Pyrethroid-linked resistance allelic mutations by molecular analysis in wild human head louse (Phthiraptera: Pediculidae) populations from schoolgirls of South Iran

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ABSTRACT

Background: Human head louse, Pediculus humanus capitis De Geer, 1767 (Anoplura: Pediculidae), is one of the most frequent ectoparasites infesting Homo sapiens worldwide. Reduced sensitivity to treatment due to genetic mutations, in particular knockdown resistance (kdr) (or target site insensitivity) allele, has led to this infestation prevalence. Molecular characterization of this resistance has a crucial impact on selecting appropriate treatment protocol. The aim of this study was to investigate kdr gene mutations on voltage-sensitive sodium channel (VSSC) among wild head lice samples from Fars province, southern Iran.

Methods: Head lice were collected using plastic detection combs on girls enrolled in public schools from 10 counties in Fars province. The specimens were screened in 10 pools (each pool per county containing 35 specimens), with three pools (30%) being positive. Following species identification with valid entomological keys, 350 (68%) out of 514 randomly collected adult head lice were analyzed after their somatic genomic DNA extraction using Sinaclon kit. Samples were investigated by polymerase chain reactions (PCR), and the amplicon was subsequently sequenced.

Results: Sequence analysis showed that the sodium channel genes in the pooled ectoparasites had two intron and three exon regions. Single (L840F), double (I836L, E837K), and triple novel point mutations (V875L, Q876P, S879V); the last involving two concomitant allelic substitutions; were discovered in the second and third exon regions of head louse DNA on chromosome II from three (30%) counties. Other exon or intron regions remained non-mutated from the remaining seven counties.

Conclusions: The detection of six amino acid substitutions from 30% of examined head lice among infested schoolgirls reveal that mutants are minutely developing. These findings provide further incentive to recapitulate the legitimacy of current control measures and resolve dynamics of resistance in human head louse populations.

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1. Background

Human head louse infestation, known as head pediculosis, due to the species of *Pediculus humanus capitis* De Geer, 1767; (Anoplura: Pediculidae) is one of the irritating public health problems in most regions, including high income- and middle- to low-income countries. Although all age groups are susceptible to this infestation, it is primarily present among schoolgirls globally (Candy et al., 2018). This infestation becomes a significant health concern while its frequency rises since interventions to suppress and mitigate these ectoparasites pose even more significant risks to susceptible human populations than the infestation itself (Pollack et al., 2000).

The head lice, *P. h. capitis*, feed exclusively by piercing and sucking on blood from sub-epidermal capillaries of the head with sharp proboscis. They are highly host-specific and spend their entire metabolic life history on humans. Their predominant transmission route is through direct contacts, particularly among 4-13 years old children (Moradiasl et al., 2018). Although this infestation does not categorically lead to specific pathogenic infections, the bite reactions from head lice cause intense pruritus that may lead to secondary microbial wound infections (Verax and Raoult, 2012). The World Health Organization (WHO) has recently added “scabies and other ectoparasites” (including head lice) to the priority list of neglected tropical diseases (Casulli, 2021).

While the prevalence of head louse infestations varies across regions, a recent meta-analysis-based systematic review has determined that the global rate of these ectoparasites is about 19% (Hatam-Nahavandi et al., 2020). In addition, whereas all age groups are susceptible to infestation, head lice primarily affect children and adolescents, with particular dominance in girls (Moradiasl et al., 2018). Its prevalence rate among infested local schoolchildren in Iran is 7.4–10.5% (Nejati et al., 2018; Moosazadeh et al., 2015). These rates express the scope and variability of this menace.

A range of interventions is available to control head lice as they are not invulnerable to physical or chemical practices. The mainstay of therapy has explicitly been insecticides (Moosazadeh et al., 2015). *P. h. capitis* control depends primarily on the topical application of pharmaceutically based products. These are usually available as over-the-counter (OTC) medicines that may have different classes of insecticides, in particular pyrethroids. Large-scale application of these OTC products is inevitable since the infestation is widely spread worldwide. Consequently, increasing therapeutic resistance in different head lice populations is widely reported (Downs et al., 2002; Hunter and Barker, 2003; Kristensen et al., 2006; Moemenbellah-Fard et al., 2016; Kalari et al., 2019).

Treatment with the pyrethrins- and pyrethroid-based products has led to a high degree of resistance even though organochlorine insensitivities were effectively operative (Mohammadi et al., 2021). Subsequently, it is essential to use efficient drugs with different active ingredients to treat this infestation. Pyrethroids, such as permethrin, attack the site of voltage-sensitive sodium channels (VSSC) on the nodes of Ranvier along the nerve axon. This action causes long opening of the gates and extensive influx of sodium ions which results in uninterrupted depolarization (reduction or reversal of the potential difference across the nerve cell membrane at rest) and muscle seizure, and finally, a reversible sudden “death-like effect” of head lice (Hemingway et al., 1999). Excessive use of permethrin induces through direct epigenetic mechanism single-base point mutations in the VSSC coding gene subunits, which leads to reduced neuronal sensitivity in the head louse (Firooziyan et al., 2017).

### Table 1

The allelic and amino acid substitutions tabulated for each head lice pool geographical sampling area alongside their abundance in each case. The mutated alleles are in color.

| County     | Coordinates       | Number of head lice collected | Adult male head lice | Adult female head lice | Number of homogenized samples | Sample weight (mg) | Mutations | Amino acid substitution                | Nucleotide substitutions                  |
|------------|-------------------|-------------------------------|----------------------|------------------------|-------------------------------|--------------------|-----------|---------------------------------------|-------------------------------------------|
| Shiraz     | 29°36’36”N, 52°32’33”E | 62                             | 25                    | 37                     | 35                            | 20                 | L840F     | Leucine-Phenylalanine               | CTT/TTT                                   |
| Marvdasht  | 29°87’87”N, 52°82’06”E | 89                             | 51                    | 38                     | 35                            | 22                 | V875L, Q876P, S879V.      | GTC/CTC, CAG/CGG, TCG/GTG                 |
| Fasa       | 28°94’40”N, 53°63’39”E | 41                             | 24                    | 17                     | 35                            | 18                 |                                      |                                           |
| Larestan   | 27°67’41”N, 54°33’58”E | 47                             | 16                    | 31                     | 35                            | 24                 |                                      |                                           |
| Kazeroun   | 29°62’71”N, 51°65’18”E | 46                             | 13                    | 33                     | 35                            | 19                 |                                      |                                           |
| Abadeh     | 31°15’95”N, 52°64’40”E | 40                             | 16                    | 24                     | 35                            | 22                 |                                      |                                           |
| Firuzabad  | 28°50’38”N, 52°34’10”E | 60                             | 31                    | 29                     | 35                            | 23                 |                                      |                                           |
| Zarindasht | 28°20’00”N, 54°20’00”E | 39                             | 18                    | 21                     | 35                            | 20                 |                                      |                                           |
| Noorabad   | 34°06’65”N, 47°97’63”E | 37                             | 17                    | 20                     | 35                            | 21                 | L836L, L837K.           | ATT/CTT, GAA/AAA                           |
| Sepidan    | 30°10’00”N, 52°00’00”E | 53                             | 23                    | 30                     | 35                            | 22                 |                                      |                                           |
Knockdown resistance (kdr) gene mutation is the most typical resistance mechanism to insecticides through target site insensitivity of the VSSC α-subunit gene (Mohammad Rezaei et al., 2019), where single-base mutations are used as biomarkers. Since kdr mutation could be closely associated with the lack of proper response to pyrethroids, its existence as a mechanism in the field has grave consequences for sustained use of these chemicals in head lice control. There is an essential need to monitor these mutations through geographically-specific genetic biomarkers due to increasing failures to first-line treatment (Fox et al., 2020). This failure took place since allele mutations related to pyrethroid resistance differ between regions. This study aimed to explore permethrin-associated kdr gene mutations through molecular analysis of human head lice in schoolgirls in south Iran.

2. Materials and methods

2.1. Sample collection

P. h. capitis specimens were collected from primary schools in ten different counties which included Shiraz, Zarindasht, Sepidan, Firoozabad, Fasa, Kazeroon, Larestan, Noorabad, Marvdasht, and Abadeh (Table 1, Fig. 1). The specimens were screened in 10 pools with three pools (30%) being positive. As previously reported, standard plastic detection combs (PDC) were used (Kalari et al., 2019). P. h. capitis specimens were examined for morphological characteristics using a stereomicroscope according to a valid diagnostic key for the adult stage (Control CfD, Prevention, 2003). Specimens were collected in accord with their accessibility. The Directorate of Shiraz University of Medical Sciences (SUMS), School of Health, Ethics Committees, confirmed the head louse collection protocol under the IR license.SUMS.SCHEANUT.REC.1400.011. The parent or guardian of each louse-provider gave written informed consent. The collected P. h. capitis specimens were taken to the laboratory and preserved in 70% ethanol preceding identification and molecular analysis.

2.2. Primer design

The partial genome of kdr gene in P. h. capitis, sequenced by other researchers, was retrieved from the National Center for Biotechnology Information (NCBI) database. First, the DNA sequences of VSSC genes, including AY191157.1, KX302005.1, KX301992.1, KX301991.1, and KX301988.1, were aligned using the MEGA6.0 software (Version 6.0). Following analysis, two regions

Fig. 1. Map of Fars province within Iran shows the counties (enlarged map on the left) where samples were collected.
were chosen to design gene-specific primers (GSPs) (Alipour et al., 2017). Two forward and reverse primers, including HLF (GAATTTGTGGCCTTACTTGTATTCGA) and HLR (TAAATTACCCAAAGCTCCAACAGTTC), were allocated as start and end primers, respectively, to identify the partial sequence of the target gene. The expected size amplicon was 582 bp. This study implemented Gene Runner 0.04, Oligo 0.7, and BLAST (online tool) to design primers on the exon regions (Ebrahimi et al., 2021a).

2.3. DNA extraction

Randomly selected head lice were arranged into 10 pools, each pool of 35 specimens per county, transferred to sterile micro-tubes, and subjected to DNA extraction and molecular analysis. Each sample was homogenized with a tissue homogenizer (Thomas Scientific, United States) at 12000 g for 600 s. DNA extraction was conducted using the commercial kit (Sinaclon Co., Sina Gene, Tehran, Iran) following the manufacturer’s instructions and stored at −20 °C.

2.4. Polymerase Chain Reaction (PCR) assay

The PCR assay was conducted on 350 out of 514 (68.1%) head lice specimens obtained from the surveyed counties. The total volume of PCR mixtures was 20 μl and included 10 μl of Master Mix Red, 7 μl double distilled water (2H2O), 1 μl of 10 pmol/μl of each primer, and 1 μl of DNA template (100–200 ng/μl). All PCR components were purchased from Sinaclon Company, Iran. Negative control was run 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, 60 °C, and 72 °C, with a terminal extension for 10 min at 72 °C (Alipour et al., 2017). All PCR constituents were exposed to agarose gel electrophoresis in 0.5 × Tris-Acetate EDTA buffer. The amplified fragments were seen after staining using the DNA Safe stain (Sinaclon Co.) with UV light Gel Documentation systems (Bio-Equip, UK).

2.5. DNA Extraction from agarose gel

According to the manufacturer’s protocols, the expected size bands were recovered from the agarose gel using the GF1 gel extraction kit (Vivantis, Malaysia). Then, sequencing in both directions was performed according to the forward and reverse GSP primers obtained from Pishgam Company in Iran.

2.6. Sequencing

One PCR production sample from each population (#10) with sizes close to the predicted range (582 bp) was sequenced using the GSPs forward (HLF) and reverse (HLR) primers. Then, sequencing in both directions was performed according to the forward and reverse GSP primers obtained from Pishgam Company in Iran. EditMan and SeqMan (Version 7.10, 2006) then performed their analyses by assembling. The resulting nucleic acid sequence was aligned with ClustalW (MEGA 6) then the sequences were BLAST in NCBI. Finally, the samples were characterized in accord with the saved sequences in GenBank. Using Sanger sequencing, the desired region was sequenced by Pishgam company/ Iran in the present study.

Fig. 2. The NCBI-derived kdr allelic DNA and RNA coding sequences of 582 bp amplicon (above) contain two introns and three exons (below).
3. Results

From a total of 514 adult lice (P. h. capitis), collected in ten counties of Fars province, only 350 (68.1%) were analyzed using their genomic DNAs. Ten pools, each with 35 lice per county (total 350), were thus analyzed. A survey of 582 bp nucleotide genes involving kdr allele of sodium channel receptor on chromosome number II of P. h. capitis was investigated for its sequence. They were compared with the GenBank AY191157.1 as a kdr-sensitive gene and the diamondback moth (DBM), Plutella xylostella (L.), as a gold standard strain permethrin-resistant gene model. The results of nucleic acid sequence analysis from 6902 to 7424 amplified by specific primers revealed two intron and three exon regions (Fig. 2). There were no mutations found in intron regions. Mutations were identified in head lice from Marvdasht, Noorabad, and Shiraz counties.

The sequence band size of the exon II region included 173 bp nucleotides. Point mutations from specimens collected in Shiraz and Noorabad resided in this exon II region. The substitution in amino acids from the head lice of Shiraz was due to L840F, corresponding to the replacement of leucine with the amino acid phenylalanine (an aromatic amino acid). Those from Noorabad were due to en bloc mutations of I836L and E837K, indicating isoleucine replaced with leucine and glutamic acid substituted with lysine, respectively (Fig. 3).

The sequence band size of the exon III region comprised 33 bp nucleotides. Triple mutations from head lice specimens collected in Marvdasht closely lay in this exon III region. The amino acid substitutions from head lice sodium channels were V875L, Q876P, and S879V (Fig. 3). The first two mutations occurred en bloc in permethrin-resistant field populations of head louse, while the third mutated substitution concomitantly involved two nucleotide conversions instead of one (Table 1). These changes were due to valine substituted with leucine, glutamate with proline (an imino or cyclic amino acid), and serine with valine, respectively. Other exon or intron regions revealed no point mutations from the remaining seven counties.

4. Discussion

The preceding results revealed that pooled adult head lice from three (30%) out of the 10 sampled areas had one or more gene mutations in the alpha subunit of the VSSC target receptor-coding gene involving a 582 bp amplicon. The unprecedented demonstration of six permethrin-linked gene mutations in lice, half of them from Marvdasht County next to the highly visitor-attractive ancient site of Persepolis, could be conjectured to be conducive to the spread of transmission. However, this speculation is unsupported by the obligatory and permanent ectoparasitic lice cycle, their close-contact transmission mechanism, and their prevalence in children. In addition, these gene mutations may have variable selective values over one another during the evolutionary course of the transmission cycle. For instance, it has been shown by various research groups that a TI (threonine to isoleucine) conversion constituted 50% of all globally reported mutations so far (Mohammadi et al., 2021). The significance of this conversion and the mutations found in the present study (e.g., leucine to phenylalanine in Shiraz), corroborated by previous reports (Ebrahimi et al., 2021b), appear to be more influential in forging refractoriness or susceptibility to pyrethroid insecticides than other mutations during the evolutionary history. More research is thus indispensable to elucidate the selective or associative value of each of these mutations in region-specific lice communities among hotspot human populations. The current study revealed that the substitution in amino acids from the head lice of Shiraz was due to L840F, corresponding to the replacement of leucine with the amino acid phenylalanine (an aromatic amino acid). This substitution is considered to reduce or postpone the insecticidal efficacy of permethrin.

Although these findings may appear normal at first, it signals an alarm that non-functional gene mutations are rapidly propagating through head louse populations. This study, however, corroborates previous reports of permethrin resistance in head louse populations from the United States (Yoon et al., 2014), the United Kingdom (Downs et al., 2002; Downs et al., 2000), Turkey (Karakuş et al., 2020),...
and recently in northwest Iran (Firooziyan et al., 2017). In the last report, researchers likewise identified six novel mutations in the head louse’s VSSC α-subunit gene of the nerve axon. These findings revealed that resistance-related alleles operated through *kdr* gene mechanisms with most recorded haplotypes of RS heterozygotes (Firooziyan et al., 2017).

Head pediculosis is indeed a neglected disease both at the national and individual family level in Iran, where several other infectious diseases could concomitantly occur in high-risk groups of people (Fakoorziba et al., 2006; Azizi et al., 2016; Neghab et al., 2014; Neghab et al., 2006). Its resistance patterns to pyrethroid-based medications differ geographically (25) since its developmental process has many individual steps, at each of which interaction may occur between the environment and the genotype or between various elements of the louse genotype. In a recent systematic review and meta-analysis, the *kdr* gene frequencies of only about a third of human head louse populations were susceptible to the pyrethroid-based OTC products in different countries of the world (Mohammadi et al., 2021). The remaining two-thirds were, however, resistant to the treatments. Most insensitivity to pyrethrin- and pyrethroid-based pediculicides (and to a lesser extent to the organochlorine DDT) was due to the *kdr*-type allelic mutations in the VSSC α-subunit target receptor-coding gene of head lice. Moreover, this resistance phenomenon is a reversible event following recessive allele mutations at one or more gene loci due to persistent and uncontrolled exposure to DDT, a pyrethroid, or both, at some point in their humanoid-associated co-evolutionary history.

These recessive resistance alleles appear to be rare in intervention-free populations of the head louse. It is, therefore, conceivable that increased abundance of diagnosis and treatment would precede more severe selection pressure on pediculicide reduced sensitivity, causing heterozygotes to propagate first through gene flow, while reversion of head louse target receptor coding genes to fully-sensitive allele status against a particular pediculicide medication could originate from its disuse during the long evolutionary history (Mohammadi et al., 2021).

Six new gene mutations located in the IIS5 (L927F, L928A, R929V, L930M, and L932M) and IIS1–2 (H813P) of the α-subunit of VSSC of the nerve axon have been reported from northwest Iran. These findings revealed resistance-linked alleles performed through *kdr* gene mutation mechanisms, with most haplotypes recorded as RS heterozygotes (Firooziyan et al., 2017). Another recent study in Turkey reported three amino acid mutation sites at L920F, M815I, and T917I, all identified using real-time PCR (Karakuş et al., 2020). 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 new gene mutations in the sodium channel was possibly due to frequent treatment failures against head lice based on the uncontrolled use of permethrin insecticides (Firooziyan et al., 2017). It suggests that failures commonly occurring in these countries may be due to the permethrin resistance observed in these populations (Ghahvechi Khaligh et al., 2021). However, one cannot state that a specific gene mutation determines a particular character or vice versa.

The strength of this study was that a sufficient sample size (350) was collected from 10 climatically and topographically diverse localities in Fars province, leading to the attainment of three pools with possibly permethrin-linked mutant head lice. The salient outcome of getting triple mutations from wild head louse specimens collected in the county of Marvdasht points unprecedentedly to the likely severity of this disease in this corner of the world.

5. Limitations

One of the fundamental limitations in this research study was that this work was not set to discriminate overall genotype as outlined in other studies since homozygous or heterozygous states could be identified using RFLP-PCR or ARMS-PCR, which were not implemented due to a predefined proposal. Using pools of 35 specimens per county was another hurdle in this work which was imposed due to the minimum sample collection size of 37 head lice from one county. The existence of SARS-CoV-2 (Covid-19) pandemic added another hurdle to our research progress.

6. Conclusions

Since the triple mutations exist *en bloc* as a permethrin-resistant haplotype, the two adjacent novel mutations (V875L and Q876P) may function to compensate for any fitness disadvantage associated with the validated T917I or T929I substitutions in permethrin-resistant human head lice from other reports. In contrast to the other seven counties in the Fars province of Iran, the detection of six amino acid mutations in human head lice in Shiraz, Noorabad, and Marvdasht could point to the region-specific treatment failures from the constant use of permethrin in recent years. Regional health executives should thus be warned. They are advised to monitor the increasing frequency of allele mutations before any resistance trend changes into the fixation state, which will be very hard to eradicate in vulnerable hot spot regions.

Declaration of Competing Interest

Authors declare that they have no conflict of interests.

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