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Research Paper

Theileria and *Babesia* Infections in Sheep and Goats With Abortion Histories in East Azerbaijan Province, Northwest Iran

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How to cite this article Shahbazi P, Ayoubi R, Khordadmehr M, Sadri H, Shirazi J, AkbariH, et al. *Theileria* and *Babesia* Infections in Sheep and Goats With Abortion Histories in East Azerbaijan Province, Northwest Iran. Archives of Razi Institute Journal. 2026; 81(2):445-452. https://doi.org/10.32598/ARI.81.2.3683

https://doi.org/10.32598/ARI.81.2.3683

Article info:
Received: 19 Sep 2025
Accepted: 18 Nov 2025
Published: 01 Mar 2026

Keywords:
Small ruminants, Hemoparasites, Anemia, Abortion, Iran

ABSTRACT

Introduction: *Theileria* and *Babesia* belong to a group of protozoan parasites known as Apicomplexa within the Piroplasmida order. Both *Theileria* and *Babesia* disrupt normal hematological functions in their respective hosts, causing hypoxia, anemia and systemic disease. Immunosuppressive effects associated with such infections may increase the susceptibility of pregnant animals to secondary infections and abortion. This study investigated the frequency of *Theileria* and *Babesia* infections in small ruminants with a new abortion evidence in East Azerbaijan Province, Iran.

Materials & Methods: The blood samples (n=373) were collected from 43 flocks of goats and sheep across nine cities of East-Azerbaijan Province during calving seasons (autumn and winter seasons 2023). Following DNA extraction from whole blood, a gradient polymerase chain reaction (PCR) method using the specific primers was employed to detect the *Theileria* and *Babesia* genomes.

Results: Molecular findings revealed the infection rates of 70.5% for *Theileria* and 8.5% for *Babesia* infections. Besides, the species identified in the positive samples included *Theileria ovis* (64.5%), *Theileria lestoquardi* (6%), and *Babesia ovis* (8.5%).

Conclusion: Taken together, the detection of *Theileria* and *Babesia* infections with a much higher rate, particularly during the autumn and winter, suggests that the resulting hypoxia and anemia, can play indirectly notable roles in the abortion of animals in this province. Thus, effective tick management strategies are critical to preventing these infections and protecting livestock health.

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1. Introduction

Vector-borne illnesses are caused by various pathogens, including bacteria and viruses, which depend on blood-sucking arthropods to transmit diseases effectively through their bites into host organisms. Certain pathogens known as haemoparasites, exhibit a tendency to infiltrate and harm the host's bloodstream. Common instances involve *Babesia* and *Theileria* species, which are tick-borne haemoprotozoan parasites impacting livestock in tropical and subtropical areas [1, 2]. *Theileria* and *Babesia* belong to a group of protozoan parasites known as Apicomplexa within the Piroplasmida order. They are primarily tick-transmitted, affecting ruminants, leading to prominent parasitic diseases in Iran [3]. The primary causative agents of Theileriosis, in small ruminants include *Theileria ovis*, *Theileria uilenbergi*, *Theileria lestoquardi*, and *Theileria luwenshuni* [4-8]. *Babesia motasi*, *Babesia ovis*, and *Babesia crassa* are important parasites of sheep, causing a disease known as ovine babesiosis. The disease, which has varying infection rates in Iran, not only increases the mortality rate among affected animals but also significantly reduces their productivity [9-12]. Both *Theileria* and *Babesia* disrupt normal hematological functions in their respective hosts, causing anemia and systemic disease. These conditions result in alterations of vascular dynamics that impede uteroplacental blood circulation. The immunosuppressive effects associated with such infections, along with stress and ecological conditions, may increase the susceptibility of pregnant animals to secondary infections, which can also lead to abortion [1, 13-15]. Thus, Theileriosis and Babesiosis continue to pose a major challenge in livestock management, resulting in substantial economic losses despite the use of various control methods [15, 16]. Thus, various methodologies have been employed in identifying *Babesia* and *Theileria* species, such as examining blood smears and performing serological assays. However, in recent years, molecular methods like polymerase chain reaction (PCR) have been frequently used in veterinary parasitology to identify blood protozoans [17]. The aim of the current study was to determine the frequency of *Babesia* and *Theileria* infections in sheep and goats with histories of abortion using molecular method in East Azerbaijan Province, northwest Iran.

2. Materials and Methods

2.1. Area and sampling

The present study was carried out in nine cities withinthe East-Azerbaijan Province, northwest Iran, including, Tabriz, Khoda Afrin, Jolfa, Marand, Charuymaq, Heris, Mianeh, Bostan Abad, and Hashtrud. These findings regarding *Babesia* and *Theileria* infections as part of a larger investigation into the infectious and non-infectious agents of abortion in small ruminants (sheep and goats) in East Azerbaijan Province, Iran. For this purpose, a total of 373 blood samples were collected from sheep and goats between November 2023 and February 2024 from farms where owners had referred or contacted for abortion. We studied a total of 43 sheep flocks, all of which were managed under traditional conditions. Sampling was carried out using a non-probability sampling method (i.e. convenience sampling) due to limited data on prevalence of these infections. Here, two mL of blood samples treated with anticoagulant were obtained from aborted animals and stored at -70 ˚C for further molecular analyses.

2.2. Molecular study (DNA extraction and PCR assay)

The nucleic acid (genomic DNA) was extracted from the whole blood using commercial kits (DNA Extraction Kit, Pishgaman Sanjesh, Iran) based on the manufacturer's instructions [18]. The quality and quantity of the extracted genome were analyzed using a NanoPhotometer® NP80 (IMPLEN, Germany). All PCR reactions were performed using Taq DNA Polymerase Master Mix RED® (Ampliqon, Denmark) with 3 µL of DNA and a final volume of 25 µL. The amplified products were evaluated via electrophoresis on 2% agarose gels stained with a safe DNA stain (SinaClon, Iran). The primers and reaction conditions are presented in Table 1 [19-20].

Specifically, in the first step, specific primers were used to identify *Theileria* and *Babesia* genus. Then, the 1 µL of the positive PCR products were used to detect *T. lestoquardi*, and *B. ovis* species via Semi nested-PCR. Additionally, DNA from *Theileria* positive samples was used for *T. ovis* detection using species- specific primer. The target gene in all PCR reactions is *18SrRNA gene*. As more detail, the semi-nested PCRs for *Theileria* and *Babesia* species are as follows: In step 1, 18S primers were used to detect genus *Theileria* (430-426bp) and *Babesia* (389-402bp). with Thei.18S as sense or the forward primer and Bab.18S as antisense or the reverse primer. In step 2 (semi-nested) for *T. lestoqurdi* detection, Thei.18S

Table 1. Primers and PCR conditions

Gene	Sequence	Product Size (bp)	Annealing Tempreture (°C)	Cycles	Ref.
Thei.18S (sense)	5′ CACAGGGAGGTAGTGACAAG 3′	430-426 Theileria	56	38 (45″)	[19]
Bab.18S (antisense)	5′ AAGAATTTCACCTCTGACAG 3′	389-402 Babesia			
B.ovis (sense)	5′ TGCGCGCGGCCTTTGCGT3′	181	58	35 (60″)	[20]
T. lestoquardi (antisense)	5′ ATTGCTTGTGTCCCTCCG 3′	235	57	38 (45″)	[20]
T. ovis	F: 5′ TCGAGACCTTCGGGT 3′ R: 5′TCCGGACATTGTAAAACAAA3′	520	53	40 (30″)	[21]

was sense or forward primer and *T. lestoquardi* was antisense or reverse primer (In short: thei.18S, Bab.18S and *T. lestoqurdi*). For *B. ovis* detection, the *B. ovis* was sense or forward primer and Bab.18S was antisense or reverse primer (In short: thei.18S, Bab.18S and *B. ovis*). *T. ovis* had two specific primers as F (forward) and R (reverse).

The PCR cycle conditions were determined based on the reference recommendation and also within the temperature range recommended by the primer manufacturer using a gradient thermocycler.

2.3. Statistical analyses

Statistical analysis of the obtained data was performed using SPSS software version 18.0 (IBM, NY, USA). The evaluation outcomes were presented as Mean±SD, and the data were assessed using a 95% confidence interval (CI).

3. Results

The results of the molecular study are presented in Figure 1 and Table 2. Infection rates were different across the nine cities from 37.7-100% and 0-20% in *Theileria* and *Babesia*, respectively, showing the targets with 426-430 bp and 389-402 bp in PCR results, respectively (Figure 1). The overall prevalence in this province for *Theileria* and *Babesia* was 70.5% (95% CI, 0.7%, 0.4%) and 8.5% (95% CI, 0.085%, 0.02%), respectively. Notably, 7.5% of the examined samples were positive for both infections. Furthermore, the prevalence rates for *T. lestoquardi* and *T. ovis* were 64.5% and 6%, respectively.

All *Babesia*- positive samples were identified as *B. ovis* (8.5%). Samples positive for *T. ovis* infection showed a 529 bp band, while those positive for *T. lestoquardi* showed a 235 bp species-specific band and a 430 bp *Theileria* genus-specific band. The 430 bp band was obtained in the semi nested PCR assay due to the reaction of the external primers present in the PCR product (Figure 2).

4. Discussion

The present study demonstrated a much higher prevalence of both *Theileria* (70.5%) and Babesia (8.5%) infections, particularly the presence of *Theileria* genome was notable. Of note, the present study examined samples were collected from sick animals with histories of abortion, even though the sampling period was not the peak or maximum time of presence of blood parasitic diseases. Although peak tick activity occurs in summer, sampling was conducted in autumn and winter, which are the calving seasons of sheep and goats. Another important point is that the animals sampled did not exhibit clinical symptoms related to *Theileria* and *Babesia* infections. These present results underscore the importance of diagnosing, controlling and preventing these infections in this province. Notably, the incidence of *T. lestoquardi* (6%) was lower than that of *T. ovis* (64.5%); it is more pathogenic than *T. ovis* (64.5%). In this regard, some parameters such as genetic diversity, strain virulence, and host immunity can affect the differences in pathogenicity. Sporozoites of *Theileria* spp., initially penetrate host leukocytes, ultimately impairing normal cellular functions, which results in unregulated cell proliferation and the development of schizonts. This often leads to significant destruction of the lymphoid tissues, leading to identifiable impairment of immune function with far-reaching consequences [13-15]. Infections caused by *Theileria* spp. on particular counts, manifest as swollen lymph nodes and jaundice, and often result in abortions in pregnant animals during the later stages of gestation [22]. In the present study, although clinical symptoms and morbidity rate were not recorded for *Theileria* or *Babesia* infections in the affected animals,

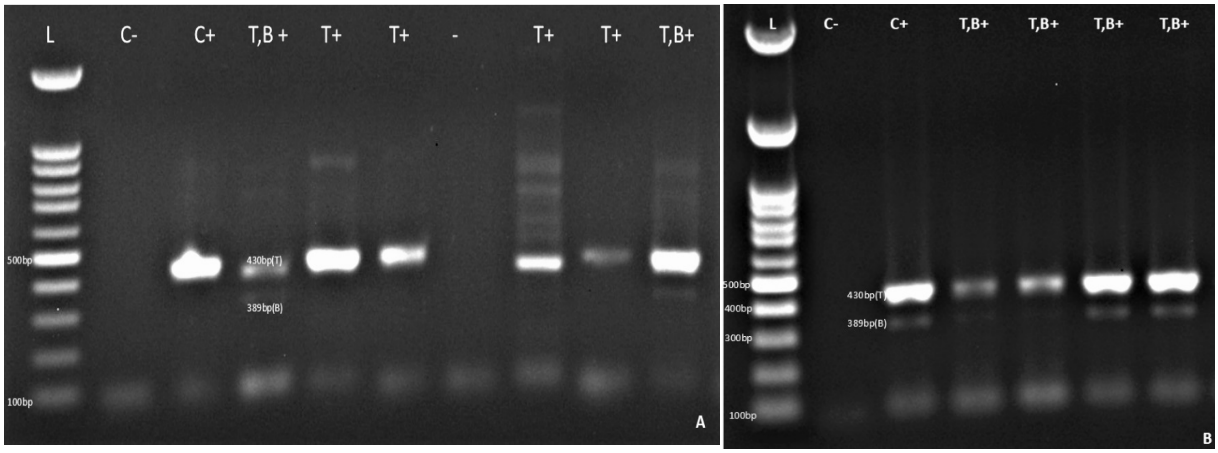

Figure. 1. PCR findings for detecting the *Theileria* and *Babesia* genomes in the blood samples

Note: The PCR products with a 426-430 bp and a 389-402 bp bands for *Theileria* and *Babesia* genomes, respectively. L: Ladder 100 bp; C-: Negative control; C+: Positive control; T+: The samples with positive results with a 430 bp band for *Theileria* sp. and T, B+: the samples showed coinfection with a 430 bp and a 398 bp amplifications for *Theileria* sp. and *Babesia* sp. respectively.

it appears that these infections might indirectly impact abortion rate.

The prevalence of infection with *T. ovis* was previously reported to be as high as 55.6% in Khorasan Razavi Province between 2009 and 2011 [23]. Similarly, *T. ovis* were found in 88% of inspected sheep in the Ahvaz region (southwest of Iran) via PCR, with 67.8% of them were also detected microscopically [24]. In Sistan and Baluchestan Province [25], sheep showed the highest prevalence of 71%, while in North Khorasan and Razavi Khorasan [23], the prevalence was 70% and 55.6%, respectively. Our findings align closely with the reports from Sistan-Baluchestan and North Khorasan provinces. *Babesia* species infect erythrocytes and proliferate inside the host cell. The resulting parasitemia can lead to a decrease in the number of red blood cells, accompanied by various types of anemia. The principal pathogenic consequence arises from the breakdown of erythrocytes, leading to significant hemolytic anemia and ensuing dysfunction across multiple organ systems [5, 6]. Investigation into tick-borne diseases in Iran, especially

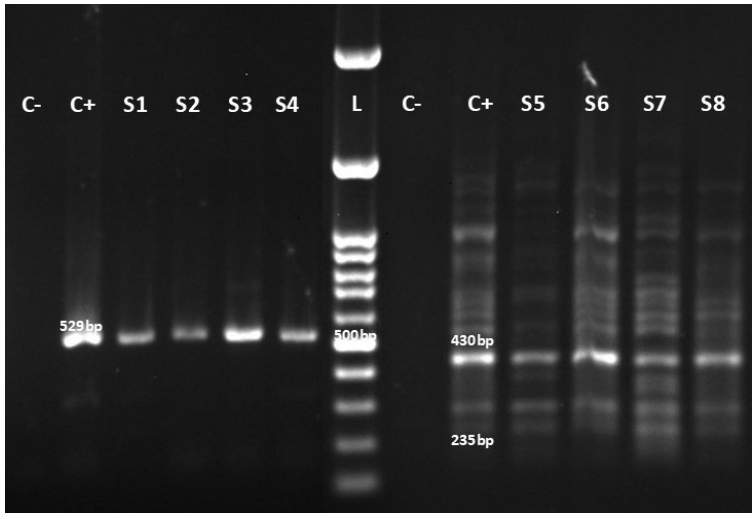

Figure. 2. PCR findings for detecting the Theileria species in the blood samples

Note: The PCR products with a 529 bp and a 235 bp bands for *T. ovis* and *T. lestoquardi* genomes, respectively. L: Ladder 100 bp; C-: Negative control; C+: Positive control; S1-S4: The positive samples for *T. ovis* infection with a 529 bp band and S5-S8: The positive samples for *T. lestoquardi* with a 235 bp species-specific band and a 430 bp *Theileria* genus-specific band has obtained in the nested PCR assay due to the reaction of the primary primers present in the PCR product.

Table 2. The PCR findings of *Theileria* and *Babesia* infections in the blood samples (n=373)

City	Number of the Samples	*Theileria*		*Babesia*	
		% Positive Samples	95% CI	% Positive Samples	95% CI
Tabriz	20	37.5	0.37, 0.21	20	0.2, 0.17
Marand	15	100	1, 0	14.28	0.14, 0.17
Charuymaq	128	68.65	0.68, 0.08	10.44	0.1, 0.05
Bostan Abad	36	52.17	0.52, 0.16	0	0, 0
Mianeh	73	80.55	0.8, 0.09	5.55	0.055, 0.052
Heris	21	57.14	0.57, 0.21	14.28	0.14, 0.14
Khoda Afarin	19	91.66	0.91, 0.12	16.66	0.16, 0.16
Jolfa	30	71.42	0.71, 0.16	9.52	0.09, 0.1
Hashtrud	25	83.33	0.83, 0.14	0	0, 0
Total	373	70.5	0.705, 0.4	8.5	0.085, 0.02

those caused by *B. ovis* and *B. motasi*, has presented several dissimilar rates. The cited infection rates for *B. ovis* range from 6.31% up to 44.9%, while *B. motasi* infections are comparatively lower, ranging between 0.5% and 14% [7-9]. In this regard, the infection rate of *B. ovis* has been reported at approximately 24.6% in sheep and 4.3% in goats in Khorasan Province [23], while *B. ovis* had a low prevalence (6[6.6%]) among sheep (n=90) in North Khorasan Province [26]. Also, the Kuhdasht region in Lorestan Province showed 4.3% infection in sheep and 0.4% in goats [12].

Reports from Zabol in southeastern Iran recorded a rate of around 4% in sheep [11]. A previous study highlighted the occurrence of blood protozoan infections among sheep in Bane, Kurdistan Province (Iran), where prevalence rates were 86.6%, 42.5%, and 24.9% for *B. ovis*, *T. ovis*, and *T. Annulata*, respectively [3]. Significantly, 86.4% of asymptomatic sheep were positive for *B. ovis* using the PCR method [3] this high rate of subclinical infection warrants attention and may be influenced by the sampling season, hygiene levels, and the sensitivity of the laboratory assays used.

Similar evidence in Turkey reported that 86.12% of animals were affected by one or more pathogens, with *B. ovis* being the most prevalent [14]. The individual infection rates for *B. ovis*, and *T. ovis* were 70.81% and 21.05%, respectively. Infection of solely *B. ovis* was more frequent (31.11%) than that caused by *T. ovis* (1.67%),while co-infection of *B. ovis* and *T. ovis* was 1.11% [14]. In another study, genomic DNA was analyzed from blood, ticks, and egg masses using 18S rRNA PCR and reverse line blotting (RLB) identified three *Theileria* species and one *Babesia* species. Among these, *T. ovis* was the most prevalent at 35.4%, followed by *B. ovis* (5.4%), and *T. annulata* (3.9%). Co-infection in this study also included those infected with both *T. ovis* and *B. ovis* [15]. These result indicated a remarkable prevalence of protozoan infection among sheep, however, the parasitic load for *T. annulata* (3.9%), *T. ovis* (35.4%), and *B. ovis* (5.4%) are low, indicating that such animals may be carriers identifiable only through a PCR test [16]. As mentioned previously, both *Theileria* and *Babesia* disrupt normal hematological functions in their respective hosts, causing anemia and systemic disease. These conditions result in alterations in vascular dynamics that impede uteroplacental blood circulation. Immunosuppressive effects associated with such infection may increase the susceptibility of pregnant animals to secondary infections, which can also cause abortion [1, 13]. Besides, stressors, whether due to the disease process or occurring independently of environmental conditions, often exacerbate the chances of aborting in affected animals [14, 15]. Additionally, ecological factors and implemented tick management strategies play critical roles in the frequency and intensity of Theileriosis and Babesiosis; hence, the efficient management practices are essential for protecting livestock health and productivity [15, 16].

5. Conclusion

In conclusion, both *Theileria* and *Babesia* cause anemia, hypoxia, and immunosuppression in their respective hosts, which may act to increase the susceptibility of pregnant animals to abortion. While the high infection rate suggests a plausible role in abortion, establishing definitive causality requires further investigation of parasite loads, placental pathology, and controlled cohort studies.

Tick control remains a prudent strategy given the economic burden of hemoparasites, even if their direct impact on abortion is uncertain. In this regard, implemented tick management strategies can play essential role in the frequency of these infections. Also, further studies to investigate this issue in apparently healthy livestock populations could help support these results.

Acknowledgements

The authors express their gratitude to Amir Reza Jafarizadeh, and Saeed Babazadeh for collecting the samples. The authors appreciate the support of the University of Tabriz, and also the Veterinary Organization, East Azerbaijan Province, Tabriz, Iran.

Compliance with ethical guidelines

All relevant international, national, and institutional guidelines for the care and use of animals, including the protocol approved by the Animal Research Ethics Committee of the University of Tabriz, Tabriz, Iran, were followed (Code: IR.TABRIZU.REC.1403.049).

Data availability

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

Funding

This work was supported by the University of Tabriz, Tabriz, Iran, and also the Veterinary Organization, East Azerbaijan Province, Tabriz, Iran.

Authors' contributions

Conceptualization: Parisa Shahbazi, Monireh Khordadmehr, Hassan Sadri, and Jafar Shirazi; Methodology: Parisa Shahbazi, Reza Ayoubi, Monireh Khordadmehr, Hassan Sadri, Hamid Akbari, Alireza Hakimnejad, and Ali Abdolmaleki; Software: Parisa Shahbazi, Monireh Khordadmehr, and Ali Abdolmaleki; Writing the original draft: Monireh Khordadmehr, Hassan Sadri, and Ali Abdolmaleki; Review, editing, and final approval: All authors; Supervision, project administration and funding acquisition: Monireh Khordadmehr.

Conflict of interest

The authors declared no conflict of interest.

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