared. Figure 3 presents the results. This study demonstrated that head lice collected from the city of Shiraz changed their amino acid No. 820 from aspartic acid (D) to glutamic acid (E). A new mutation was also substituted in amino acid No. 840, in which leucine (L) was replaced with phenylalanine (F). In addition, at amino acid No. 874 in head lice samples taken from Shiraz, asparagine (N) changed to amino acid glycine (G). This study revealed that head lice samples from Shiraz had mutated most probably due to noncompliant persistent exposure to permethrin. Furthermore, the present study exposed that samples of head lice provided from Firuzabad, Sepidan, and Zarin-Dasht did not exhibit any mutations in their amino acid sequence and therefore appeared to be entirely susceptible to permethrin.

Discussion

This study demonstrated that human head lice species caught from Shiraz have three mutations in amino acids Nos. D820E, L840F, and N874G, while a change in the amino acid at site No. 840 from leucine to phenylalanine was a new mutation compared to the reference moth species, DBM-R. This report corroborates other reports of permethrin refractoriness in head lice populations from the United Kingdom,[41] Palestine,[31] the United States,[31] and recently in northwest Iran.[42] In the previous study conducted by Firooziyan *et al.* in 2017, they

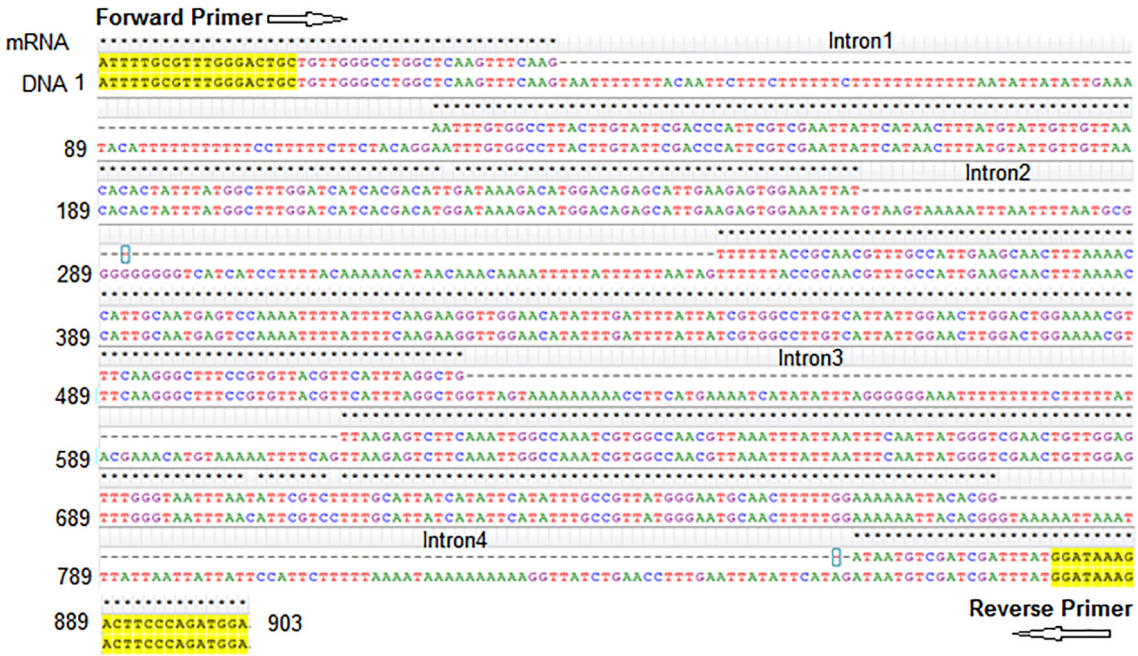

Figure 2: Sequence of amplified nucleotide bases of human head lice in school children in Fars province, Iran. Forward and reverse primers are highlighted at the start and end of the sequence. The amplified region of this genome has 4 introns and 5 exons.

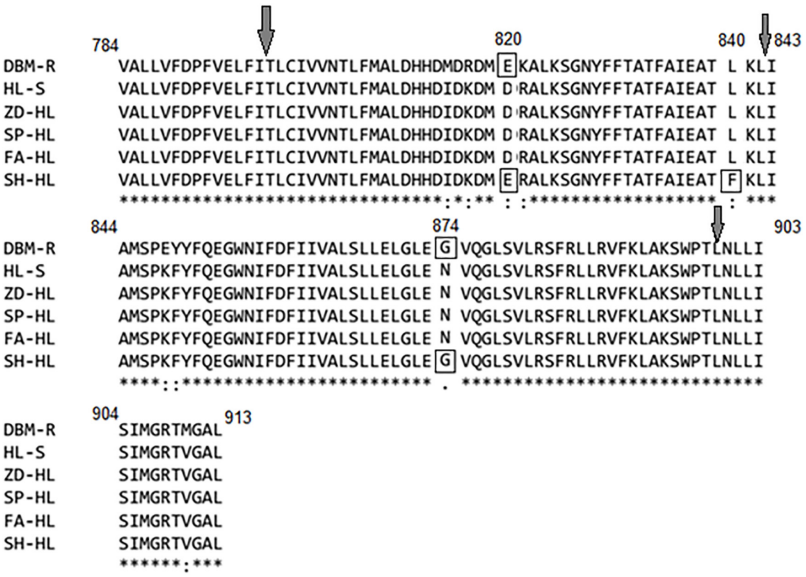

Figure 3: Alignment of amino acid 784 to 913 in human head lice species from the cities of Shiraz [SH-HL], Sepidan [SP-HL], Zarrin Dasht [ZD-HL], and Firuzabad [FA-HL]. In addition, permethrin-resistant diamondback moth species [DBM-R], and permethrin-susceptible human head lice species [HL-S] have been shown for comparison. The head lice in Shiraz have mutations in amino acids 820, 840, and 874 (square boxed), which most likely reflect resistance to permethrin. *Symbols*: downward arrows indicate the locations of introns in genomic DNA. Stars reflect homogeneity, and semicolons show heterogeneity in amino acids between reference species (DBM-R) and head lice samples from four different localities in Fars province, Iran.

reported six novel mutations placed in the IIS5 (I927F, L928A, R929V, L930M, and L932M) and IIS1-2 (H813P) of the α-subunit of VSSC of the nerve axon. These data exhibited that resistance-related alleles performed through *kdr* mechanisms with most haplotypes recorded RS heterozygotes.[42]

Another recent study conducted by Karakus *et al.* in Turkey reported three amino acid mutation sites at L920F, M815I, and T917I, all identified using real-time PCR.[43] Of the studied specimens, resistance allele frequency (RAF) was 0.99 for L920F, 1.00 for M815I, and 0.98 for T917I. The presence of *kdr*-related novel mutations in the sodium channel is likely to be the reason for the frequent treatment failures against human head lice in this area due to the uncontrolled use of permethrin insecticides.[32] It suggests that these failures commonly occurring in these countries may be dueto the permethrin resistance observed in these populations.

Within our head lice genomic fragment, we recognized four introns and five exons. Their locations for the mentioned specimens are illustrated in Figure 3. The nucleotides GT splice donor-acceptor sequences were well maintained in all introns. The heterogeneity in amino acid sequence between the reference moth species (DBM-R) and other human head lice species appeared at only 6 sites along the alignment sequence (indicated by a semicolon underneath each letter). Our study revealed that head lice specimens provided from the other nearby cities, including Firuzabad, Sepidan, and Zarin-Dasht, did not have any mutations in their amino acid sequence, and therefore appeared to be thoroughly sensitive to permethrin.

Pediculosis capitis is one of the significant public health problems among school children, and the cost of damages it incurs on the economy is far greater than what can be estimated. More than 100 research studies on head lice infestations have been performed in Iran from 2010 to 2019, and all were conducted in primary schools. The infestation rate differs between 0.54%–34.1%, and compared to boys; girls always exhibited a higher infestation rate in previous Turkish head lice studies.[43, 44]

Recent Iranian studies[45, 46] reported that families of infested children usually involve re-infestation even if successful treatment is implemented.[47, 48] The absence of a nurse as the first responsible person to notice the head lice infestation in primary schoolsin Iran, makes it harder to control.[41] This situation is exacerbated when considering the concomitant susceptibility and exposure of most of these children to one or more endemic vector-borne infectious diseases coupled with indoor residual spraying (IRS) of insecticides in this tropical part of the world.[49-53]

Conclusion

in contrast to the other three cities in Fars province in Iran, the detection of three amino acid mutations in human head lice in Shiraz, , points to the region-specific treatment failures due to the constant use of permethrin in recent years. Regional health executives should thus be alarmed and advised to monitor the increasing frequency of allele mutations before any resistance trend moves into the fixation phase which will be very hard to eradicate in vulnerable hot spot regions.

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Conflicts of interest: None declared.

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