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Detection of pyrethroids resistance alleles in goat biting louse *Bovicola caprae* (Phthiraptera: Trichodectidae) in west and northwest of Iran

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Abstract

Pyrethroid insecticides target voltage-gated sodium channels (VGSCs) that are essential for electrical signaling in the nervous system of insects. Three-point mutations at the corresponding amino acid sequence positions M815I, T917I, and L920F located in domain II conferring the knockdown resistance (kdr) are the most important mutations in pyrethroid-resistant lice worldwide. In addition, six new mutations have been reported in the extracellular loops IIS1-2 (H813P) and IIS5 (I927F, L928A, R929V, L930M, L932M) in the α- subunit of the sodium channel in lice. The aim of this study was to detect alleles resistant to pyrethroids in the domain II (S5-S6) of the VGSC gene in goat biting louse. Goat biting lice were collected from five provinces in the west and northwest of Iran. Genomic DNA was extracted from goat biting lice and *Bovicola (Damalinia) caprae* species was confirmed by amplifying the cytochrome oxidase subunit I (COXI) gene. A fragment in the domain II (S5-S6) of the VGSC gene was amplified using the specific primers and the resultant polymerase chain reaction products were sequenced. Substitutions T917I, L920F, I927F, L928A, R929V and L930M were identified in the examined sequences. The results showed that all the examined lice had at least one mutation in their VGSC gene associated with pyrethroid resistance or new mutations. The presence of these mutated alleles in the VGSC gene may be due to the long-term and multiple use of pyrethroids against arthropods. Thus, the molecular detection of resistance to pyrethroid insecticides in goat chewing lice can help plot a kdr frequency map to enact effective policies to control caprine pediculosis.

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Introduction

Bovicola (Damalinia) caprae is a goat chewing lice that have been reported in many countries as an external and obligate parasite of goats. It causes caprine pediculosis, mostly occurring in winter.[1,2] Caprine pediculosis is often associated with alopecia, skin irritation, papulocrustous dermatitis, self-excoriation, anemia, stress-associated production loss and growth reduction. Infestation with *Bovicola* biting lice stimulates T helper 2 cells immune response, causing pro-oxidative hematobiochemical damage in goats.[3]

Various insecticides are used to control lice in animals,[4] among which pyrethroids are highly selective insecticides that have been widely used in the past three decades to control a wide range of agricultural, animal, and public health pests.[5] Nonetheless, the extensive use of pyrethroid-based products has caused resistance in some lice species, including *Bovicola ovis, Bovicola ocellatus, Bovicola bovis, Haematopinus tuberculatus,* and *Haematopinus suis*.[6] The primary target sites of pyrethroids are voltage-gated sodium channel (VGSC) genes.[7] The VGSCs play a key role in creating action potential in the neurons of vertebrates and invertebrates. A sodium channel is a large and complex membrane protein (4,260 kDa). The main body of this channel comprises a single polypeptide (alpha subunit) that consists of four repeated domains (I-IV), each consisting of six membrane regions. These 24 segments are folded in the membrane to form the selective orifice of the sodium channel.[8] By altering the natural function of VGSC, these insecticides affect the nervous system of insects and cause uncontrolled action potential, fatigue, paralysis, and death.[9]

The knockdown resistance (kdr), is a key mechanism of pyrethroid resistance that diminishes nervous sensitivity to these insecticides. The kdr trait was first detected in

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houseflies, in which single nucleotide polymorphism (SNP) in the VGSC gene led to the substitution of leucine with phenylalanine amino acid in the 1014 position. The second mutation of M918T, along with L1014F (M918T+L1014F), is a genotype that causes higher levels of pyrethroid resistance and is known as the super-kdr phenotype. The kdr mutation has been recorded worldwide in numerous disease-carrying arthropods, and mutations in the sodium channel are currently known to be responsible for kdr.[10] The L1014F mutation that was first reported in houseflies has been documented in various species of *Anopheles*,[11] *Triatoma infestans*,[12] *Cimex lectularius*,[13] and *Rhipicephalus microplus*.[14] In addition to the L1014F mutation, several point mutations associated with super-kdr exist in the S4-S6 region of domain II in the VGSC sequence in several vectors, including the *Blattella germanica*,[15] *Pediculus humanus capitis*, [16] *Cimex lectularius*, *Cimex hemipterus,* and *Ctenocephalides felis*.[17] In the 1990s, reports emerged in the USA about the failure of permethrin to treat lice infestations.[18] Subsequently, Three point mutations in the coding positions of amino acids M815I, T917I, L920F were reported in the VGSC gene of permethrin-resistant human head lice[19] and were functionally confirmed as a kdr-type mutation.[18]

Identifying mutations associated with pyrethroid resistance in lice provides precise molecular markers for the rapid assessment of the frequency of resistance alleles in the field populations.[10] The early detection of pyrethroid resistance is crucial for selecting effective lice management strategies and can delay the further development of resistance. Therefore, establishing a rapid and precise resistance monitoring system using molecular markers is critical to maximizing the lifespan of the available insecticides; otherwise, the development and stabilization of resistance in lice will become a serious problem in pediculosis control. The current study counts as a first step toward the assessment of resistance alleles in lice collected from goats throughout five provinces of Iran.

Materials and Methods

Sampling. In this study, 509 chewing lice from five provinces in Iran including West Azerbaijan (37.5498° N, 45.0786° E), Kurdistan (35.3219° N, 46.9862° E), Kermanshah (34.1397° N, 45.9206° E), Lorestan (33.4647° N, 48.3390° E), and Hamedan (34.9083° N, 48.4393° E) were collected by direct separation from the goats' body (Fig. 1). The collected lice samples were stabilized in 70.00% ethanol and transferred to the Parasitology Laboratory of Faculty of Veterinary Medicine, Urmia University, Iran. The lice species were initially identified *B. caprae* using valid diagnostic keys (round head and antenna with three segments, the ventral surface of the thorax with a dark-colored plates, all legs of similar size and with a single claw, abdominal segments with tergal and paratergal plates, antennae in males are slightly taller than females and have transverse bands).[20,21] Subsequently, 11 lice samples from Kermanshah (n = 3), West Azerbaijan (n = 2), Kurdistan (n = 2), Lorestan (n = 2), and Hamedan (n = 2) were used for molecular characterization.

Fig. 1. Geographical map of locations where *B. caprae* samples were collected in the present study. 1: West Azerbaijan; 2: Kurdistan; 3: Hamedan; 4: Kermanshah; and 5: Lorestan.

DNA extraction. Genomic DNA was extracted using a commercial DNA extraction kit (MBST, Tehran, Iran) according to the manufacturer's instructions. DNA quality and concentration were evaluated using a Nano-Drop spectrophotometer (2000c; Thermo Fisher Scientific, Waltham, USA). The extracted DNA samples were kept at –20.00 ˚C until the next steps.

COX1 and VGSC genes amplification and nucleotide sequencing. To confirm the detection of the *B. caprae* species, a 669-bp fragment of the cytochrome oxidase 1 gene was amplified. A polymerase chain reaction (PCR) reaction was performed on the extracted DNA samples using primers described by *Folmer et al*.[22] The PCR was performed in a volume of 50.00 µL and each microtube contained 5.00 µL DNA Template, 5.00 µL 10X PCR buffer, 1.00 µL dNTPs (200 µM), 4.00 µL $MgCl_2$ (50.00 mM), 1.00 µL of each primer (20.00 µM) and 1.00 µL Taq polymerase (Sinaclon, Tehran, Iran), and 32.00 µL ddH_2O. The PCR setup consisted of an initial DNA denaturation stage at 95.00 ˚C for 5 minutes, and then 35 repetitions, each cycle involving denaturation at 95.00 ˚C for 45 sec, primer annealing at 55.00 ˚C for 45 sec, extension at 72.00 ˚C for 45 sec, and a final extension step to complete polymerization at 72.00 ˚C for 10 min. The PCR product was visualized using 1.50% agarose gel and UV-Transilluminator (20M; BTS, Tokyo, Japan). Finally, the PCR product was purified and sent along with forward and reverse primers to Takapou Zist Company (Tehran, Iran) for sequencing. A fragment in the domain II (S5-S6) of the VGSC gene was amplified and mutation sites T917I, L920F, I927F, L928A, R929V and L930 were investigated. Point

mutations at the corresponding amino acid sequence positions T917I, L920F located in domain II are the most important mutations in pyrethroid-resistant lice. To identify the mutation sites in the VGSC gene, a 322-bp length fragment in the domain II (S5-S6) of the VGSC gene was amplified using the primers 5'-AAATCGTGGCCAA CGTTAAA-3' (sense) and 5'-TGAATCCATTCACCGCATAA-3' (antisense).[23] This fragment was amplified in a PCR reaction volume of 50.00 µL containing 5.00 µL DNA, 5.00 µL 10X PCR buffer, 1.00 µL Taq polymerase (Sinaclon), 1.00 µL of each primer (20.00 µM), 1.00 µL of dNTP (200 µM), 4.00 µL $mgcl_2$ (50.00 mM), and 32.00 µL ddH_2O. The PCR thermal condition was an initial DNA denaturation step at 95.00 ˚C for 10 minutes, followed by 40 cycles of denaturation at 95.00 ˚C for 40 sec, annealing at 56.00 ˚C for 40 sec, elongation at 72.00 ˚C for 60 sec, and a final elongation step to complete polymerization at 72.00 ˚C for 10 min. The PCR products were electrophoresed and stained using DNA Safe Stain (Sinaclon) on 1.50% agarose gel and then visualized on the UV-Trans-illuminator (BTS). Finally, the PCR products were purified and sent to Takapou Zist Company for nucleotide sequencing.

Bioinformatic analysis. The sequences were analyzed using NCBI BLAST, Chromas Software (version 2.2.1, Technelysium Pty Ltd., Brisbane, Australia) and ClustalW Software (UCD, Dublin, Ireland). The final sequences were aligned in MEGA (version 6.0; Biodesign Institute, Tempe, USA) with the haplotype sequence representing VGSC in GenBank®.[24] The corresponding phylogenetic tree was plotted in MEGA Software using maximum probability and bootstrapping (1,000 replicates).

Results

COX1 and VGSC gene amplification. In this study, 509 samples of lice were collected and identified after morphological examination. All collected specimens were found to be *B. caprae*. Using primers (HCO2198, LCO1490), a 669 bp fragment of the cox1 gene was amplified and then purified for nucleotide sequencing. The sequences of cox1 gene were blasted in NCBI and compared with the reference sequences in the GenBank®. The lice species were identified as *B. caprae*. Using the specific primers for partial amplification of VGSC gene, a fragment of 322 bp in size was successfully amplified. To examine the mutation sites in the VGSC gene, PCR products of VGSC gene were sent to Takapou Zist Company for nucleotide sequencing.

Mutation detection of VGSC gene sequences. Sequence alignments of VGSC gene of eleven *B. caprae* collected from five provinces showed six substitutions in the second domain of S5 region of the VGSC sequences. In three isolates of Kermanshah and two isolates of Kurdistan in amino acid position 917, isoleucine was replaced by threonine, and in two isolates of Kurdistan and two isolates of Kermanshah in amino acid position 920, phenylalanine replaced by leucine. Also, in two isolates of Hamedan in amino acid position 927 replacement I927F and in two isolates of West Azerbaijan and one Lorestan isolate in amino acid position 928 substitution L928A was identified. In two isolates of Hamedan, two isolates of Kurdistan, two isolates of Kermanshah and one isolate of Lorestan in amino acids of 929, replacement of R929V was observed and in one isolate of Kermanshah in amino acid, position 930 and replacement of L930M was observed. Figure 2 shows the alignment of the VGSC amino acid sequence in the second region S5-S6 in *B. caprae.*

Phylogenetic analysis. The illustrated phylogenetic tree is composed of four branches based on the comparison of the partial sequence of domain II of VGSC gene of *B. caprae* obtained in the present study and those retrieved from GenBank® (Fig. 3).

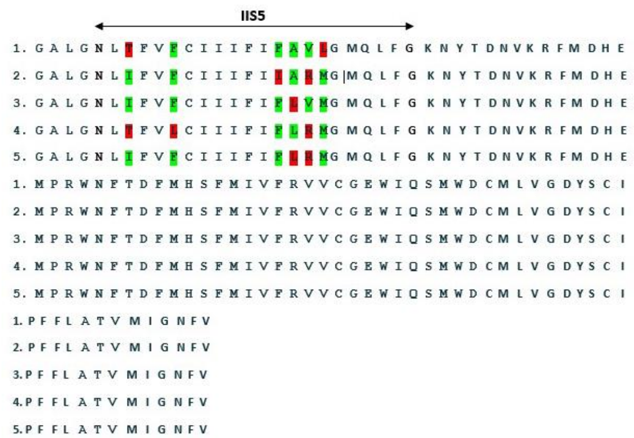

Fig. 2. Alignment of VGSC amino acid sequence in domain II S5-S6 region in *B. caprae.* Sequence 1 (isolate from Kermanshah), Sequence 2 (isolate from Hamedan), Sequence 3 (isolates from West Azerbaijan and Lorestan) Sequence 4 (isolates from Kurdistan and Kermanshah) and Sequence 5 (isolate from Lorestan).

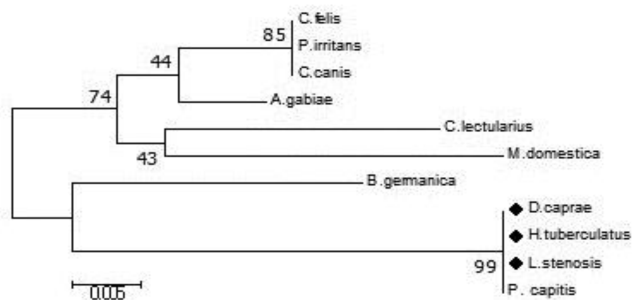

Fig. 3. The phylogenetic tree based on the sequence of the S5-S6 segment in domain II in the VGSC gene of different arthropods. Branch length shows the magnitude of change in the genetic distance between different sequences. *B.caprae, Haematopinus tuberculatus, Linognathus stenopsis,* and *Pediculus capitis* lice belong to the *Blattella germanica* sub-branch. *Pulex irritans, Ctenocephalides canis, and Ctenocephalides felis* fleas belong to the same branch. *Anopheles gambiae* belong to the sub-branch of fleas. *Cimex lectularius* bug and *Musca domestica* fly belong to the next branches, respectively.

Based on the analysis of the amino acid sequence, five identified *B. caprae* belonged to five haplotypes (Fig. 4). From a total of 11 sequences studied haplotype IV had the highest frequency (36.36%) with the substitution of T917I, L920F, and R929V, followed by haplotype III with the substitution of L928A (27.27%) and haplotype II with the substitution of I927F and R929V (18.18%). The frequency of haplotype I (T917I, L930M) and haplotype V (L928A, R929V) was 9.09%.

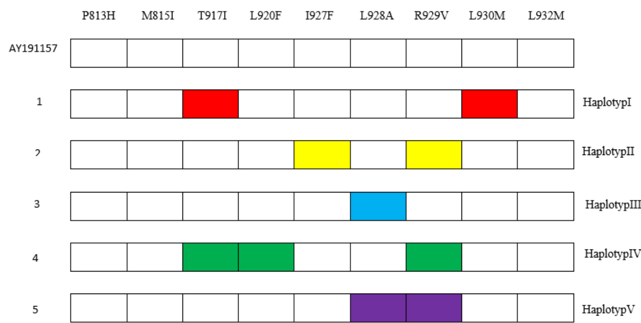

Fig. 4. Representation of five haplotypes in *B. caprae* lice. Haplotype I was collected from Kermanshah, haplotype II from Hamedan, haplotype III from West Azerbaijan and Lorestan, haplotype IV from Kurdistan and Kermanshah provinces and haplotype V from Lorestan province.

Discussion

Various insecticides are used to control lice in animals.[4] Typically, topical insecticides such as amidines, organophosphates, carbamates, and pyrethroids are used to control chewing lice in animals. Meanwhile, synthetic pyrethroids have become extensively popular due to their high efficacy, ease of use, and rapid dispersion on the body.[3] In a 1998 study, *Garg et al.* examined the effect of Flumethrin on *B. caprae*. Their results showed that flumethrin 1.00% was 100% effective in treatment of this infestation.[25] The excessive use of pyrethroid-based products has led to the appearance of deltamethrin-resistant *B. bovis* lice in the UK, *B. ovis* lice resistant to cypermethrin, alphamethrin, and deltamethrin in Australia, and *B. ocellatus* resistant to cypermethrin and permethrin in the UK.[6] Pyrethroid resistance in human head lice has also been reported in many countries, including the US, the UK, Argentina, France, the Czech Republic, and Japan.[26]

Over the last two decades, more than 50 mutations or combinations of mutations have been reported in the VGSC gene that are associated with or responsible for kdr in various arthropods and disease vectors.[27] Three-point mutations at the corresponding amino acid sequence positions M827I, T929I, and L932F of the housefly para-orthologous VSSC (M815I, T917I, and L920F in the numbering of the head louse amino acid sequence) located in domain II indicate the kdr trait. These are the most relevant mutations in head lice resistant to pyrethroids and DDT worldwide. These mutations are found together in blocks as a resistant haplotype.[19,23,28] In addition, when the M815I and L920F mutations are expressed alone, permethrin sensitivity is reduced two to three-fold, but the T917I mutation eliminates permethrin sensitivity, whether alone or in combination with T917I mutation was thus identified as the main cause of permethrin resistance in head lice. The frequency of these mutations has been previously reported by several authors with values ranging from 10.00% in Japan (Kasai *et al.*) to 90.00% in Denmark and France.[23,26,29,30] Substitutions of T917I and L920F were observed in the sequences studied in the present research. In studies, the L920F mutation along with the T917I mutation has been reported as a haplotype in *Pediculus humanus* capitis.[23,31] An association has been reported between T917I and L920F mutations in VGSC gene and permethrin resistance in head lice.[19,28,32] *Drali et al.* reported T917I substitution as the main factor involved in permethrin resistance in human body lice.[33] According to *Kwon et al.*, the T917I mutation is a proper indicator of pyrethroid resistance in head lice.[34] T929I mutation has been detected in pyrethroid-resistant *Plutella xylostella* which causes kdr-like resistance in this species.[35] The high prevalence of kdr-like haplotype in head lice has been reported in several countries, including the USA,[36] the UK[37] and Denmark.[29] In these countries, the frequency of kdr-like alleles ranges from 44.00% (Wales, UK) to 96.00% (Florida) indicating that pyrethroid insecticides are less effective in these regions.[36,37] In a study by *Bass et al.* in the UK and US to investigate pyrethroid resistance in *Ctenocephalides felis*, T929V mutation along with L1014F mutation were observed in the VGSC gene, which made fleas resistant to pyrethroids.[5] *Ghavami et al.* also reported T929V mutation along with L1014F mutation in the VGSC gene of *Pulex irritans*, which causes resistance to pyrethroids.[29] The analyses in this study indicated that in addition to T917I and L920F, new mutations of L928A, R929V, L930M, and I927F were also present in the studied sequences. Firooziyan *et al*. have recently shown that besides T917I and L920F in human head lice and L920F in human body lice, there are new mutations of A928L, V929R, and M930L in the IIS5 membrane of the VGSC of human head lice and body lice.[27] According to them, it is unclear whether or not these new mutations are related to pyrethroid resistance. The new mutations observed in this study included A928L, V929R, and M930L, as previously reported by Firooziyan *et al*. The I927F mutation found in *B. caprae* lice in this study, however, was not observed by Firooziyan *et al*. in human head and body lice.[27]

The results of this study showed that all the samples had at least one mutation associated with pyrethroid resistance in lice or new mutations. Therefore, the presence of kdr-like and new mutations in the sodium channel is likely due to the widespread use of pyrethroid

insecticides. To establish a proper management plan, the early detection of insecticide resistance is crucial, while preventive measures to prevent spread of resistance should also be promoted. Thus, the molecular detection of resistance to pyrethroid insecticides in goat chewing lice in all Iranian provinces along with toxicology studies can help plot a kdr frequency map to enact effective policies to control caprine pediculosis. However, the results of this study are preliminary, and due to the small number of sample size, the actual frequency of mutated alleles in these areas may differ from the results obtained in this study. Further studies are recommended on more extensive geographical areas and larger population.

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Conflict of interest

The authors declare that they have no competing interests.

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