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Research Paper

Permethrin Resistance in Field Populations of *Rhipicephalus sanguineus* sensu lato (Latrielle, 1806) Collected From Dogs in Eastern Iran

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How to cite this article Valizadeh A, Shirali S, Solgi R, Khaksar E. Permethrin Resistance in Field Populations of *Rhipicephalus sanguineus* sensu lato (Latrielle, 1806) Collected From Dogs in Eastern Iran. Archives of Razi Institute Journal. 2025; 80(6):1543-1550. https://doi.org/10.32598/ARI.80.6.3577

https://doi.org/10.32598/ARI.80.6.3577

Article info:
Received: 14 Jun 2025
Accepted: 15 Jul 2025
Published: 01 Nov 2025

Keywords:
Acaricide resistance, Diagnostic concentration, Permethrin, *Rhipicephalus sanguineus*

ABSTRACT

Introduction: The high level of acaricide resistance in ticks has become a challenge for dog owners in Iran.

Materials & Methods: This study was conducted in South Khorasan Province of Iran in 2024. In this study, the resistance status of *Rhipicephalus sanguineus* (Acari: Ixodidae) to permethrin at various concentrations was evaluated using the larval packet test (LPT) method recommended by the Food and Agriculture Organization (FAO). Polymerase chain reaction (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.

Results: The LPT discriminating dose bioassays confirmed the pyrethroid resistance phenotype of the analyzed strains. The mortality rate at LC99 ranged between 38.1 and 49.1%. At the discriminating dose, survival rates ranged from 48.3% to 60.1%. Additionally, of the 40 ticks analyzed, mutations *C2130T* and *T2134C* were detected in 38(95%) ticks.

Conclusion: The presence of permethrin resistance in *R. sanguineus* 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.

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1. Introduction

Ticks are one of the most important arthropod vectors of disease-causing agents in both humans and animals. *Rhipicephalus sanguineus* is an important tick species that feeds mainly on dogs but can also infest other mammalian hosts [1]. *R. sanguineus* feeds on the blood of their hosts and transmit a wide range of pathogens, including viruses, bacteria, and protozoans [2]. *R. sanguineus* is the most commonly found tick worldwide 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 in *R. sanguineus* [3, 4]. Understanding the potential 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 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 acaricides, resulting in decreased binding of acaricides and reduced effectiveness in killing ticks. Metabolic detoxification involves the overexpression of enzymes that break down acaricides, making them less effective. Changes in behavior and physiology involve alterations in the tick's behavior, such as reduced exposure to acaricides, and changes in the tick's physiology, such as altered cuticle permeability, which can reduce the uptake of 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. Materials and Methods

2.1. Sample collection

During June 2022 to May 2023, brown dog ticks were collected from sheepdogs of four locations in rural areas of South Khorasan Province, east of Iran. The engorged and/or partially engorged female ticks were collected from naturally infested dogs using tick collection 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 was 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 laying. 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 World Health Organization (WHO) guidelines [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) was used as the active ingredient (AI) 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 a previous study, which 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% AI was also tested. Bioassays were conducted in three replicates with 100 larvae per packet 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) according to 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 DNA extraction. The quality and concentration of the DNA obtained were assessed by agarose gel electrophoresis and using a Nanodrop spectrophotometer. Polymerase chain reaction (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), 12.5 µL of 2X Taq PCR MasterMix (Takara, Japan), and 8.5 µL of nuclease-free water. The primers FG-228 (5'- CTA

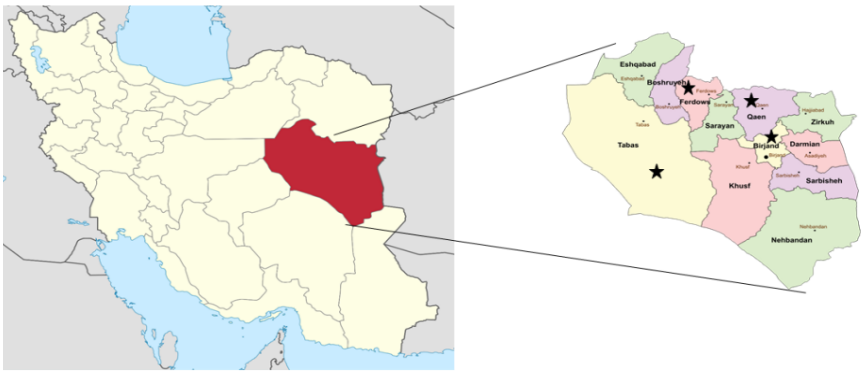

Figure 1. The collection site of ticks

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 analyses.

2.4 Statistical analysis

The evaluation of mortality was conducted at 24 hours. The adjustment of control mortality was calculated based on Abbott's formula [16]. The percentage survival was recorded for each multiple of the diagnostic concentration. The classification of resistant phenotypes was divided into 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 in LC99×2) [17].

3. Results

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

In these study, only four populations of *R. sanguineus* were reared successfully and provided sufficient numbers of larvae, and were subsequently subjected to bioassay to test their susceptibility to permethrin. The field-caught *R. sanguineus* strains were evaluated for mortality with permethrin concentrations one and two times the diagnostic concentrations, i.e. 0.09 and 0.19%. The mortality rate at LC99 ranged between 40.5 and 49.1% (Table 1).

At 2×LC99 (0.19% AI), lethal rates ranged from 48.3% to 65.1%. To screen for mutations in 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).

The analysis revealed four genotypes on domain III among the *R. sanguineus* population from eastern Iran, by comparing the susceptible (GenBank KU886031) and permethrin-resistant (KU886032) *R. sanguineus* larvae. Out of 40 studied ticks, two 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 (Figures 3 and 4).

4. Discussion

This study provides the first laboratory-confirmed permethrin resistance data for brown dog ticks from the eastern Iran. *R. sanguineus* is one of the most prevalent tick species infected with different pathogens in Iran [18]. The results of this study provide important preliminary insights into the efficacy of permethrin on the *R. sanguineus* population in eastern. The findings show that the mortality rates of *R. sanguineus* populations varied significantly when subjected to different concentrations of permethrin. At 2×LC99 (0.19% AI), lethal rates ranged from 48.3% to 65.1%, indicating that this concentration is not effective for controlling the tick population. Previous studies in Iran have also shown high levels of resistance to pyrethroids 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 *R. sanguineus*

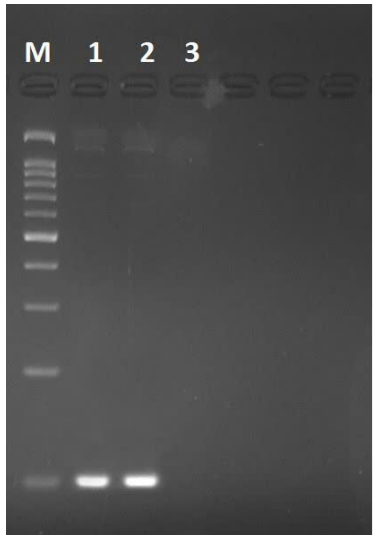

Figure 2. Agarose gel separation of representative PCR products of the voltage-sensitive sodium channel gene

Note: Lanes 1–2: Positive isolates; Lane 3: Negative control, Lane M: DNA ladder (100 bp).

in this area. Previous studies from around the world have also demonstrated resistance to pyrethroid pesticides among *R. sanguineus* [12, 13]. Importantly, our bioassay findings highlight the need for careful consideration of appropriate acaricide concentrations to achieve effective tick control, suggesting that higher doses may be required to obtained satisfactory outcomes. Overall, these results represent an important step toward the development of more effective and targeted approaches for tick control in Iran.

In this study, Only four populations 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

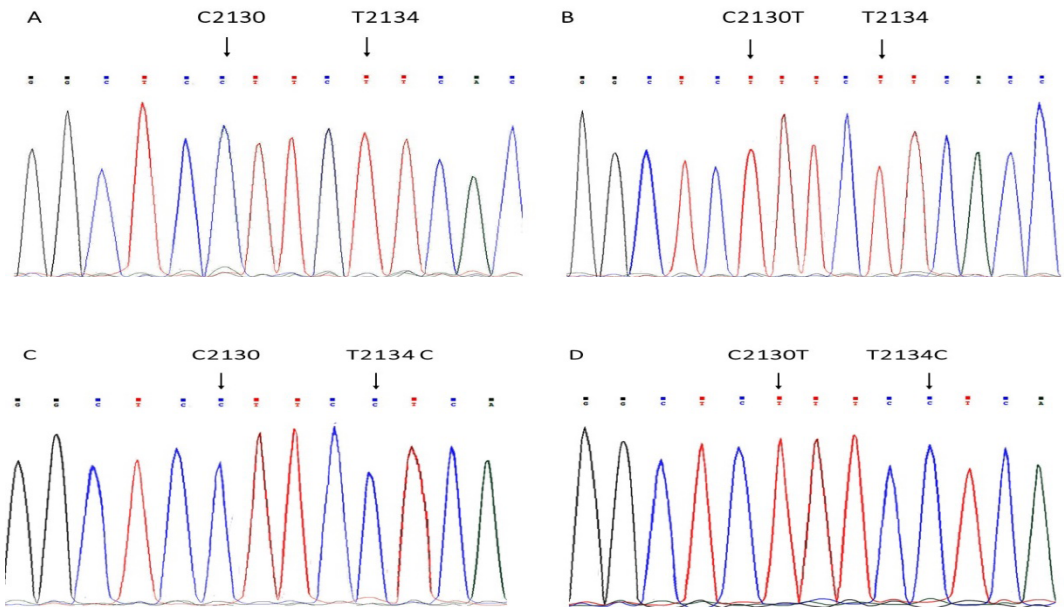

Figure 3. Chromatograms showing kdr genotypes of *R. sanguineus*

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*

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

Strain	Location	% Mortality	
		LC99 (0.09% AI)	2×LC99 (0.19% AI)
B1	Birjand	40.5	49.6
B2	Ferdows	42.5	48.3
B3	Ghaen	49.1	60.1
B4	Tabas	38.1	65.1

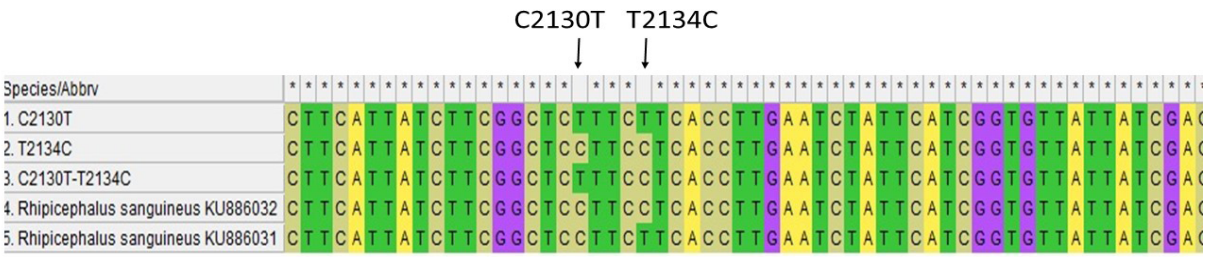

Figure 4. Gene sequences of the voltage-gated sodium channel of *R. sanguineus* were aligned with the wild sequences (GenBank accession number: KU886031) showing the mutations *C2130T* and *T2134C* (three haplotypes were reported)

could explore how these sub-lethal concentrations of permethrin impact the reproductive capacity of adult female *R. sanguineus* 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* mutation in this gene is associated with resistance to pyrethroid in *R. sanguineus* [3]. The findings reveal that out of the 40 ticks examined, 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, which could explain 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].

5. Conclusion

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 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 resulting in a smaller sample size and fewer gene targets being analyzed than initially desired.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of the Science and Research Branch, Islamic Azad University, Tehran, Iran (Code: IR.IAU.SRB.REC.1403.330).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

The present study did not receive any grant from public, commercial, or not-for-profit funding agencies.

Authors' contributions

Conceptualization: Abolfazl Valizadeh, Saloomeh Shirali, and Rahmat Solgi; Project administration: Abolfazl Valizadeh; Methodology and investigation: Saloomeh Shirali and Ehsan Khaksar; Data curation: Abolfazl Valizadeh and Rahmat Solgi; Visualization and validation: Rahmat Solgi and Saloomeh Shirali; Supervision and resources: Rahmat Solgi and Saloomeh Shirali; Software: Ehsan Khaksar; Funding acquisition: Saloomeh Shirali; Formal analysis and writing the original draft: Abolfazl Valizadeh, Rahmat Solgi, and Ehsan Khaksar; Review and editing: All authors.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

Some parts of this research were performed at Birjand University of Medical Sciences, and we would like to express our gratitude to all our colleagues in these centers who kindly helped us during the study.

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