Molecular and parasitological assessment of *Theileria* species and tick vectors in sheep (*Ovis aires*) from Kerman, Southeastern Iran

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Abstract

This study assessed the prevalence of *Theileria* species in sheep (*Ovis aires*) from Kerman, Southeastern Iran, using semi-nested PCR. Fifty blood samples were collected from the jugular vein of sheep, prepared for microscopic examination, and stored at −20 °C for molecular analysis. Simultaneously, ticks were collected and identified. Molecular diagnostics detected *Theileria* DNA in 44% (22/50) of samples, all identified as *Theileria ovis*, compared to 20% prevalence detected by microscopy, demonstrating the superior sensitivity of molecular methods. The tick Hyalomma anatolicum was confirmed as the primary vector. The high prevalence of pathogenic *T. ovis* emphasizes the importance of molecular surveillance and targeted control strategies to reduce the impact of theileriosis on sheep health and productivity in the region.

Keywords: *Theileria*, Sheep, Semi-nested PCR, Kerman, Iran

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Introduction

Theileriosis is a tick-borne parasitic disease caused by protozoa belonging to the genus *Theileria*, which are of considerable concern in veterinary medicine due to their role in causing a significant disease in livestock, particularly sheep (*Ovis aires*) and cattle (Onizawa and Jenkins, 2024). Theileriosis can cause severe economic losses in the sheep farming industry worldwide, owing to high morbidity and mortality rates associated with the disease (Valente *et al*., 2022). The impact of *Theileria* infections is exacerbated by the potential for rapid spread within livestock populations, making effective management and control measures imperative. The clinical manifestations of theileriosis in sheep vary widely and can range from subclinical infections, where animals show minimal or no symptoms, to severe clinical disease characterized by fever, anemia, weakness, and in some cases, death. Affected animals may exhibit signs of depression, inappetence, and a general decline in health (Kiara *et al*., 2018, Onizawa and Jenkins, 2024). The production losses associated with theileriosis include reduced wool and meat yield, control costs, as well as increased veterinary costs for treatment of affected animals (Inci *et al*., 2007), highlighting the economic importance of managing this disease effectively within sheep husbandry systems.

Traditional methods for diagnosing *Theileria* infections have relied on clinical symptoms and morphological detection through microscopic examination of blood smears. However, these techniques often yield inconclusive results, especially in subclinical infections where low parasite densities can evade detection. The limitations of these conventional methods have prompted a shift towards molecular techniques, such as Polymerase Chain Reaction (PCR), which offer higher sensitivity and specificity for the detection of *Theileria* species. Semi-nested PCR has gained popularity as an advanced molecular technique that combines the advantages of conventional PCR with increased sensitivity and specificity (Hsu *et al*., 2021).

This method not only enhances detection capabilities but also allows for the simultaneous identification of multiple *Theileria* species within a single assay. The ability to accurately differentiate between species is critical, as various *Theileria* species can present differing clinical outcomes and levels of virulence. This study aims to employ semi-nested PCR for the molecular identification of *Theileria* species in sheep. Enhanced diagnostic capabilities, coupled with targeted control measures, are essential for reducing the impact of *Theileria* infections and ensuring the sustainability of sheep farming operations.

Materials and methods

Study area and sample collection

Kerman City, the capital of Kerman Province, is located in southeastern Iran, and situated at an altitude of approximately 1,750 meters above sea

level, Kerman enjoys a unique climate characterized by arid and semi-arid conditions. The climate in Kerman is predominantly arid with little rainfall, averaging about 120 mm (4.7 inches) annually, primarily occurring between late autumn and early spring. A total of 50 sheep were randomly selected from various farms in the area. The blood samples were collected from the jugular vein of each sheep using sterile techniques and transferred into tubes containing EDTA as an anticoagulant. A thin blood smear was prepared for each sample for microscopic examination and the remaining was stored at -20°C until molecular analysis. At the same time, the animals were thoroughly examined, and any ticks found on them were gathered into properly labeled specimen tubes.

Microscopic and tick examination

The smears were fixed with methanol and stained in 5% Giemsa solution for 30 min, and then examined microscopically to screen for the presence of *Theileria* species under oil immersion lens (×1000). The isolated ticks were counted and identified using available diagnostic keys (Estrada-Peña, 2004).

Molecular examination

Total DNA was extracted from blood collected in EDTA tubes using a DNA isolation kit from GeneAll, (South Korea). Subsequently, a semi-nested PCR procedure was conducted, following the protocol outlined by Shayan and Rahbari in 2005 (Shayan and Rahbari, 2005). In the initial amplification round of the semi-nested PCR, two oligonucleotide primers were employed: the forward primer (P1) 5′-CACAGGGAGGTAGTGACAAG -3′ and the reverse primer (P2) 5′-AAGAATTTCACCTATGACAG -3′, which were specifically designed to differentiate between *Theileria* and *Babesia*. For the second round of amplification, internal primers were utilized to target *T. lestoquardi*, employing the forward primer (P2) 5′-AAGAATTTCACCTATGACAG -3′ alongside the reverse primer (P3) 5′-ATTGCTTGTGTCCCTCCG -3′. To detect *T. ovis*, the same forward primer (P2) 5′-AAGAATTTCACCTATGACAG -3′ was used in conjunction with a distinct reverse primer (P4) 5′-TTGCTTTTGCTCCTTTACGAG -3′. Amplification was performed in 20 µL reaction volumes using the Accupower PCR premix kit (Bioneer®, South Korea). Each reaction contained a final concentration of 250 µM for each dNTP, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl2, and 1 U of Taq DNA polymerase, along with 10 pmol of each PCR primer provided by Takapouzist Co. (Iran). To initiate the PCR, 1 µL of DNA template was introduced into the reaction mixture, which was then adjusted to a final volume of 20 µL with sterile distilled water. The thermal cycling conditions were programmed on a Bio-Rad thermal cycler as follows: an initial denaturation step at 95°C for 5 minutes, followed by 36 cycles consisting of denaturation at 94 °C for

45 seconds, primer annealing at temperatures between 54-58°C for 45 seconds, and extension at 72°C for 45 seconds. A final extension step was performed at 72°C for 10 minutes, after which the products were cooled to 4°C

To analyze the PCR products, electrophoresis was conducted on a 1.5% agarose gel using TBE buffer, and the bands were visualized under ultraviolet light after staining with ethidium bromide. The initial round of PCR was expected to generate a 431 bp band, indicating the presence of *Theileria* spp. A second round of amplification was performed on the products that tested positive in the first round, maintaining the same reaction conditions. Electrophoresis was repeated to identify specific bands for T. lestoquardi and T. ovis. Positive control samples for the PCR included genomic DNA of *T. ovis* and *T. lestoquardi* graciously provided by Dr. Saeed Yaghfoori (derived from sheep blood in a related study conducted at the Faculty of Veterinary Medicine at Ferdowsi University of Mashhad) (Yaghfoori *et al*., 2013). Additionally, each PCR amplification series incorporated a negative control that contained only water.

Results

The molecular analysis conducted by the PCR technique revealed a significant presence of Theileria in the sampled sheep population. Out of 50 sheep analyzed, 22 samples (44%) tested positive for *Theileria*, as revealed by the amplification of a 431bp product (Fig. 1), all of which were specifically identified as *Theileria* ovis by the Semi-nested PCR analysis, with a 238 bp band (Fig. 2).

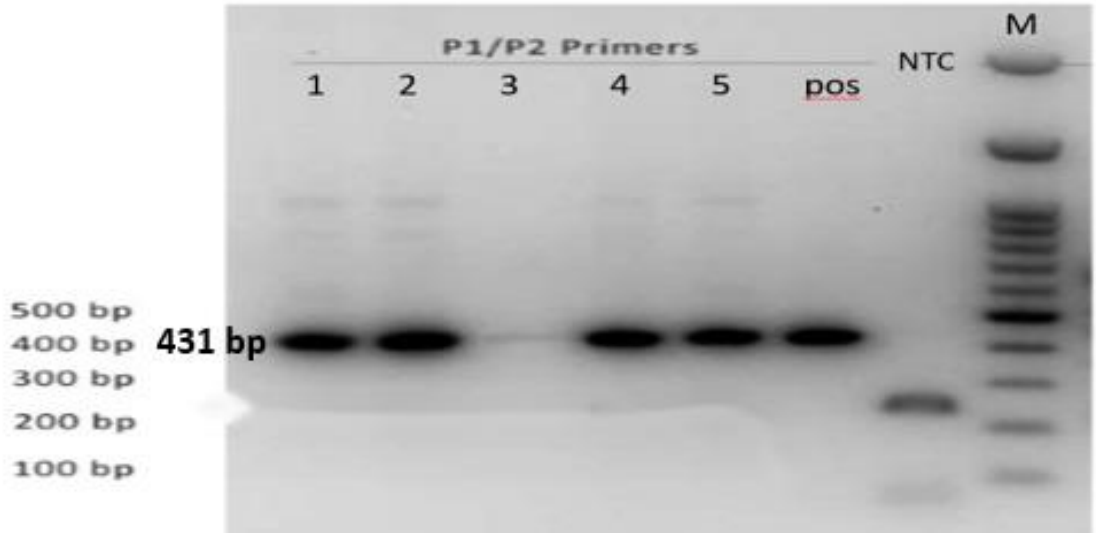

Figure 1: Agarose-gel electrophoresis of sheep (*Ovis aires*) DNA samples amplified in the first round of semi-nested PCR. Ladder marker (lane M), Negative control (lane NTC), Positive control (lane pos, 431bp), *Theileria* spp. (lane 1, 2, 4, 5, 431bp), negative sample (lane 3).

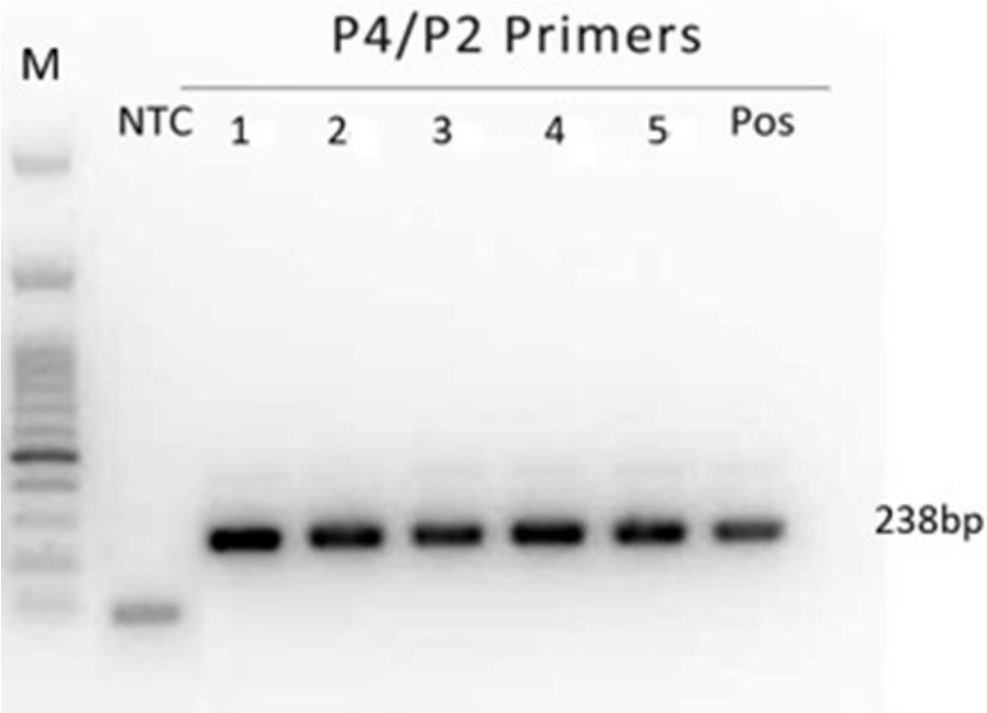

Figure 2: Agarose-gel electrophoresis of DNA from *Theileria*-positive domestic sheep (*Ovis aires*) samples amplified in the second round of semi-nested PCR. Ladder marker (lane M), Negative control (lane NTC), T. ovis (lane 1, 2, 3, 4, 5: 238bp), Positive control for T. ovis (lane Pos, 238bp).

Discussion

This finding is in agreement with previous studies carried out in Iran that reported *T. ovis* as the only *Theileria* species affecting sheep in the north-west and the eastern half of the country (Heidarpour Bami *et al*., 2010, Imani Baran *et al*., 2020). However, several studies have reported the presence of additional species of *Theileria* in sheep (Habibi *et al*., 2020). In a study conducted by Zaeemi *et al*. (2011), a nested PCR-RFLP method was employed to identify various species of *Theileria* in sheep from certain regions in the western part of Iran. Their findings revealed that *Theileria* infection was present in 32.8% of the examined samples, with 54.8% identified as *T. lestoquardi* and 40.2% as *T. ovis*. Additionally, mixed infections involving *T. annulata* and *T. lestoquardi* were observed in 4.8% of the cases (Zaeemi *et al*., 2011). This inconsistency can be attributed to some factors including: i; different geographical locations, which may harbor varied tick populations and environmental conditions that facilitate the transmission of multiple *Theileria* species. In regions where other *Theileria* species are found, such as *T. annulata* or *T. lestoquardi*, the vector-tick interactions and the overall epidemiological landscape may support a broader diversity of *Theileria* infections, ii; differences in sampling methodologies, which can also account for the isolation of additional species. Studies employing larger sample sizes or including diverse populations might

inadvertently capture other *Theileria* species due to differences in host exposure and immune responses, and finally, iii; seasonal and temporal factors, that may influence the prevalence of *Theileria* species, as some species may have different seasonal peaks or are more prevalent at certain times of the year (Soosaraei *et al*., 2018, Alanazi *et al*., 2019).

Additionally, just as in our study, *T. ovis* was found to be the most prevalent *Theileria* species in sheep in different parts of Iran including, Fars (43%), Sistan and Baluchistan (36.2%) (Zarei *et al*., 2019), , Khorasan Razavi (55.6%) (Razmi *et al*., 2013), and Semnan (62.5%) (Heidarpour Bami *et al*., 2010) provinces, reinforcing the ongoing challenge posed by this pathogen in sheep farming. In contrast, a lower prevalence of *T. ovis* has been documented by (HashemzadehFarhang *et al*., 2011) (5.5%).

The combination of favorable ecological conditions, effective transmission dynamics, and the specific susceptibility of local sheep breeds contributes to *T. ovis* being the most frequent *Theileria* species in Iran. Furthermore, studies conducted in other countries such as Pakistan (9%) (Riaz *et al*., 2024), , Turkey (54%) (Altay *et al*., 2005), and Saudi Arabia (18.5%) (Almahallawi *et al*., 2024), have also reported different prevalence rates of *T. ovis*. These findings illustrate that *T. ovis* is a significant pathogen not only in Iran but also across parts of the Middle East and Africa, reflecting ecological and agricultural practices that favor the transmission of this pathogen. In addition, the differences in prevalence of *Theileria* infection in different regions may be partly related to differences in livestock management, herd movement, environmental and climatic changes, host susceptibility, breed of animals studied, seasonal frequency of vectors, tick resistance to insecticides, inadequate preventive measures, number of samples tested and sensitivity of diagnostic methods *(*Riaz *et al.,* 2017, Cui *et al*., 2024).

When compared to the results from microscopic examination, which indicated a lower prevalence of *Theileria* at 20% (10 out of 50 sheep), the disparity highlights the sensitivity and specificity variations between diagnostic methods. Previous studies also confirmed that molecular approaches including PCR are more effective and accurate than traditional methods. Microscopic examination, while effective, can miss lower infection levels due to factors such as the stage of infection and technician skill. In contrast, the semi-nested PCR method provides a more sensitive and comprehensive approach, capable of detecting small amounts of the pathogen's DNA even at early infection stages (Hsu *et al*., 2021, Mohanta *et al*., 2023).

In the present study, 57 hard tick belonging to the genus *Hyalomma* were isolated from the studied animals. *Hyalomma* is the most abundant tick in Iran and has been reported in most parts of the country (Arghavani *et al*., 2022). The compelling association of *T. ovis*

with *Hyalomma* ticks, reinforces the ecological dynamics of *Theileria* transmission. All collected ticks in the current study were identified as *H. anatolicum*, which is known for its role as an efficient vector for various pathogens, including *Theileria* species, and other tick-borne diseases in livestock and human (Wang *et al*., 2024). Previous studies in Iran, such as those by Biglari *et al*. (2018), reported high incidences of *H. anatolicum* ticks in sheep (Biglari *et al*., 2018, Mohanta et al., 2024). Given the high prevalence of *T. ovis* associated with *H. anatolicum*, implementing effective tick control measures is crucial for managing *Theileria* infections within sheep populations. Integrated pest management strategies, which include acaricides, strategic pasture management, and regular tick population monitoring, could significantly reduce the prevalence of *T. ovis* in susceptible sheep herds. In conclusion, this study confirms a significant presence of *T. ovis* in sheep in Kerman, with a PCR prevalence rate of 44% compared to a 20% rate from microscopic examination. The strong association between *T. ovis* and *H. anatolicum*, coupled with the extensive documentation of *T. ovis* in various Iranian studies and neighboring countries, underscores the importance of ongoing surveillance and comprehensive control strategies to mitigate the health impacts of *Theileria* infections in sheep farming. Additionally, more comprehensive studies will be essential to evaluate the prevalence of *Theileria* species and the associated risk factors in sheep of the study area.

Ethics

The study design was approved by the ethics committee of the Islamic Azad University, Kerman, Iran.

Authors' contribution

Study concept and design: A. A.
Acquisition of data: M. M.S.
Analysis and interpretation of data: A. A.
Drafting of the manuscript: R. M.
Critical revision of the manuscript for important intellectual content: A. A
Statistical analysis: B. K
Administrative, technical, and material support: M. M

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Conflict of interest

The authors certify that they have no conflicts of interest.

Data availability

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

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