Running title: Pyrethroid resistance of brown dog ticks

Permethrin Resistance in Field Populations of *Rhipicephalus Sanguineus* Sensu Lato (Latrielle, 1806) Collected from Dogs in eastern of Iran

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ABSTRACT

The high level of acaricide resistance in ticks becomes a challenge for dog owners in Iran. This study was conducted in South Khorasan province of Iran at 2024. In this study, the resistance status of *Rhipicephalus Sanguineus* (Acari: Ixodidae) to permethrin at various concentrations were evaluated using the Larval Packet Test (LPT) method recommended by the Food and Agriculture Organization (FAO). PCR assays were conducted to investigate the mechanisms of resistance to acaricides. We used PCR to amplify segment 6 of domain III of the voltage-sensitive sodium channel protein from both pyrethroid-susceptible and pyrethroid-resistant tick strains. The LPT discriminating dose bioassays confirmed the pyrethroid resistance phenotype of the analyzed strains. The mortality rate at LC_{99} was ranged between 38.1 to 49.1%. At discriminating dose, survival rates ranged from 48.3% to over 60.1%. Additionally, of the 40 ticks analyzed, mutations C2130T and T2134C were detected in 38 (95%) ticks. The presence of permethrin resistance in *R. sanguineus* s.l. populations in Iran highlights the need for alternative control strategies, and the identification of genetic mutations provides valuable information for understanding the mechanisms of resistance.

Keywords: *Rhipicephalus sanguineus*; acaricide resistance; diagnostic concentration; permethrin

1.INTRODUCTION

Ticks are one of the most important arthropod vectors of disease-causing agents in both humans and animals. The *R. sanguineus* is an important tick species that feeds mainly on dogs but can also infested other mammalian hosts. [1] *R. sanguineus* feed on the blood of their hosts and transmit a wide range of pathogens, including viruses, bacteria, and protozoans. [2] *R. sanguineus*, the most commonly found tick around the world due to its biological flexibility. One of the primary methods of controlling tick infestations is through the use of acaricides. However, the excessive and often inappropriate use of acaricides has led to the emergence of acaricide resistance, including *R. sanguineus*. [3, 4] Understanding the probable acaricide resistance in *R. sanguineus* populations in Iran is crucial for developing effective strategies to control tick infestations and prevent the transmission of tick-borne diseases. [5] Acaricide resistance is a complex phenomenon that involves various genetic and physiological mechanisms. These mechanisms can result in decreased sensitivity to the acaricides used to control tick populations. [6] Recent studies have suggested that acaricide resistance in tick populations is multifactorial and involves several mechanisms, including target-site insensitivity, metabolic detoxification, and changes in behavior and physiology. [7] Target-site insensitivity involves mutations in the genes that code for the target sites of the acaricides, resulting in decreased binding of the acaricides and reduced effectiveness in killing the ticks. Metabolic detoxification involves the overexpression of enzymes that can break down the acaricides, making them less effective. Changes in behavior and physiology involve alterations in the tick's behavior, such as reduced exposure to the acaricides, and changes in the tick's physiology, such as altered cuticle permeability, which can reduce the uptake of the acaricides. The emergence of acaricide resistance in *Rhipicephalus* populations in Iran is a major concern for both animal and public health(8). Further research is needed to elucidate the molecular and physiological mechanisms underlying acaricide resistance in *R. sanguineus* populations in Iran.

2. MATERIAL AND METHODS

2.1. Sample Collection

During June 2022 to May 2023, brown dog ticks were collected from sheepdog of four locations in rural areas located in South Khorasan provinces, east of Iran. The engorged and/or partially engorged female ticks were collected from naturally infested dogs using tick infestation methods, tick drags, and visual searches. The collected ticks were transported immediately to the laboratory in vials containing moist filter paper. The morphological identification of collected samples were confirmed under a stereo-microscope using the standard keys [9]. From each colony, 30 engorged females were incubated in an environmental chamber at 26–27 °C and 85±5% relative humidity for 3-4 weeks to allow egg lying. The 14-21 day old tick larvae were utilized for the bioassay experiments. The female adult specimens that had been depleted of eggs were isolated, rinsed with distilled water, and then dried using paper towels. Each individual was then frozen separately at a temperature of -80°C for future use in molecular analysis.

2.2 Acaricide bioassays

The sample size calculation was based on WHO guidline (10).The efficacy of permethrin was assessed using the larval packet test (LPT) developed for acaricide testing of tick populations. [11] Technical-grade 92% permethrin (Mumbai, India) were used as the active ingredients for the LPT. A stock solution was prepared by dissolving permethrin in a 2:1 ratio using trichloroethylene (TCE) (Merck, Germany), and olive oil. [12] In Iran, the standard susceptible indigenous strain of *R. sanguineus* was not available. Therefore, in this study, the discriminating concentration of acaricide-susceptible brown dog tick strain was acquired from previous study that was set as 0.19% . [13]The DC used was calculated by doubling the lethal concentration 99.9% (LC99) derived from a series of tests conducted with a susceptible strain. [14]The LC99 of 0.09% active ingredient (AI) was also tested. Bioassays were conducted on three replicates with 100 larvae per pocket for each concentration.

2.3 Molecular analysis

The genomic DNA of 10 *R. sanguineus* larvae from each location was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN) as the manufacturer's guidelines. Each larva was homogenized in 50 microliters of distilled water and incubated at 56°C for 6 hours before being transferred to the column for preparation. The quality and concentration of the DNA obtained were assessed through agarose gel electrophoresis and a Nanodrop spectrophotometer. PCR amplification was conducted in a total volume of 25 µl, containing 2 µl of template

DNA, 1 μl of each primer (forward and reverse primers), 12.5 μl of 2X Taq PCR MasterMix (Takara, Japan), and 8.5 μl of nuclease-free water. The primers FG-228 (5'- CTA ACA TCT ACA TGT ACC -3)' and BDT-227 (5'- TTG TTC ATT GAA ATT GTC AA-3') were utilized for amplification of the domain III segment VI of the sodium channel gene. [15] The PCR amplification was carried out with an initial denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. In total, 20 samples demonstrating phenotypic susceptibility and 20 samples displaying phenotypic resistance were used for the sequence analysis.

2.4 Statistical Analysis.

The evaluation of mortality was conducted at 24 hours. The adjust of control mortality was calculated based on the formula of Abbott. [16] The percentage survival was recorded for each multiple of the diagnostic concentration. The classification of resistant phenotypes will be placed in three classes: low resistance (60 to 90% mortality in LC99×2), moderate resistance (13 to 50% mortality in LC99×2), and severe resistance (1 to 12 Mortality percentage in LC99×2). [17]

3. RESULTS

This study represents the initial assessment of acaricides efficacy on *R. sanguineus* population in South Khorasan provinces (Figure 1).

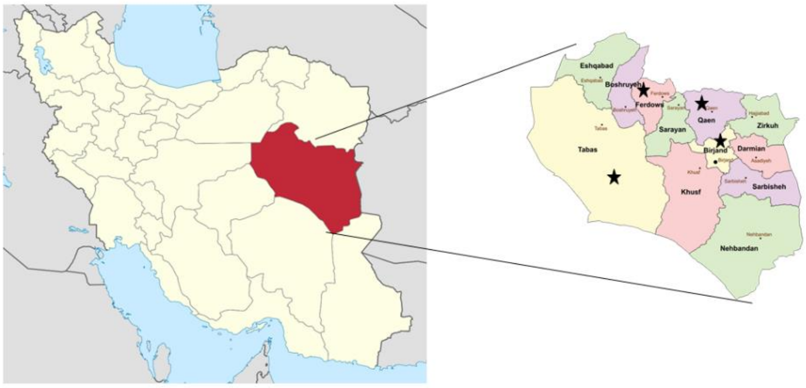

FIGURE 1. The collection site of ticks were shown.

Of these study, Only 4 population of *R. sanguineus* were reared successfully and provided sufficient numbers of larvae and subsequently subjected to bioassay to test their susceptibility to permethrin. The field cached *R. sanguineus* strains were evaluated for mortality with permethrin concentrations 1and 2 times the diagnostic concentrations, i.e. 0.09 and 0.19%. The mortality rate at LC99 was ranged between 40.5 to 49.1% (Table.1).

Table 1. The average lethal rate of *Rhipicephalus sanguineus* (Latreille) strains, collected from various regions in the east of Iran, when exposed to permethrin

Strain	Location	LC_{99} (0.09% AI) % Mortality	$2\times LC_{99}$ (0.19% AI) % Mortality
B1	Birjand	40.5	49.6
B2	Ferdows	42.5	48.3
B3	Ghaen	49.1	60.1
B4	Tabas	38.1	65.1

At $2\times LC_{99}$ (0.19% AI), lethal rates ranged from 48.3% to over 65.1%. To screen for mutations on the sodium channel's domain III segment VI, sequencing was conducted on 10 random samples from each phenotypically resistant population of brown dog ticks (Figure 2).

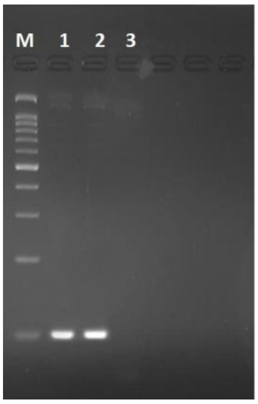

FIGURE.2 Agarose gel separation of representative PCR products of the voltage-sensitive sodium channel gene. Lane 1–2, positive isolates; Lane 3 negative control, DNA ladder 100 bp

The analysis revealed four genotypes on domain III among the *R. sanguineus* population from east of Iran by comparing the susceptible (GenBank KU886031) and permethrin-resistant (KU886032) *R. sanguineus* larvae. Out of 40 studied ticks, 2 ticks (5%) were wild strains for all loci; In this study, two ticks (5%) exhibited homozygosity for a silent mutation known as C2130T. One tick carried the C2130T mutation along with the T2134C mutation, while the remaining ticks (90%) showed homozygosity for the T2134C mutation (Figure 3, 4).

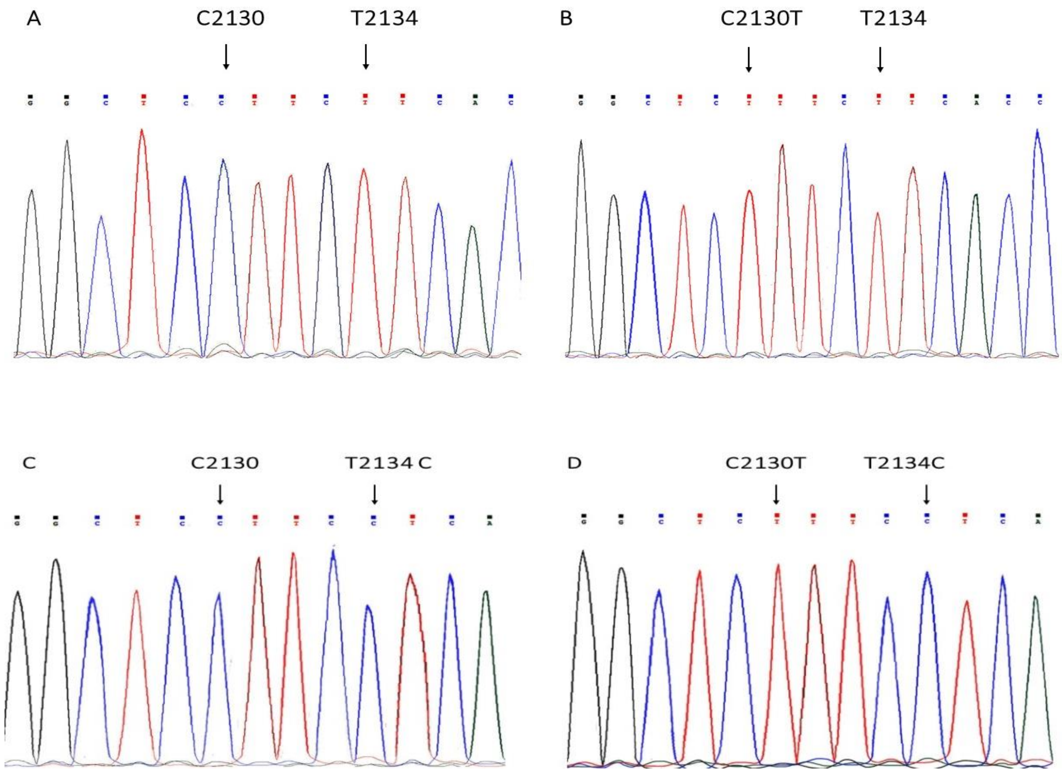

FIGURE 3. Chromatograms showing kdr genotypes of *Rhipicephalus sanguineus*. A: wild strain; B: C2130, transitions without change in amino acid; C: T2134C, transitions with change in amino acid from phenylalanine (F) to leucine (L); D: C2130T and T2134C.

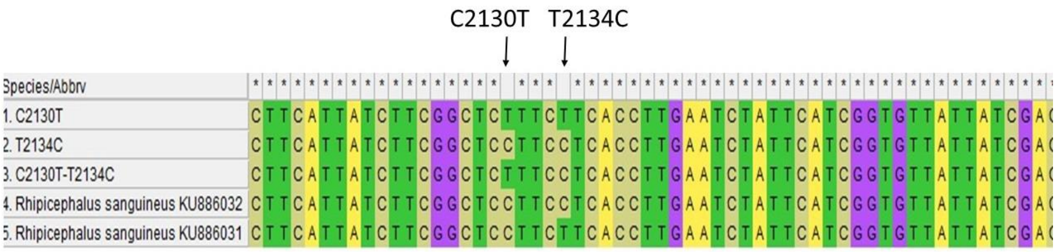

FIGURE 4. Gene sequences of the voltage-gated sodium channel of *Rhipicephalus sanguineus* aligned with that of wild sequences (GenBank accession number:KU886031) showing the mutations C2130T and T2134C .Three haplotypes were reported.

4. DISCUSSION

This study provides the first laboratory-confirmed permethrin resistance data for brown dog ticks from the East of Iran. *R. sanguineus* is one of the the most prevalent infected ticks for different pathogen in Iran. [18] The results of this study provide important preliminary insights into the efficacy of permethrin on the *R. sanguineus* population in east of Iran. The findings show that the mortality rates of *R. sanguineus* populations varied significantly when subjected to different concentrations of permethrin. At 2×LC_{99} (0.19% AI), lethal rates ranged from 48.3% to over 65.1%, indicating that this concentration is not effective for controlling of the following tick population. Previous studies in Iran have also shown high levels of resistance to pyrethroid insecticides among populations of *Rhipicephalus* [8, 19]. Limited studies have been carried out on the resistance of ticks to pyrethroid in Iran, [20, 21] and the present study is the first comprehensive investigation of the *R. sanguineus* in this area. Previous studies from around the world also showed resistance to pyrethroid pesticides among *R. sanguineus*[12, 13]. Importantly, our bioassay findings highlight the need for careful consideration of appropriate concentrations of acaricides to achieve effective tick control, and suggest that higher concentrations may be necessary to achieve satisfactory results. Overall, these results constitute an important step towards the development of more effective and targeted approaches for tick control in Iran.

Of these study, Only 4 population of *R. sanguineus* were reared successfully. An important consequence of resistance development in tick populations may be a decline in overall fitness. According to Roma et al. (2010), exposure to sub-lethal levels of permethrin adversely affects reproductive success (22). Subsequent research could explore how these sub-lethal concentrations of permethrin impact the reproductive capacity of adult female *R. sanguineus* with SNPs in comparison to their susceptible counterparts.

The current study identified a mutation on domain III segment VI of the sodium channel that was responsible for resistance to insecticides in the tick population. [3, 23] In previous studies, it has been shown that T2134C mutations in this gene is associated with resistance to pyrethroid resistance in *R. sanguineus*. [3] The findings reveal that out of the 40 ticks examined, just 5% were wild strains, suggesting that the majority of ticks had been subjected to selection pressure and had acquired resistance to insecticides. In this study, 38 out of 40 samples (90%) carried the T2134C mutation that could be the explained the high levels of permethrin resistance. However, it is possible that other mechanisms, such as metabolic detoxification, sequestration, reduced penetration, or additional mutations in the sodium channel, may be related to insecticide resistance. [24, 25]. Overall, this study underscores the importance of bioassay and genetic studies in understanding and controlling brown dog ticks populations. The number of samples collected may not fully represent the genetic diversity of the tick populations across the eastern regions of Iran. A larger sample size from various geographical locations could provide a more comprehensive understanding of resistance patterns. The study primarily focused on permethrin resistance, which may not reflect the overall resistance profile of the tick populations to other classes of acaricides. A broader assessment of resistance to multiple insecticides would provide a more complete picture. Limited funding restricted the scope of the sequencing project, potentially leading to a smaller sample size and fewer gene targets being analyzed than initially desired.

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Conflict of interest

The author declare no conflict of interest

Authors' Contribution:

A. V: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. R. S: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. E Kh: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis. S. Sh: Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Ethics

Research ethics committee of islamic azad university, science and research branch (IR.IAU.SRB.REC.1403.330).

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Data Availability

Should there be a need for data that support the findings of this study, they are available from the corresponding author upon reasonable request.

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