# Prevalence of *Theileria* and *Babesia* infections in sheep and goats with a recent abortion history in East Azerbaijan Province, northwest Iran

**Running title: *Theileria* and *Babesia* infections in sheep and goats**

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## Abstract

*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 act to increase the susceptibility of pregnant animals to secondary infections and abortion. Thus, this study focused on the prevalence of *Theileria* and

*Babesia* infections in sheep and goats with a recent abortion history in East Azerbaijan province, northwest Iran. For this purpose, a total of 373 blood samples from 43 flocks were collected from sheep and goat in nine cities of East-Azerbaijan province, in calving seasons (fall and winter 2023). The conventional PCR method associated with using the specific primers was employed for detection of the *Theileria* and *Babesia* genomes after extracting DNA from the whole blood samples. Molecular findings revealed the infection rates of 70.5% and 8.5% for *Theileria* and *Babesia* infections, respectively. Besides, the species including *Theileria ovis* (64.5%)*, Theileria lestoquardi* (6%)*,* and *Babesia ovis* (8.5%) were detected in the PCR positive samples. In conclusion, the detection of *Theileria* and *Babesia* infections with a much higher rate (particularly in the autumn and winter seasons), which can result in hypoxia and anemia, can play indirectly notable roles in the abortion of animals in East Azerbaijan. Therefore, the importance of efficient management practices (tick management strategies) to prevent and control of these infections aims at protecting the health and productivity of the small ruminants in this province.

**Keywords:** Small ruminants, hemoparasites, anemia, abortion, Iran.

## 1. Introduction

Vector-borne illnesses stem from a variety of pathogens, such as bacteria and viruses that rely upon blood-sucking arthropods to spread through bites to host bodies effectively transmitting diseases. Certain pathogens known as haemoparasites exhibit a tendency to infiltrate and harm the host bloodstream. Common instances involve *Babesia* and *Theileria* species which are tick-borne haemoprotozoan parasites impacting livestock, in tropical and subtropical areas (1). *Theileria* and *Babesia* belong to a group of protozoan parasites known as *Apicomplexa* within

the Piroplasmida order. Are mainly transmitted by ticks impacting ruminant species, leading to prominent parasitic diseases in Iran (2). The primary causative agents of Theileriosis, in small ruminants, include *Theileria ovis, Theileria lestoquardi*, *Theileria uilenbergi*, and *Theileria luwenshuni* (3-8).

*Babesia ovis (B. ovis)*, *Babesia motasi (B. motasi)*, and *Babesia crassa (B. crassa)*, are important parasites of sheep causing a disease known as ovine babesiosis. The disease with various infection rates in Iran, doesn't just increase the death rate in the animals it affects but also reduces their production notably (9-13). Both *Theileria* and *Babesia* disrupt normal hematological functions in their respective hosts, causing anemia and systemic disease. These result in alterations of vascular dynamics that impede uteroplacental blood circulation. Immunosuppressive effects associated with such infection, stress, and ecological conditions may act to increase the susceptibility of pregnant animals to secondary infections, which also can cause abortion (1, 14-17) (18-20). Thus, Theileriosis and Babesiosis continue to pose a major challenge in livestock management with the evidencing of substantial economic losses, notwithstanding the use of various control methods (20, 21).

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 such as PCR have been widely used in veterinary parasitology to identify blood protozoans (22). The aim of the present study was to investigate the identification of *Babesia* and *Theileria* infections in sheep and goats with a recent abortion history by molecular method in East Azerbaijan province, northwest Iran.

## 2. Materials and methods

### *2.1. Study area*

This study was conducted in Tabriz, Marand, Charuymaq, Khoda Afrin, Jolfa, Heris, Bostan Abad, Mianeh, and Hashtrud cities located in the East-Azerbaijan province in northwest Iran. Indeed, this study presents findings on *Babesia* and *Theileria* infections as part of a larger investigation into the infectious and non-infectious causes of abortion in small ruminants in East Azerbaijan province, northwest Iran. For this purpose, from November 2023 to February 2024, a total of 373 blood samples were collected from sheep and goats in the mentioned regions, which their owners had referred or contacted for abortion in their farms. We studied a total of 43 sheep flocks, which were kept in the traditional conditions. Sampling was carried out using a non-probability sampling method (i.e. convenience sampling) due to a lack of information on viral infection prevalence in the area.

### **2.2.** ***Sampling method***

Here, five milliliters of blood samples that had been treated with anticoagulant were obtained from aborted and pregnant animals. To separate the whole blood was centrifuged at 6000 rpm for 10 minutes and then the v was transferred to a sterile 1.5-ml microcentrifuge tube, which was stored at -70 °C for further molecular analyses.

### *2.3. Molecular study (DNA extraction and PCR assay)*

The nucleic acid (DNA) was isolated from the whole blood using the commercial kits (DNA Extraction Kit, Pishgaman Sanjesh, Iran). Briefly, a volume of 200 µL of sample and 20 µL of proteinase K were added to a 1. 5 ml microtube, followed by 300 µL of Lysis Buffer and 10 µL of Carrier RNA. Following a 15-minute incubation at 60°C, a Binding Buffer was introduced before the mixture was moved to a column. The column underwent washing with Wash Buffers I, II, and

III and was subsequently eluted using Elution Buffer. The genome's quality and quantity were analyzed using NanoPhotometer® NP80 (IMPLEN, Germany).

All PCR assays were performed in a final volume of 25 µL with Taq DNA Polymerase Master Mix RED® (Ampliqon, Denmark) and 3µl DNA/cDNA, using the T100 Thermal Cycler (Bio-Rad, USA). The amplified products were detected through electrophoresis on 2% agarose gels stained with a safe DNA stain (SinaClon, Iran). The primers and reaction conditions for each PCR protocol are presented in Table 1. In more detail, at first step, the specific primers were used to identify *Theileria* and *Babesia* genus. Then, the 1 µl of the positive PCR products were used to detect of *T. lestuquardi*, and *B. ovis* species in the Semi nested-PCR. Also, the DNA of the *Theileria* positive samples used for *T. ovis* detection with species specific primer.

**Table 1.** Primers and thermocycler conditions used in this study.

| **Gene** | **Sequence (5'-3')** | **Product size (bp)** | **Annealing temperature (°C)** | **Cycles** (duration of per step-seconds) |
|---|---|---|---|---|
| Thei.18S (sense) | 5′ CACAGGGAGGTAGTGACAAG 3′ | 426-430 *Theileria* | 56 | 38 (45") |
| Bab.18S (antisense) | 5′ AAGAATTTCACCTCTGACAG 3′ | 389-402 *Babesia* | | |
| *B.Ovis* (sense) | 5′ TGCGCGCGGCCTTTGCGT 3′ | 181 | 58 | 35 (60") |
| *T. lestoquardi* (antisense) | 5′ ATTGCTTGTGTCCCTCCG 3′ | 235 | 57 | 38 (45") |
| *T. ovis* | F: 5′ TCGAGACCTTCGGGT 3′<br>R: 5′ TCCGGACATTGTAAAACAAA 3′ | 520 | 53 | 40 (30") |

### *2.4. Statistical analyses*

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

## 3. Results

The results of the molecular study are presented in Figure 1 and Table 2. Briefly, the infection rates were different in nine cities from 37.7-100% and 0.0-20% in *Theileria* and *Babesia*, respectively, which showed the targets with 426-430bp and 389-402bp in PCR results, respectively (Figure 1). The overall prevalence in this province for *Theileria* and *Babesia* were 70.5% (95%CI: 0.70±0.40) and 8.5% (95%CI: 0.085±0.020), respectively. Of note, 7.5% of the examined samples demonstrated positive results for both infections. Also, the prevalence of *T. lestoquardi* and *T.ovis* were 64.5% and 6% respectively. All *Babesia* positive samples were *B.ovis* (8.5%).The positive samples for *T.ovis* infection showed a 529 bp band and the positive samples for *T. lestoquardi* showed a 235 bp species-specific band and a 430 bp *Theileria* genus-specific band. The 430 bp band has obtained in the semi nested PCR assay due to the reaction of the external primers, present in the PCR product (Figure 2).

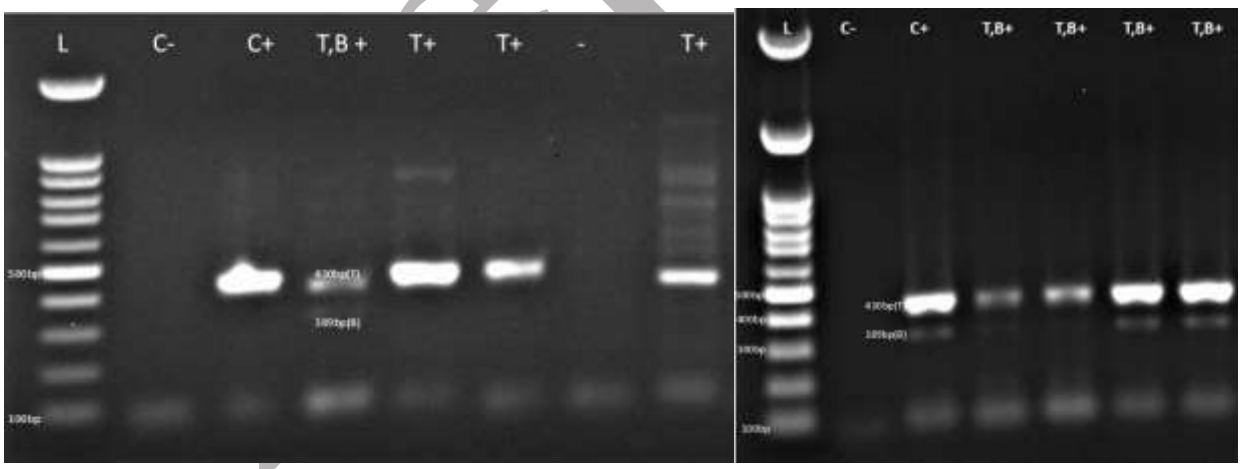


**Figure. 1.** PCR findings for detecting the *Theileria and Babesia* genomes in the blood samples. The PCR products with a 426-430 bp and a 389-402bp bands for *Theileria* and *Babesia* genomes, respectively, were detected through electrophoresis on agarose gels stained with a safe DNA stain. L: ladder 100 bp; C$^-$: negative control; C+: positive control; T+: the samples with positive results with a 430bp band for *Theileria* sp. and T, B+: the samples showed coinfection with a 430bp and a 398bp amplifications for *Theileria* sp. and *Babesia* sp. respectively.

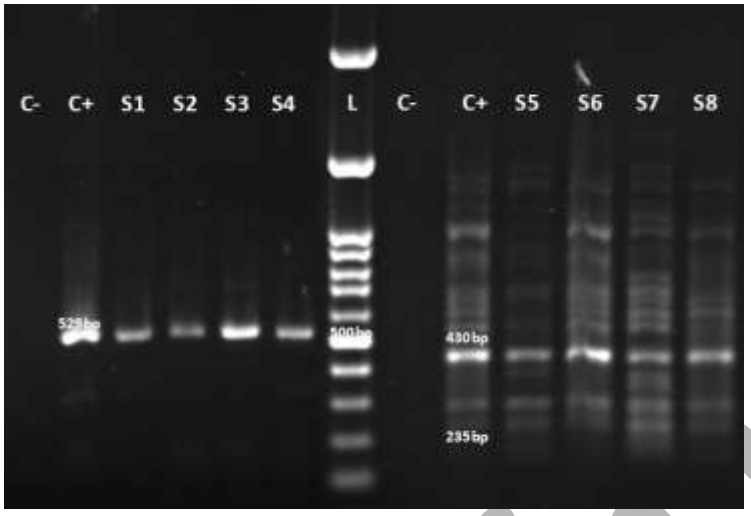


**Figure. 2.** PCR findings for detecting the *Theileria* species in the blood samples. The PCR products with a 529 bp and a 235 bp bands for *T. ovis and T. lestoquardi* genomes, respectively, were detected through electrophoresis on agarose gels stained with a safe DNA stain. L: ladder 100 bp; $C^-$: 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 1.** The PCR findings of *Theileria* and *Babesia* infections in the blood samples (n = 373).

| | | Theileria | | Babesia | |
|---|---|---|---|---|---|
| **City** | **No. the samples** | **% positive samples** | **CI95%** | **% positive samples** | **CI95%** |
| **Tabriz** | 20 | 37.5 | 0.37±0.21 | 20 | 0.20±0.17 |
| **Marand** | 15 | 100 | 1.0±0.0 | 14.28 | 0.14±0.17 |
| **Charuymaq** | 128 | 68.65 | 0.68±0.08 | 10.44 | 0.10±0.05 |
| **Bostan Abad** | 36 | 52.17 | 0.52±0.16 | 0.0 | 0.0±0.0 |
| **Mianeh** | 73 | 80.55 | 0.80±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±0.10 |
| **Hashtrud** | 25 | 83.33 | 0.83±0.14 | 0.0 | 0.0±0.0 |
| **Total** | 373 | 70.5 | 0.705±0.40 | 8.5 | 0.085±0.020 |

## 4. Discussion

The present study demonstrated a much high prevalence in both *Theileria* (70.5%) and *Babesia* (8.5%) infections, particularly the presence of *Theileria* genome was notable. Of note, the present examined samples were taken from sick animals with a recent abortion history. Although the sampling period was not the peak or maximum time of presence of blood parasitic diseases. The time of highest presence and activity of parasitic diseases is usually in the warm season of the year (mostly summer). While the sampling of the present study was in the fall and winter, which is the calving season of sheep and goats. Another important point is that the animals sampled did not have clinical symptoms related to Theileria and Babesia infections. The present results show the importance of diagnosing, controlling and preventing these infections in this province.

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 results in significant destruction of the lymphoid tissues, leading to identifiable impairment of immune function with far-reaching consequences (16). Infections via *Theileria* spp., on particular counts, depict swollen lymph nodes and jaundice, and they often result in abortions in pregnant animals during the later stages of gestation (23). In the present study, we did not record clinical symptoms and morbidity rate for *Theileria* or *Babesia* infections in the affected animals. However, it seems that these infections might impact the abortion rate, indirectly.

The prevalence of infection with *T. ovis* was reportedly as high as 55.6% in Khorasan Razavi province from 2009 to 2011 (24). On the other hand, the combined infection rate with *T. annulata* and *T. lestoquardi* was reportedly relatively low in Ahwaz: 4% in sheep (16). Emerging evidence reported that 19% of cattle in Iran are affected by theileriosis with the significant variations in

179 infection rates in different regions (19). In Sistan and Baluchestan province (25), sheep showed
180 the highest prevalence of 71%, while in North Khorasan (26) and Razavi Khorasan (24), the
181 prevalence was 70% and 55.6%, respectively. The present findings are more in agreement with the
182 reports from Sistan and Baluchestan province and North Khorasan province.
183 *Babesia* species infect erythrocytes and proliferate inside the host cell. The resulting parasitemia
184 can lead to a decrease in the number of red blood cells, accompanied by various types of anemia.
185 The principal pathogenic consequence arises from the breakdown of erythrocytes, leading to
186 significant hemolytic anemia and ensuing dysfunction across multiple organ systems (5, 6). The
187 investigation into tick-borne diseases in Iran, especially those caused by *B. ovis* and *B. motasi*,
188 has presented several dissimilar rates. The cited infection rates for *B. ovis* range from 6.31% up
189 to 44.9%, while *B. motasi* infections are comparatively lower and range between 0.5% and 14%
190 (7-9). In this regard, the infection rate of *B. ovis* has been reported around 24.6 % in sheep and 4.
191 3% in goats in Khorasan province (14), while the Kuhdasht region in Lorestan province showed
192 4.3% infection in sheep and 0.4% in goats (9). There were also reports of infections in West
193 Azerbaijan with both species affected at a rate of 17% well as the northwest region (15) with an
194 infection rate of approximately 23%, in sheep alone, and Zabol located in southeastern Iran
195 recording a rate of around 4% (8). A previous study highlighted the occurrence of blood protozoan
196 infections, among sheep in Bane, Kurdistan province (Iran); where the prevalence rates were
197 86·6%, 42.5%, and 24.9% for *B. ovis, T. ovis*, and *T. annulata*, respectively (2). Significantly,
198 86.4% of asymptomatic sheep were positive for *B. ovis* using the PCR method (2).
199 A similar evidence in Turkey demonstrated the prevalence rates of *B. ovis* 62.4%, and *Theileria*
200 *spp.* 7.0% with co-infections in 44.4% of the samples in sheep and goats (18). Another study in
201 Turkey reported that 86.12% of animals were affected with one or more pathogens, with *B. ovis*

202 being the most prevalent. The individual infection rates for *B. ovis*, and T. *ovis*, were 70.81%, and
203 21.05%, respectively. Infection of solely *B. ovis* was more frequent (31.11%) than that which *T.ovis*
204 caused (1.67%). Co-infection of *B. ovis* and *T. ovis* was 1.11% (19). Also in another study, the
205 genomic DNA was analyzed from blood, ticks, and egg masses using 18S rRNA PCR and reverse
206 line blotting (RLB), which identified three *Theileria* species and one *Babesia* species. Of these, *T.*
207 *ovis* was the most prevalent at 35.4%, followed by *B. ovis* (5.4%), and *T. annulata* (3.9%). Co-
208 infection in this study included those infected with both *T. ovis* and *B. ovis* (20). The result
209 indicated a remarkable prevalence of protozoan infection among sheep, while parasitic load for *T.*
210 *annulata* (3.9%), *T. ovis* (35.4%), and *B. ovis* (5.4%) are low, indicating that such animals may be
211 carriers identifiable only through a PCR test (21).
212 As mentioned previously, both *Theileria* and *Babesia* disrupt normal hematological functions in
213 their respective hosts, causing anemia and systemic disease. These result in alterations of
214 vascular dynamics that impede uteroplacental blood circulation. Immunosuppressive effects
215 associated with such infection may act to increase the susceptibility of pregnant animals to
216 secondary infections, which also can cause abortion (1, 14-17). Besides, stressors, whether due
217 to the disease process or occurring independently of environmental conditions, often exacerbate
218 the chances of aborting in affected animals (18-20). Additionally, ecological factors and
219 implemented tick management strategies play critical roles in the frequency and intensity of
220 Theileriosis and Babesiosis; hence, the importance of efficient management practices aims at
221 protecting the health and productivity of the livestock (20, 21). In conclusion, Both *Theileria* and
222 *Babesia* cause anemia, hypoxia, and immunosuppression in their respective hosts, which may
223 act to increase the susceptibility of pregnant animals to abortion. The high prevalence in the

present study in the autumn and winter seasons should be noted. In this regard, implemented tick management strategies can play essential role in the frequency of these infections.

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**Author's contribution**

Conceptualization: PSh, MKh, HS, JSh; Methodology: PSh, RA, MKh, HS, HA, AH, and AA; Software: PSh, MKh, and AA; Writing/preparation of original draft: MKh, HS, and AA; Writing, review and editing: PSh, MKh, HS, JSH, HA, AH, and AA; Supervision, project administration and funding acquisition: MKh; All authors have read and approved the final version of the manuscript.

**Ethics**

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 (ID: IR.TABRIZU.REC.1403.049) were followed.

**Conflicts of Interest**

There is no conflict of interest.

## Data availability

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

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