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Pyrethroids resistance in *Pulex irritans* and *Ctenocephalides canis* in west and northwest Iran

Shahin Seidy, Mousa Tavassoli*, Farnaz Malekifard

Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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Abstract

Resistance to the knockdown effect of pyrethroid insecticides occurs due to mutations at target sites of pyrethroids, meaning the voltage-gated sodium channels gene (VGSC) in the membrane of the neurons. In fleas, this mutation occurs at two sites in the sodium channel in neurons: one is the replacement of leucine with phenylalanine (L1014F) and the other is the replacement of threonine with valine (T929V). In this study, 81 *Pulex irritans* and 47 *Ctenocephalides canis* fleas were collected from five provinces in the west and northwest of Iran. Adult fleas were exposed to cypermethrin 0.75%, and the mortality rate was calculated after 1 and 8 hr, and the mutation sites in the VGSC gene were investigated. The lethality of cypermethrin 0.75% for *P. irritans* was 40.00 - 57.14% after 1 hr and 60.00 - 73.91% after 8 hr. The lethality of this dose for *C. canis* after 1 and 8 hr of exposure was 33.33 - 41.17% and 66.66 - 80.33%, respectively. The VGSC sequence analysis indicated two mutation sites in the resistant and one mutation site in the susceptible fleas. The VGSC sequence analysis of susceptible *P. irritans* showed that 5.50% of them were homozygous susceptible and 94.45% were heterozygous susceptible. Susceptible *C. canis* were 5.26% homozygous and 94.73% heterozygous susceptible. All the resistant fleas were homozygous. The development of pyrethroid resistance and high-frequency L1014F mutation in fleas suggest that pyrethroids are likely to be ineffective in controlling fleas. Therefore, monitoring pyrethroid resistance and its underlying mechanisms are necessary for controlling fleas and finding new alternative control methods.

Introduction

Pyrethroids are insecticides of choice that have been widely used over the past three decades to control agricultural pests and animal parasites and promote public health. These insecticides are relatively inexpensive and their rate of toxicity and retention in the body of mammalians are very low compared to other insecticides.[1] Pyrethroids affect the insect nervous system by altering the normal function of sodium channels. The extensive use of these insecticides has led to the development of resistance in many insect species, which is a serious obstacle to their effectiveness.

Knockdown resistance (kdr) is one of the most important types of pyrethroid-insecticide resistance which occurs through point mutations in the voltage-gated sodium channels (VGSC) and decreases susceptibility of insect nervous system to these compounds. The voltage-gated sodium channel is a large and complex membrane protein. The main body of the channel is composed of a single polypeptide containing the replication of four domains (I-IV), and each contains six alpha-helical membrane regions. These 24 fragments accumulate in the membrane and form the selective sodium channel.[2]

The kdr was first observed in houseflies in the 1950s following the use of chlorinated hydrocarbon insecticides. Subsequent studies in houseflies showed two types of pyrethroid resistance: kdr and super-kdr. Molecular analysis of nucleotide sequences of the VGSC gene in these insects shows a genetic link between nerve insensitivity and amino acid replacement in domain II of this channel.[3,4] The first replacement of leucine with phenylalanine was observed in the membrane section of domain II in the S6 segment in houseflies and cockroaches. Subsequent studies have identified the replacement of leucine with serine (L1014S), histidine (L1014H), cysteine (L1014C), and tryptophan (L1014W), which is associated with L1014F mutation in other resistant arthropods. This mutation indirectly alters

***Correspondence:**

Mousa Tavassoli. PhD
Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: m.tavassoli@urmia.ac.ir

the binding site of pyrethroids in the membrane section of domain II in the S6 segment and leads to a decrease in susceptibility to these compounds.[4,5] To date, varying levels of pyrethroid resistance have been reported in important vectors in medicine and veterinary medicine. L1014F mutation, first reported in houseflies, is now known as kdr mutation, which is reported in various species of *Anopheles,*[6,7] *Triatoma infestans,*[8] *Cimex lectularius,*[9] and *Rhipicephalus microplus*.[10-12] In addition to the L1014F mutation, there are several super-kdr-associated point mutations in domain II of the S4-S6 region in the VGSC sequence in a number of vectors including the *Pediculus humanus capitis*,[13-15] *Blattella germanica*,[16] *Cimex lectularius*, *C. hemipterus*[17-21] and *C. felis*.[22-24] Fleas of the Pulicidae family are among the most important ecto-parasites of humans and animals in the world. These parasites are among the most important and common pests that pose a serious threat to human health in many parts of the world by biting and transmitting dangerous infectious diseases.[25] Due to the importance and frequency of fleas, it is necessary to plan and monitor a control program with the knowledge of susceptibility to insecticides. Research on the susceptibility of fleas to insecticides is scarce, and reports of insecticide resistance are more common for *C. felis*,[22-24] *Xenopsylla cheopis* and *X. astia*.[26-29] Today, bioassay and molecular methods are two reliable method for determining resistance and susceptibility in flea populations. The PCR-based diagnostic assays have already indicated that L1014F and T929V mutations associated with pyrethroid resistance are common in cat flea populations in both the UK and the USA.[22,23]

Due to the medical importance of fleas, various groups of insecticides have been used to control these insects since 1950.[24] Dichlorodiphenyltrichloroethane (DDT) resistance was then reported in various areas around the world. Moreover, the mean lethal dose 50.00 (LD50) in the bioassay method in previous studies suggests that the susceptibility of target sites may be a major mechanism of resistance to pyrethroids.[24] Previous use of DDT in these areas may have caused cross-resistance to DDT and pyrethroids. The significant reduction in the effectiveness of pyrethroids is attributed to the development of resistance at the target site of these insecticides.[30]

Therefore, this study was conducted to identify the kdr mutation in the VGSC gene in *P. irritans* and *C. canis* in the west and northwest of Iran. Susceptibility was evaluated by bioassay and molecular analysis. The mortality rate below 90.00% in the bioassay method was considered as resistance to cypermethrin.

Materials and Methods

Sampling. Live fleas were collected during March to February of 2019 by human prey from houses and pens of domestic animals and by direct isolation of the samples from animals in the five provinces of Kermanshah (34.1397° N, 45.9206° E), Kurdistan (35.3219° N, 46.9862° E), West Azarbaijan (37.5498° N, 45.0786° E), Lorestan (33.4647° N, 48.3390° E) and Hamedan (34.7989° N, 48.5150° E). In this method, volunteers walked in white clothes and knee-high white socks for 3 - 5 min in the houses and pens to collect fleas. Fleas were collected using an air vacuum pump attached to an Erlenmeyer flask and a pipette through a hose. Samples were sent to the parasitology laboratory of the Faculty of Veterinary Medicine of Urmia University and were identified using valid keys for Iranian fleas.[31]

Bioassay. Bioassay is done in different ways. One of these methods is the use of filter paper. To perform this step, bioassay samples were prepared using insecticide-impregnated filter paper. pyrethroid 0.75% (100 ppm; National Agrochemical Co., Tehran, Iran) was used according to the World Health Organization (WHO) protocol.[32] The four live fleas were transferred to Erlenmeyer flasks lined with a piece of filter paper (12.00 cm in diameter) impregnated with one µL of active ingredient to assess the susceptibility to the knockdown effect of pyrethroids. A negative control was used for two species *P. irritans* and *C. canis*. In the negative control, fleas were exposed to filter paper impregnated with distilled water. The dishes were covered with a cleaning cloth and exposed to 24.00˚C and 40.00% humidity for 1 hr and 8 hr. After the bioassay, the dead and live fleas were transferred to sterile containers for molecular analysis. According to the WHO protocol, if the mortality rate is 98.00 - 100%, the population is susceptible; if the mortality rate is 90.00 - 98.00%, the resistance must be confirmed by the molecular method; and if the mortality rate is below 90.00%, the population is resistant.[33]

DNA extraction. First, DNA extraction was performed by proteinase K method using a Molecular Biological System Transfer (MBST, Tehran, Iran) kit manufactured in Iran according to the manufacturer's instructions. It should be noted that DNA was extracted from all parts of the body, live and dead samples of *C. canis* and *P. irritans* fleas. The DNA samples were stored in a freezer at – 20.00˚C after DNA extraction until they were used in PCR.

Amplification of cytochrome oxidase subunit I (COX) gene. The identity of *C. canis* and *P.* irritans fleas was confirmed through PCR performed on the extracted DNA samples with the primers designed by Folmer *et al*.for COX1.[34] The results were observed in a 700-bp band on agarose gel 1.50% (Table 1).

Amplification of voltage-gated sodium channels (VGSC). To investigate the mutation site in the resistant samples and the absence of mutations in the susceptible samples, PCR was performed with primers designed by Bass *et al*. for VGSC.[22]

Table 1. Nucleotide sequence and specificity of the primers used.

Genes	Primer nucleotide sequence	Annealing temperature (˚C)	Fragment length (bp)	Reference
COX1	Sen: GGT CAA CAA ATC ATA AAG ATA TTG G Rev: GAA GGG TCA AAG AAT GAT GT	55.00	700	34
VGSC	D1: AARYTNGCNAARTCNTGGCC D5: GCNAARTCNTGGCCNAC	50.00	600	22

The program used for the PCR consisted of an initial denaturation at 94.00˚C for 5 min followed by 30 repeated cycles, each containing 95.00 ˚C for 30 sec, 50.00 ˚C for 50 sec, 72˚C for 90 sec, and the final elongation step of 72.00 ˚C for 5 min.[22] After PCR, the reaction product was electrophoresed on agarose gel 1.50% and photographed under ultraviolet light. The PCR product was purified using the MBST kit and 22 samples were sent to Takapou zist Company, (Tehran, Iran) for sequencing.

Bioinformatics analysis. The nucleotide sequence analysis was performed using the databases and programs of the National Center for Biotechnology Information (NCBI) site. The phylogenetic tree was plotted in MEGA Software (version 6.0; Biodesign Institute, Tempe, USA) using the maximum likelihood and bootstrapping (1,000 replicates).

Results

Mortality bioassay. A total of 81 *P. irritans* and 67 *C. canis* fleas were selected for the test. There was a significant variation in susceptibility to cypermethrin in the five studied populations. Mortality rates in different populations of *C. canis* and *P. irritans* fleas are shown in Table 2. At the end of the experiment, all the negative control samples were alive. Among the five studied populations, the lowest (60.00%) and highest (73.91%) susceptibility to cypermethrin for *P. irritans* fleas were observed in Kurdistan and Kermanshah provinces, respectively. Also, the lowest (66.60%) and highest (83.30%) susceptibility to cypermethrin for *C. canis* fleas were detected in Hamedan and Lorestan provinces, respectively.

The mortality at a concentration of 0.75% was 70.00% for *P. irritans* and 79.16% for *C. canis* fleas. Among the dead *P. irritans* fleas, four (5.50%) were homozygous susceptible and 50 (94.45%) were heterozygous susceptible. Also, among the dead *C. canis* fleas, three (5.26%) were homozygous susceptible and 54 (94.73%) were heterozygous susceptible, and all the resistant homozygous fleas were alive (Table 2).

VGSC gene amplification. The use of specific primers of cytochrome oxidase I (Sen, Rev) in the extracted DNA sample caused the amplification and production of a 700-bp fragment. After sequencing the PCR product, the genera of *C. canis* and *P. irritans* fleas' identification were confirmed. After confirming the *C. canis* and *P. irritans* by the molecular methods, PCR was performed on DNA samples extracted from resistant and susceptible fleas with primers designed by Bass *et al*. (Table 1).[22] The result of this reaction was observed after electrophoresis on agarose gel 1.50% in the form of a 600-bp band.

Mutation in VGSC gene in *P. irritans.* The results of the VGSC sequencing for resistant *P. irritans* in the five studied provinces (access number MZ384379) showed two mutation sites in the sequence: A mutation in nucleotide acids 71 and 72 in which the nucleotide acids guanine and thymine were replaced with adenine and cytosine, which have resulted in the replacement of threonine with valine in the S5 segment in domain II. The next mutation in nucleotide acid was number 389, in which the nucleotide acid thymine was replaced by cytosine, converting leucine to phenylalanine in S6 segment in domain II (Fig. 1).

Table 2. The number of fleas collected from different provinces and the mortality rate after 1 and 8 hr of exposure to 0.75% concentration of cypermethrin.

Provinces	Number of samples	Flea species	Number of fleas	Mortality rate	
				1 hr	8 hr
				No. (%)	No. (%)
Kermanshah	40	*Pulex irritans*	23	10 (43.47)	17 (73.91)
		Ctenocephalides canis	17	7 (41.17)	13 (76.47)
Kurdistan	15	*Pulex irritans*	10	4 (40.00)	6 (60.00)
		Ctenocephalides canis	5	2 (40.00)	4 (80.00)
West Azarbaijan	57	*Pulex irritans*	21	10 (47.61)	15 (71.42)
		Ctenocephalides canis	36	14 (38.88)	30 (83.33)
Hamedan	20	*Pulex irritans*	14	10 (57.14)	10 (71.42)
		Ctenocephalides canis	6	2 (33.33)	4 (66.66)
Lorestan	17	*Pulex irritans*	9	4 (55.55)	6 (66.66)
		Ctenocephalides canis	8	3 (37.50)	6 (75.00)

Mutation in the VGSC gene in *C. canis*. The results of VGSC sequencing for resistant *C. canis* in the five studied provinces showed two mutation sites in the sequence: A mutation in nucleotide acids 71 and 72 in which the nucleotide acids guanine and thymine replaced the nucleotide acids adenine and cytosine, and this mutation resulted in the replacement of threonine with valine in S5 segment in domain II. The next mutation in nucleotide acid was number 389, in which the nucleotide acid thymine was replaced by cytosine, converting leucine to phenylalanine in segment S6 in domain II (Fig. 2).

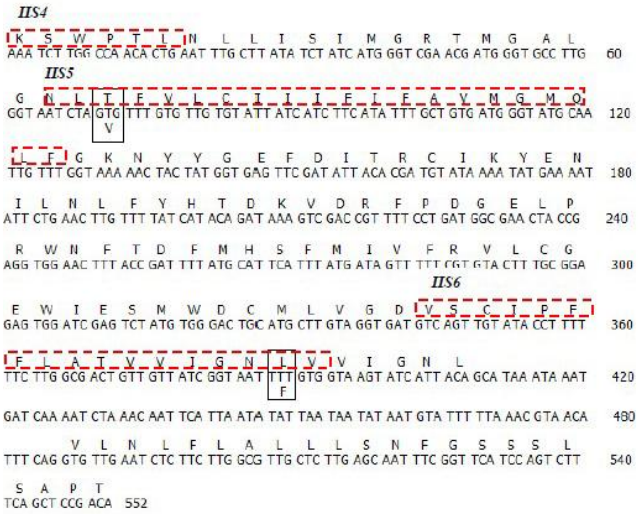

Fig. 1. Alignment of partial voltage-gate sodium channel gene and deduced amino acids of *P. irritans*. The L1014F and T929V mutation sites are indicated in vertical box.

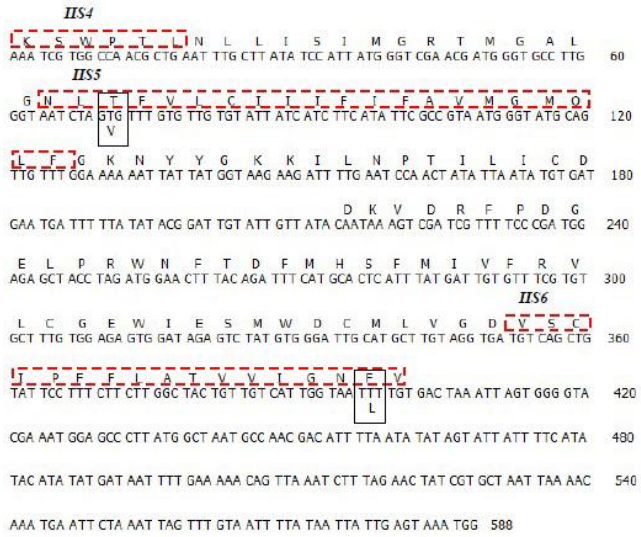

Fig. 2. Alignment of partial voltage-gate sodium channel gene and deduced amino acids of *C. canis*. The L1014F and T929V mutation sites are indicated in vertical box.

Phylogenetic tree based on mutations in the VGSC gene. The phylogenetic tree based on the similarities between the sequences of the recent study and the sequence registered in the gene bank was drawn and formed four branches. *P. irritans*, *C. canis*, and *C. felis* are in the same branch. The *Anopheles gambiae* is located under the flea branch. *Musca domestica*, *Drosophila melanogaster*, *C. lectularius*, *P. capitis*, and *B. germanica* are in the next branches (Fig. 3). The results of the protein sequence alignment of the VGSC in different arthropods show that segment S4 contains amino acids 1 to 6, and amino acids 7 to 21 constitute the distance between segments S4 and S5. The S5 segment contains amino acids 21 to 42. The distances between segments S5 and S6 had amino acids 21 to 64, and amino acids 64 to 81 show segment S6 (Fig. 4).

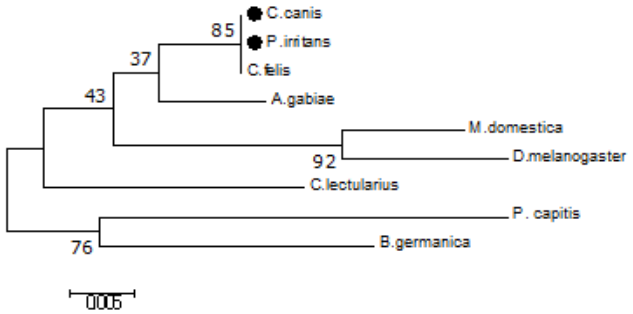

Fig. 3. The phylogenetic tree based on the sequence of the S4 - S6 segment in domain II in the partial voltage-gate sodium channel gene of different arthropods.

Discussion

The excessive use of pyrethroids for the control of insects has led to resistance to these insecticides. Monitoring resistance to pyrethroids and its underlying mechanisms is essential for flea control. This study aimed to identify the susceptibility of *P. irritans* and *C. canis* to cypermethrin and to identify mutations in the VGSC gene in 5 populations. The population studied in this study showed significant differences in pyrethroid susceptibility. Due to the low mortality and high resistance in the population of fleas in the studied regions, molecular methods were used to confirm the bioassay. To investigate whether pyrethroid resistance is related to the mutations developed in segments S4 - S6, the VGSC was sequenced and the two mutation sites of L1014F and T929V were examined, which confirmed the pyrethroid resistance in these vectors. This mutation has been reported for the first time in houseflies. So far, this mutation has been reported in twenty different species of arthropods.[4]

This study showed that kdr mutation (replacement of leucine with threonine) which has previously been associated with pyrethroid resistance in houseflies was present in a wide range of insects. This mutation has been reported in seven strains of cat fleas. The kdr mutation has been reported in domain II in segment S6 of the VGSC in eight different species of insects to date, including *M. domestica*, *Haematobia irritans*, *B. germanica*, *A. gambiae*, and *C. pipiens*.[35]

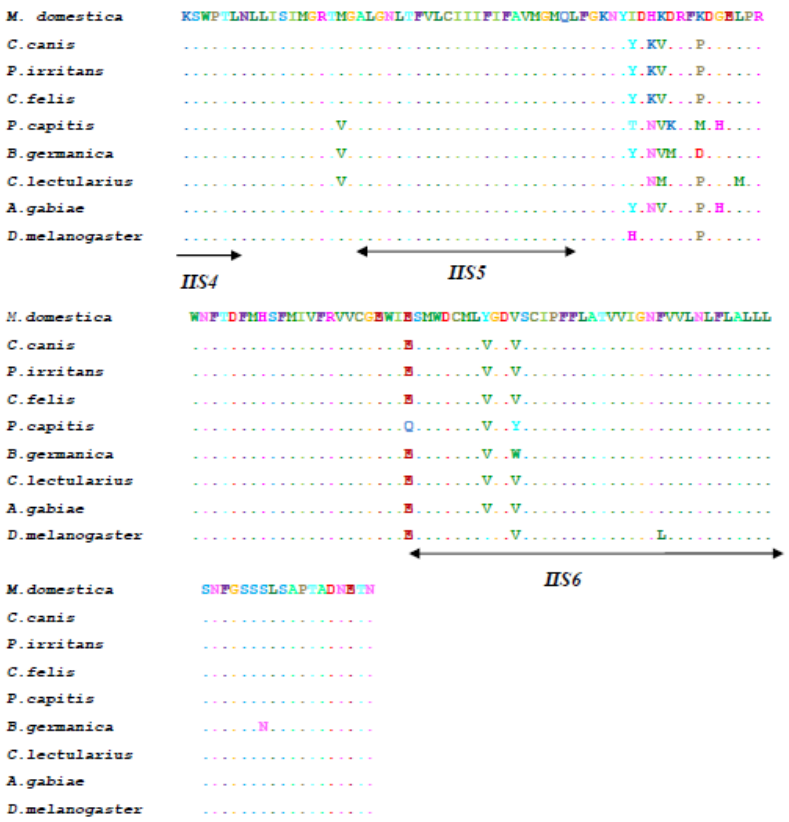

Fig. 4. The protein sequence alignment results show the partial voltage-gate sodium channel gene in *M. domestica*, *C. canis*, *P. irritans*, *C. felis*, *P. capitis*, *B. germanica*, *C. lectularius*, *A. gambiae*, and *D. melanogaster*, and amino acids constituting segments S4, S5, and S6 are marked with an arrows.

The L1014F mutation generally causes (10 to 30-fold) resistance to pyrethroids and DDT (similar function to pyrethroids). Studies performed to express the function of sodium channels in insects show that the L1014F mutation reduces the susceptibility of sodium channels to pyrethroids.[36]

In this study, similar to previous studies, a second mutation related to the replacement of threonine with valine was investigated in segment S5 in domain II. This mutation is also present in houseflies. This type of mutation was first found in the laboratory strain of cotton tail bug. This mutation has made insects highly resistant to pyrethroids.[22] Another type of mutation has been reported in head lice in which threonine is replaced with isoleucine. This mutation causes high resistance. A mutation in the Drosophila fly in which threonine is replaced by valine resulted in resistance to deltamethrin.[36]

Bass *et al*. investigated pyrethroid resistance in *C. felis* in UK and US and two mutations of L1014F and T929V were found in the VGSC, which made fleas resistant to pyrethroids.[22]

Ghavami *et al*. evaluated the resistance to pyrethroids in *P. irritans* fleas in Zanjan province (Iran) by bioassay and molecular methods and observed two mutations of L1014F and T929V in the VGSC gene. Due to the low mortality rate and the two mutation sites in the gene sequence, they concluded that resistance to insecticides is due to two mutations in the target sites of pyrethroids.[37]

In a study conducted by Samiei *et al*. to investigate the resistance to pyrethroids in *C. hemipterus* in four climatic regions of Iran, the basis for the resistance was the V419L mutation in the VGSC gene.[38]

In conclusion, the occurrence of the VGSC gene mutations, L1014F and T929V, in *P. irritans*, *C. canis* representing the target site insensitivity is the major mechanism of pyrethroid resistance in these vectors. Identifying the main effective factors in the expression of the mutant alleles and their stability in the absence of pyrethroid insecticide pressure are important issues for future studies. Since pyrethroids are still widely used in the control of fleas, point mutations in the target sites of these insecticides have led to kdr mutation; therefore, monitoring and management for controlling fleas and use of insecticides are necessary.

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Conflict of interest

The authors declare that they have no conflict interests.

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