Microscopic and Molecular Study of *Theileria ovis* and *Anaplasma ovis* in Certain Sheep Referred to the Clinic from Ilam Province, Western Iran

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Abstract

Introduction: Tick-borne diseases caused by *Anaplasma*, *Babesia*, and *Theileria* species are prevalent in tropical and subtropical regions, resulting in substantial economic losses. The present study aimed to detect *Anaplasma*, *Babesia*, and *Theileria* spp. in sheep in Ilam province, Iran, using both microscopic and molecular methods. This evaluation is of great importance given the crucial role of these diseases in livestock health and their associated economic losses, as well as the contribution of this evaluation to selecting the most appropriate diagnostic method for the region.

Materials & Methods: Blood samples (n=73) were collected from clinically symptomatic sheep between June and September 2024. Giemsa-stained blood smears were examined under light microscopy, and DNA was extracted for polymerase chain reaction (PCR) targeting *Theileria* spp. 18S rRNA, *Babesia ovis* 18S rRNA, and *Anaplasma ovis msp4* genes. The PCR products were sequenced, and phylogenetic relationships were analyzed using MEGA software. Statistical tests were used to evaluate the associations between infection rates, age, and gender.

Results: Microscopy detected *Theileria* spp. in all 73 samples and *Anaplasma* spp. in 12, while PCR identified *Theileria ovis* in 60 (82.2%) samples, *A. ovis* in 21 (28.76%), and mixed infections in 13 (17.80%). No *B. ovis* was found. PCR proved significantly more sensitive than microscopy. Sequence analysis revealed over 99% identity to reference sequences for *T. ovis* and *A. ovis*, indicating limited genetic diversity. There was no significant association between age or gender and infection with *Theileria* or *Anaplasma*.

Conclusion: The results provide important epidemiological data, highlighting the need for improved tick control and molecular diagnostics to reduce economic losses in Iran's livestock industry.

Keywords: *Anaplasma*, *Babesia*, PCR, Sheep, *Theileria* spp., Tick-borne

1. Introduction

Tick-borne hemoparasites, including *Anaplasma*, *Babesia*, and *Theileria* species, pose significant threats to livestock health worldwide, causing substantial economic losses due to reduced productivity, morbidity, and mortality [1-3]. *Anaplasma* species are obligate intracellular, Gram-negative bacteria belonging to the family Anaplasmataceae, order Rickettsiales. They primarily infect erythrocytes of various vertebrates, which serve as hosts and reservoirs. Anaplasmosis is an important disease in ruminants [4,5]. The primary vectors are hard ticks of the genera *Ixodes*, *Dermacentor*, *Rhipicephalus*, and *Amblyomma*, which have been reported to transmit *Anaplasma* spp. in various regions worldwide [5]. In contrast, *Babesia* and *Theileria* are protozoan parasites of the phylum Apicomplexa, order Piroplasmida, transmitted mainly by *Ixodes*, *Rhipicephalus*, and *Hyalomma* ticks [6,7].

In Iran, *Theileria ovis* and *Anaplasma ovis* are among the most prevalent hemoparasites in ovine populations [8,9]. It has been asserted that *A. ovis* generally exhibits mild pathogenicity and causes subclinical infections in small ruminants [10]. This disease typically manifests as mild clinical symptoms unless accompanied by stress-inducing factors [11]. Furthermore, research indicates the presence of severe anemia disease in ovine and caprine populations, attributable to this pathogen [12]. In contrast, *T. ovis* typically causes subclinical or mild infections, manifesting as fever, lethargy, weight loss, and lymphadenopathy [13]. *Babesia ovis*, the causative agent of a disease, is characterized by the presence of fever, hemolytic anemia, and hemoglobinuria in affected animals. The disease ultimately results in death and has a significant economic impact, particularly on the sheep farming industry [14]. These infections present significant economic challenges for livestock producers, given the resulting decline in animal health and productivity.

In small ruminants such as sheep, these tick-borne pathogens are particularly common in regions with favorable ecological conditions for vector proliferation, including the mountainous and pastoral areas of western Iran. Ilam province, located on the western slopes of the Zagros Mountains, features a semi-arid to Mediterranean climate with diverse vegetation, creating an ideal environment for tick

vectors and intensive livestock farming. Despite the region's significance as a livestock hub, data on the genetic characteristics and precise distribution of these hemoparasites in sheep remain limited, hindering effective disease management [15].

Conventional diagnostic approaches, such as microscopic examination of Giemsa-stained blood smears, are often limited by low sensitivity and specificity, particularly in cases of low parasitemia, carrier states, or morphological similarities among species. Molecular techniques, such as polymerase chain reaction (PCR), offer superior sensitivity and specificity, as well as the ability to detect genetic diversity, enabling precise strain identification [16].

Given the significant impact of anaplasmosis, babesiosis, and theileriosis on livestock health and the resulting economic losses, selecting an optimal diagnostic method is essential. This study aimed to evaluate and compare the effectiveness of microscopic and molecular (PCR) methods for detecting infections caused by *Anaplasma*, *Babesia*, and *Theileria* species in sheep from Ilam province, Iran. The present study aimed to microscopically and molecularly detect *Anaplasma*, *Babesia*, and *Theileria* spp. in sheep clinically diagnosed with theileriosis during the peak season to evaluate the prevalence of co-infection overlooked by routine clinical diagnosis.

2. Materials and Methods

2.1. Study area

The present study was conducted in Ilam Province, located in western Iran on the western slopes of the Central Zagros Mountains (approximately 33°38'N, 46°25'E). The province shares its longest border with Iraq to the west and is bordered by Khuzestan to the south, Lorestan to the east, and Kermanshah to the north. It exhibits a diverse climatic profile, ranging from cold mountainous to temperate and semi-arid conditions, influenced by its topography and elevation. The region experiences a Mediterranean-like climate with hot, dry summers and cool, wet winters [17]. Due to its favorable climatic conditions, abundant orographic rainfall, and lush vegetation in the northern, northwestern, northeastern, and Kabirkoh mountainous areas, Ilam Province provides ideal pastures

and groundwater resources, making it a key region for livestock farming and raising various types of animals [17].

2.2. Sample Collection

A total of 73 blood samples were collected from sheep with natural infections, referred to veterinary clinics in Ilam Province, during the tick-active season (June to September 2024). The animals were selected based on the presence of at least one clinical sign suggestive of tick-borne hemoparasitic infection, including fever, anorexia, anemia, and lethargy.

Blood samples (approximately 3–5 mL per animal) were obtained from the jugular vein using sterile vacutainer needles. A thin blood smear was prepared for each animal. The remaining blood was collected into EDTA-containing tubes, gently mixed, and stored at 4°C until DNA extraction for subsequent PCR-based detection of *Anaplasma*, *Babesia*, and *Theileria* species.

The study population comprised 73 sheep, categorized into four age groups: less than 1 year, 1–2 years, 2–3 years, and over 3 years. Among them, 46 (63%) were female, and 27 (37%) were male. No other inclusion/exclusion criteria (e.g., breed, health status beyond clinical signs, or prior treatment) were specified.

2.3. Microscopic Examination

Thin blood smears were prepared immediately after blood collection, air-dried, fixed with absolute methanol for 5 minutes, and stained with Giemsa solution (typically 5–10% dilution for 20–30 minutes). After rinsing with tap water and air-drying, the slides were examined under a light microscope (Leica DMLS) at ×1000 magnification using oil immersion objective to detect intraerythrocytic parasites. At least 200–300 oil-immersion fields per slide were systematically screened for the characteristic forms of *Anaplasma* (small, dense, marginal blue-purple inclusion bodies, 0.2–0.5 μm in diameter), *Babesia* (pleomorphic ring forms, pear-shaped or Maltese cross configurations), and *Theileria* (piroplasm stages, typically small round or oval intraerythrocytic bodies). Smears were considered negative if no parasites were observed after thorough examination of the required fields [18].

2.4. DNA Extraction

Genomic DNA was extracted from 200 µL of blood using the SinaPure™ Blood DNA Extraction Kit (SinaClon, Iran) following the manufacturer's instructions. Briefly, 200 µL of blood (collected in EDTA tubes) was mixed with an equal volume (200 µL) of lysis buffer to disrupt cells. The mixture was then supplemented with 25 µL of Proteinase K (for protein digestion) and 5 µL of Ribonuclease A (to remove contaminating RNA), vortexed vigorously for 20 seconds, and incubated at 59 °C for 30 minutes to complete lysis and digestion.

After incubation, the lysate was transferred to a SinaClon spin column and 200 µL of precipitation/binding solution was added to facilitate DNA binding to the silica membrane. The column was centrifuged at ≥12,000 × g for 1 minute, and the flow-through was discarded. The column was then washed twice with 600 µL of wash buffer (each time centrifuged as above) to remove impurities and PCR inhibitors. Finally, the column was dried by an additional centrifugation step (optional but recommended), and DNA was eluted in 100 µL of pre-warmed elution buffer (or nuclease-free water) by incubation at room temperature for 1–2 minutes followed by centrifugation. Extracted DNA was quantified and assessed for purity using a NanoDrop™ spectrophotometer (measuring A260/A280 ratio; values of 1.8–2.0 indicate good quality) and visualized on 2% agarose gel electrophoresis stained with ethidium bromide to confirm integrity (high-molecular-weight band without smearing). Purified DNA samples were stored at –20 °C until used for PCR amplification.

2.5. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) assays were performed on all extracted DNA samples to detect *Theileria* spp., *Babesia ovis*, and *Anaplasma ovis* using previously published primer pairs targeting the 18S rRNA gene for *Theileria* spp. and *B. ovi*s, and the major surface protein 4 (*msp4*) gene for *A. ovis* (Table 1). Each 25 µL PCR reaction mixture contained 3 µL of DNA template (approximately 100 ng, diluted 1:30 in nuclease-free water), 12.5 µL of 2× PCR Master Mix (Ampliqon, Denmark), 1 µL (0.5 µM final concentration) of each forward and reverse primer (Metabion, Germany), and 7.5 µL of nuclease-free water.

Amplification was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) under the following thermal cycling conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 54°C (for *Theileria* spp.), 65°C (for *B. ovis*), or 67°C (for *A. ovis*) for 60 s, and extension at 72°C for 60 s; with a final extension step at 72°C for 10 min. PCR products were separated by electrophoresis on 2% agarose gels stained with 0.5 μg/mL ethidium bromide in 1× TBE buffer, and visualized under UV transillumination using a Gel Doc XR+ system (Bio-Rad, USA). Each run included a negative control (nuclease-free water instead of template) and positive controls (confirmed DNA from *Theileria* spp., *Babesia* spp., and *A. ovis*). The expected amplicon sizes were approximately 778 bp for *Theileria* spp., 549 bp for *B. ovis*, and 347 bp for *A. ovis* (based on the specific primers used; Table 1).

Table 1 List of primers used for polymerase chain reaction assays (PCR)

Target gene	Forward (5' → 3')	Reverse (5' → 3')	Reference
Babesia ovis (18S rRNA)	TGGGCAGGACCTTGGTTCTTCT	CCGCGTAGCGCCGGCTAAATA	[3]
Anaplasma ovis (*msp4*)	TGAAGGGAGCGGGGTCATGGG	GAGTAATTGCAGCCAGGCACTCT	[3]
Theileria spp. (18S rRNA)	GAAACGGCTACCACATCT	AGTTTCCCCGTGTTGAGT	[3]

2.6. DNA Sequencing and Phylogenetic Analysis

Positive PCR products specific for *Theileria* spp. (18S rRNA gene) and *A. ovis* (*msp4* gene) were selected for sequencing analysis by Bioneer Corporation (South Korea). Sequencing was performed bidirectionally (forward and reverse primers) using an automated sequencer to obtain accurate and overlapping reads. Raw sequence data were received as chromatogram files and edited/trimmed for quality using software such as BioEdit. Consensus sequences were generated and compared to

reference sequences in the NCBI GenBank database using the BLASTn algorithm to confirm identity and similarity to known *Theileria* spp. (primarily *T. ovis*) and *A. ovis* isolates.

Phylogenetic analyses were conducted using MEGA version 12.0.11. For the analysis based on partial 18S rRNA gene sequences, sequences were aligned using the ClustalW algorithm and manually inspected to remove poorly aligned and ambiguous regions. The best-fit nucleotide substitution model was determined using the built-in model selection tool in MEGA based on the Bayesian Information Criterion (BIC), which selected the Tamura–Nei model with gamma-distributed rate variation among sites (TN93+G). The maximum likelihood (ML) tree was constructed using this model with partial deletion (95% site coverage cutoff). Nodal support was evaluated via bootstrap analysis with 1000 replicates, and representative *T. ornithorhynchi* were included as outgroups to root the tree.

Separately, phylogenetic analysis of the *Anaplasma ovis msp4* gene sequences was performed using the ML method in MEGA. Sequences were aligned with ClustalW, followed by manual inspection to ensure correct codon alignment and removal of ambiguous regions. The optimal substitution model was selected via the BIC criterion in MEGA, and the ML tree was constructed using the selected model with invariable (I) rate variation and partial deletion (95% site coverage cutoff). Bootstrap support (1000 replicates) was calculated, and representative *Anaplasma phagocytophilum* sequences served as outgroups.

2.7. Statistical Analysis

To statistically assess the prevalence of *Theileria* and *Anaplasma* infections across different age groups of the studied sheep, as well as to examine the potential association between infection rates of *Theileria* and *Anaplasma* in male and female sheep, the Chi-square (χ^2) test was employed. Data analysis was performed using SPSS software, version 16. A p-value of less than or equal to 0.05 was considered statistically significant.

3. Results

3.1. Microscopic Examination Findings

In this study, a total of 73 Giemsa-stained thin blood smears were microscopically examined. All samples were identified as positive for *Theileria* spp., and 12 (16.4%) samples were confirmed positive for *Anaplasma* spp., but *Babesia* parasites were not detected in any of the examined smears.

3.2. Molecular Detection (PCR Analysis)

PCR analysis of the 73 blood samples from sheep exhibiting clinical signs of theileriosis revealed that 60 samples (82.2%) were positive for *Theileria* spp. while *A. ovis* was detected in 21 samples (28.8%), with mixed infections of *Theileria* spp. and *A. ovis* observed in 13 samples (17.8%). *Babesia ovis* was not detected in any of the samples. Overall, 12 sheep (16.4%) were negative for all three screened pathogens by PCR.

3.3. Comparison with Microscopic Examination

All 73 samples were positive for *Theileria* spp. by microscopic examination, whereas PCR detected the parasite in only 60 samples (82.2%). The 13 discrepant samples (microscopy positive/PCR negative) may have indicated technical limitations or differences in the accuracy and skill of the technician in microscopic diagnosis.

3.4. Sequencing and Phylogenetic Analysis

A total of 60 *Theileria*-positive and 21 *Anaplasma*-positive samples were successfully sequenced. BLAST analysis of the partial 18S rRNA gene sequences confirmed the exclusive presence of *Theileria ovis*, exhibiting 100% identity with reference sequences in GenBank. Likewise, sequencing of the *msp4* gene from the *Anaplasma*-positive samples revealed 100% nucleotide identity with *Anaplasma ovis*. Representative sequences generated in this study have been deposited in GenBank under accession numbers PZ028036. and PZ044073 for *T. ovis* and *A. ovis*, respectively.

In the phylogenetic tree, all local *T. ovis* isolates clustered tightly within a well-supported clade containing global *T. ovis* reference strains (Figure 1). Similarly, the *A. ovis* isolates formed a distinct monophyletic group with high bootstrap support alongside previously reported *A. ovis* sequences from diverse geographic regions (Figure 2).

No intraspecies nucleotide variations were detected among the local isolates of either pathogen, indicating limited genetic diversity within the circulating populations in the study area.

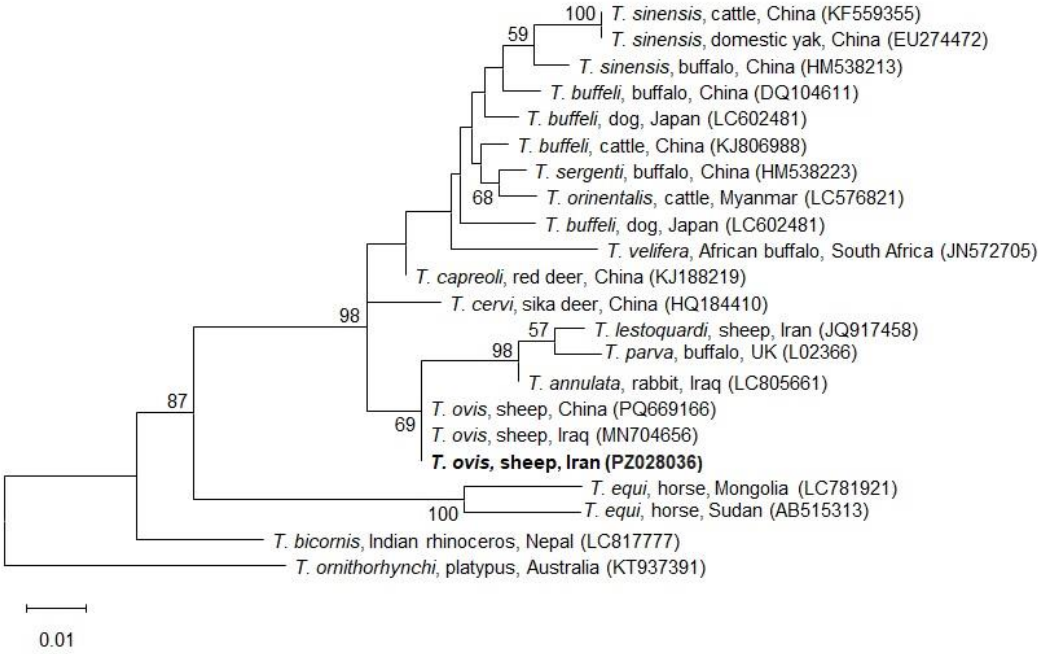

Figure 1. Maximum likelihood phylogenetic tree based on partial 18S rRNA gene sequences showing the relationship of *Theileria ovis* isolates with reference sequences retrieved from GenBank. The analytical procedure encompassed 22 nucleotide sequences. The partial deletion option was applied to eliminate all positions with less than 95% site coverage, resulting in a final data set comprising 732 positions. Bootstrap values (1000 replicates) ≥50% are shown at branch nodes. *T. ornithorhynchi* was used as the outgroup. Scale bar indicates 0.01 substitutions per site.

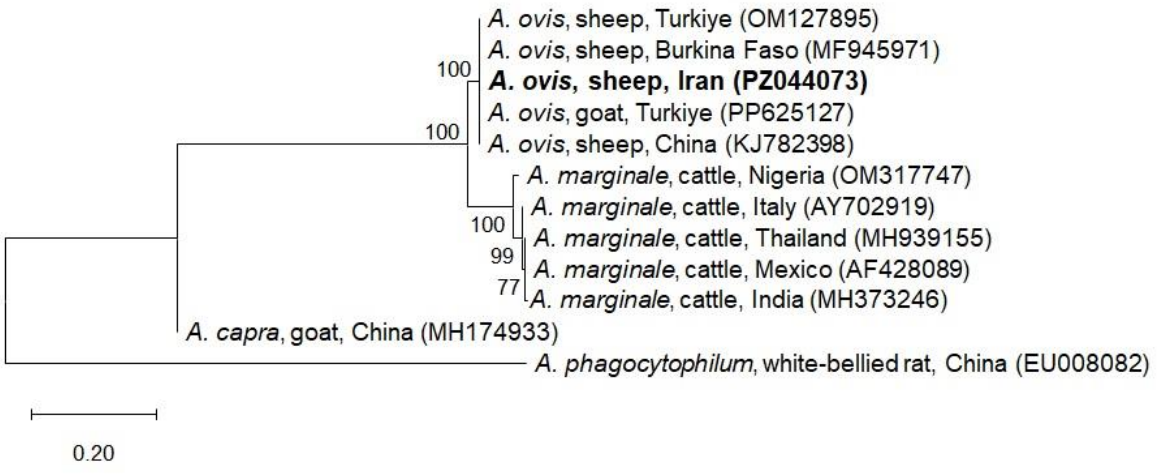

Figure 2. Maximum likelihood phylogenetic tree based on partial *msp4* gene sequences showing the genetic relationships among *Anaplasma ovis* isolates and reference sequences retrieved from GenBank. The analytical procedure encompassed 12 nucleotide sequences. The partial deletion option was applied to eliminate all positions with less than 95% site coverage, resulting in a final data set comprising 298 positions. Bootstrap values (1000 replicates) ≥50% are shown at branch nodes. *Anaplasma phagocytophilum* was used as the outgroup. Scale bar represents 0.20 substitutions per site.

3.5. Statistical analysis

No significant association was observed between gender and infection with *T. ovis* (80.8% in males [n=26] vs. 83% in females [n=47]; χ^2 test, P = 0.813) or *A. ovis* (38.5% in males vs. 23.4% in females; χ^2 test, P = 0.174) (Figure 3). Similarly, age group was not significantly associated with *T. ovis* infection (infection rates: 76.31% in <1 year [n=38], 83.33% in 1–2 years [n=12], 87.5% in 2–3 years [n=16], 100% in >3 years [n=7]; χ^2 test, P = 0.435). For *A. ovis*, infection rates were 29% (<1 year), 17% (1–2 years), 38% (2–3 years), and 29% (>3 years), with no significant differences across age groups (χ^2 test, P = 0.693) (Figure 4).

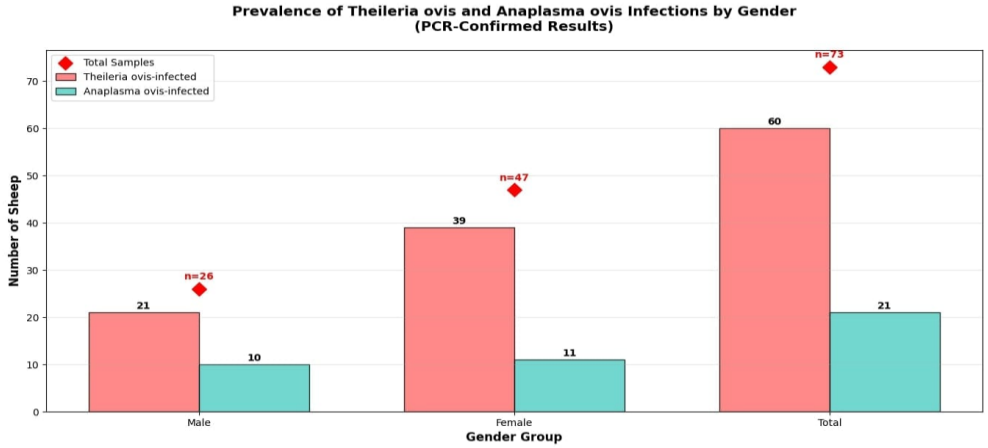

Figure 3. Percentage of *T. ovis* and *A. ovis* infections by gender based on PCR-confirmed results

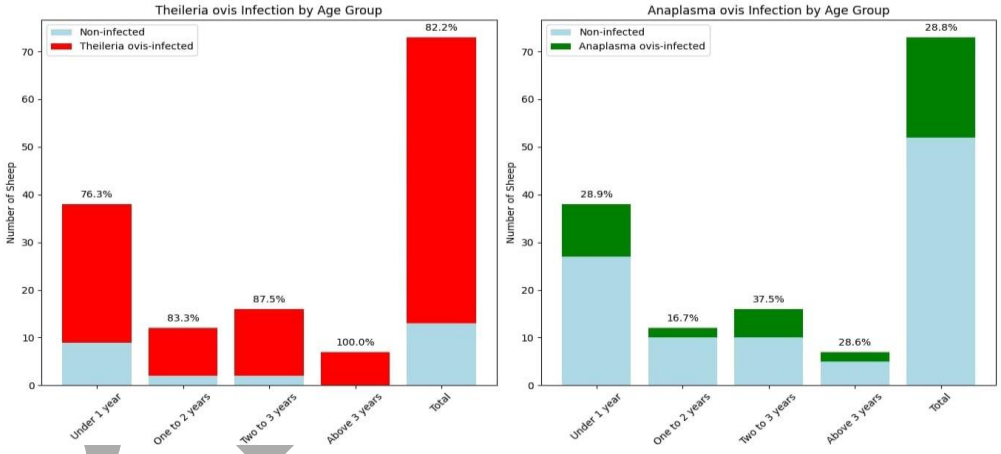

Figure 4. Percentage of *T. ovis* and *A. ovis* infections by age based on PCR-confirmed results

4. Discussion

These findings suggest that *T. ovis* is the predominant hemoprotozoan parasite in the studied population, while *A. ovis* plays a secondary role, and *B. ovis* appears absent or negligible in Ilam province during the study period.

A notable discrepancy was observed between the two diagnostic methods for *T. ovis* detection: microscopy identified the parasite in all clinically affected sheep, whereas PCR was positive in only 82.2% of the same samples. This finding contradicts the majority of previous reports, in which molecular methods generally yield higher infection rates compared to microscopy due to their enhanced sensitivity in low-parasitemia or carrier states. For instance, Zaeemi et al. [19] observed a rate of 9.2% by microscopy compared to 32.8% by nested PCR in western Iran. However, comparable high rates were reported by Rahravani et al. [20] in Kurdistan province (91.2% by microscopy and 86.8% by semi-nested PCR).

In contrast, the molecular method exhibited superior sensitivity in detecting *A. ovis*, identifying the pathogen in 21 samples (28.8%) in comparison to the 12 samples (16.4%) identified through microscopic examination. This pattern aligns with the findings of the majority of previous studies, which indicate that PCR-based techniques consistently identify higher infection rates for *Anaplasma* spp. than microscopy. In a study by Jalali et al. [21], polymerase chain reaction (PCR) identified *Anaplasma* infections in 87.4% of the samples. This is in contrast to the routine blood smear examination, which revealed inclusion bodies in only 33.6% of the samples. This pattern of significant underestimation by microscopy is further corroborated by Rahravani et al. [20] in Kurdistan province, who reported an *Anaplasma* prevalence of 23.2% via microscopy compared to 78.8% using semi-nested PCR.

The epidemiological landscape in neighboring countries provides important context for our findings. Molecular studies on *Theileria* reveal considerably lower prevalence rates in the surrounding regions compared to our study, including 13.43%, 17.91% in sheep by microscopic and PCR, respectively, in Kurdistan, Iraq [22]. Similarly, for *A. ovis* infections, substantial regional variation is evident, with

molecular prevalence ranging from 12.5% in Pakistani sheep [28] to 43.9% in Turkish flocks [23]. Notably, a large-scale survey in southern Kazakhstan detected *Anaplasma* spp. in 39.8% of ewes, identifying altitude below 500 meters as a significant risk factor [24]. The consistent and substantial gap between microscopic and molecular results across these different studies and regions unequivocally highlights the inherent limitations of blood smear analysis for detecting *Anaplasma* infections and establishes PCR as the essential tool for accurate surveillance. Microscopic detection of blood protozoan parasites is often challenging due to factors such as the morphological similarities among species, low parasitemia levels, and the requirement for skilled personnel and optimal staining techniques to achieve accurate diagnosis [16]. These comparative data highlight the endemic nature of these pathogens across the region while underscoring the substantial geographical variation in infection pressures.

The higher accuracy of PCR addresses a significant research gap by providing a reliable diagnostic tool for detecting hemoparasite infections in sheep. This capability is vital for effective disease control in livestock-dependent regions such as Ilam.

No significant differences were found in the infection rates of *T. ovis* or *A. ovis* between male and female sheep (P = 0.813 and P = 0.174, respectively). Although males showed a numerically higher infection rate for *A. ovis* (38.5% vs. 23.4%), this difference was not statistically significant (Fig. 3). Similarly, no significant age-dependent patterns were observed for either pathogen (P = 0.435 for *T. ovis*; P = 0.693 for *A. ovis*) (Fig. 4). The consistently high infection rates of *T. ovis* across all age groups (83–100%) confirm its endemic status in the studied population. The absence of significant risk factors related to gender or age suggests that other factors- such as tick exposure or management practices- may play a more important role in transmission. Previous investigating *Anaplasma* infection rates in sheep of different sexes and ages reported no significant differences between males and females or between immature and mature sheep [21]. However, Abbas Zadeh [25] found a significant differences males or females or between age, breed, and *A. ovis* infection in the studied goats. Furthermore, the infection rate of *A. ovis* was significantly higher in female goats than in males.

316 In a phylogenetic analysis based on the 18S rRNA gene sequence, the *Theileria ovis* strain isolated
317 in this study (from sheep, Iran) was placed with 100% bootstrap support in a common clade with the
318 reference *T. ovis* strain from sheep in Iraq (accession number: MN704656). The Iranian strain was
319 subsequently linked to a UK strain (accession: PQ686916) with 50% support. This grouping indicates
320 high genetic proximity and a likely common local origin in the Middle East region, consistent with
321 previous reports of high *T. ovis* prevalence in Iranian sheep (such as studies in Iran) [6,21]. The *T.*
322 *ovis* clade is positioned in proximity to *T. velifera* and more pathogenic species, including *T. parva*,
323 *T. annulata*, and *T. lestoquardi*. However, it distinctly separates from bovine-associated clades, such
324 as *T. orientalis/buffeli*. This finding confirms that the present strain unequivocally belongs to the
325 species *T. ovis* and does not exhibit significant genetic divergence compared to Asian and African
326 strains. These findings underscore the importance of *T. ovis* as a dominant and typically benign
327 species in Iranian sheep and may be useful for epidemiological control strategies. However, due to
328 the conserved nature of the 18S rRNA gene, its resolution for assessing intra-specific genetic diversity
329 is limited, and the observed low variation among *T. ovis* isolates may partly reflect this
330 methodological constraint rather than true genetic homogeneity.

331 The phylogenetic tree constructed based on the *msp4* gene reveals that the *A. ovis* isolate from sheep
332 in the present study (*A. ovis*, sheep, Iran) forms a single clade with 100% bootstrap support together
333 with reference strains from Türkiye (from sheep and goat), China, and Burkina Faso. This robust
334 clustering corroborates the species identity of the isolate as *A. ovis* and clearly distinguishes it from
335 closely related species, including *A. marginale*, *A. centrale*, and *A. phagocytophilum*.

336 The *msp4* gene is known to be relatively conserved in *A. ovis*, exhibited limited genetic variation.
337 Therefore, the complete sequence identity observed with strains from geographically distant regions
338 (Asia and Africa) likely reflects common transmission routes through tick vectors (e.g.,
339 *Rhipicephalus* spp.) or animal trade and movement. These results align with previous studies in Iran
340 and neighboring countries, demonstrating the circulation of highly similar *A. ovis* strains among sheep

populations. This finding highlights the need for continuous epidemiological monitoring and effective tick control strategies to prevent ovine anaplasmosis.

Despite the valuable insights provided, the present study has certain limitations. The relatively modest sample size (n = 72) was primarily determined by financial constraints and logistical challenges associated with extensive field sampling and molecular analyses. Nevertheless, this dataset provides meaningful preliminary epidemiological and molecular information from an understudied region, serving as a foundation for future investigations.

5. Conclusion

In conclusion, a substantial rate of *Anaplasma* co-infections (28.8%) was detected among sheep clinically diagnosed with theileriosis, highlighting significant underdiagnosis by routine microscopic methods. These findings underscore the critical need for implementing molecular diagnostic techniques, such as PCR, to accurately identify mixed tick-borne infections. Microscopic detection of hemoprotozoan parasites remains challenging due to morphological similarities among species, low parasitemia levels, and dependence on skilled personnel and optimal staining procedures. The superior sensitivity and specificity of PCR enable reliable detection of these infections, addressing a key gap in ovine hemoparasite diagnostics. This approach is particularly vital in livestock-dependent endemic regions like Ilam Province, where it can facilitate targeted treatment, improve disease management, and ultimately reduce substantial economic losses associated with tick-borne diseases. Future studies should incorporate larger sample sizes and include both symptomatic and asymptomatic sheep to obtain a more comprehensive epidemiological understanding of tick-borne hemoparasitic infections in the region. Longitudinal sampling to assess seasonal trends in *Theileria*, *Babesia*, and *Anaplasma* prevalence would further strengthen surveillance efforts. Additionally, investigating tick species distribution and their role as vectors in Ilam would enhance understanding of transmission dynamics. Further genetic analysis of *T. ovis* and *A. ovis* strains across Iran could clarify regional differences and guide control measures. Additionally, assessing the economic impact of these infections on livestock productivity would yield useful data for policymaking. These findings

offer important insights into hemoprotozoan infections in a key livestock-producing region of Iran, helping to address a significant gap in regional epidemiological data.

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Conflict of interest: The authors declare no conflicts of interest regarding the publication of this paper.

Ethics

This paper does not involve research related to experimental animals or specific human diseases.

Data Availability

The data supporting the findings of this study are not publicly available because they are not necessary for the public. However, they can be made available upon request from the corresponding author.

Authors' Contributions

1- Study concept and design: M. N., and F. P.

2- Acquisition of data: M. N., and R. F.

3- Analysis and interpretation of data: M. N., F. P., and R. F.

4- Drafting of the manuscript: M. N. and R. F.

5- Critical revision of the manuscript for important intellectual content: M. N., and F. P.

6- Statistical analysis: M. N., and R. F.

7- Administrative, technical, and material support: M. N., and F. P.

8- Study supervision: F. P.

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