Journal of the Hellenic Veterinary Medical Society

Vol 77, No 2 (2026)

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doi: 10.12681/jhvms.37484

To cite this article:

Karakus, A., & Tanrikuli, İ. (2026). Molecular Characterization of Babesia ovis Isolated from Sheep in Van Province, Turkey. *Journal of the Hellenic Veterinary Medical Society*, *77*(2), 10337–10344. https://doi.org/10.12681/jhvms.37484

J HELLENIC VET MED SOC 2026, 77 (2): 10337-10344
ΠΕΚΕ 2026, 77 (2): 10337-10344

Research article
Ερευνητικό άρθρο

Molecular Characterization of *Babesia ovis* Isolated from Sheep in Van Province, Turkey

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ABSTRACT: This study investigated *Babesia ovis* infection in sheep in Van Province, Turkey, using microscopy and polymerase chain reaction (PCR). Blood samples were collected from 155 sheep (66 clinically affected and 89 apparently healthy). Seven samples (4.51%) were positive by microscopy, whereas 21 samples (13.54%) were PCR-positive for the 18S rRNA gene (549 bp). Four representative amplicons were sequenced and submitted to GenBank (ON137543.1, ON139020.1, ON139021.1, ON139024.1), all showing 100% identity with *B. ovis*. Phylogenetic analysis confirmed clustering with reference *B. ovis* isolates from different geographical regions. Importantly, these findings provide region-specific molecular evidence on the genetic identity, sequence homology, and phylogenetic placement of *B. ovis* in eastern Turkey, rather than merely reporting detection.

Keyword: Sheep; *Babesia ovis*; PCR; 18S rRNA; Molecular diagnosis; Turkey

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Date of submission: **18-4-2024**
Date of acceptance: **19-2-2026**

INTRODUCTION

Babesiosis is an important tick-borne hemoprotozoan disease caused by protozoa of the genus *Babesia*, affecting domestic and wild animals in tropical, subtropical, and temperate regions worldwide (Kuttler et al., 1988). These apicomplexan parasites are transmitted primarily by ixodid ticks and develop within erythrocytes, leading to substantial health problems and productivity losses in livestock. Ovine babesiosis represents one of the most significant tick-borne diseases of small ruminants and is responsible for considerable economic losses due to morbidity, mortality, and reduced production performance (Sadeghi Dehkordi et al., 2010).

In sheep and goats, babesiosis is most commonly attributed to *Babesia ovis, B. motasi, B. crassa, B. taylori,* and *B. foliata*; however, the epidemiology, pathogenicity, and genetic diversity of these species vary markedly among geographic regions (Schnittger et al., 2022). During the last two decades, several novel species or genotypes affecting small ruminants have been described, including *Babesia* sp. Xinjiang (Guan et al., 2010), *Babesia aktasi* (Ozubek et al., 2023), and *B. motasi*-like genotypes (Lintan, Tianzhu, and Hebei types) (Wang et al., 2023). Moreover, based on morphological, serological, pathogenic, and genetic characteristics, *B. motasi* has been subdivided into two main groups: *B. motasi lintanensis* and *B. motasi hebeinensis* (Ozubek and Aktas, 2018). Among ovine piroplasms, *B. ovis* is considered the most pathogenic species, causing acute hemolytic anemia, fever, hemoglobinuria, jaundice, weight loss, decreased productivity, and, in severe cases, death. *Rhipicephalus bursa* is recognized as its principal vector (Wang et al., 2023). Although *B. ovis* has also been reported from atypical hosts, its epidemiological significance outside sheep and goats remains unclear (Schnittger et al., 2022; De Waal, 2000; Stuen, 2020). These clinical manifestations account for the major economic and veterinary impact of *B. ovis* infections in endemic regions.

Diagnosis of ovine babesiosis has traditionally relied on clinical signs and microscopic examination of Giemsa-stained blood smears. While microscopy is useful in acute infections, it frequently fails to detect low parasitemia or carrier animals and may lead to misidentification due to the morphological similarity among *Babesia* species. Serological assays have also been applied; however, their diagnostic value may be compromised by cross-reactivity, persistence of antibodies after parasite clearance, and limitations in sensitivity and specificity (d'Oliveira et al., 1995; Aktas et al., 2002; Gubbels et al., 1999; Saki et al., 2008). Importantly, reliance on conventional or serological approaches alone does not allow reliable discrimination of closely related species nor assessment of genetic variability and evolutionary relationships among *Babesia* isolates.

Polymerase chain reaction (PCR)-based methods enable sensitive and specific detection of *Babesia* DNA, even in subclinically infected or carrier animals. Among molecular markers, the small subunit ribosomal RNA (SSU 18S rRNA) gene has been widely used for the identification, classification, and phylogenetic analysis of *Babesia* species and genotypes (Aktas et al., 2002; Wang et al., 2019). Comparative sequence analysis of this conserved genomic region provides valuable information on genetic variability, evolutionary relationships, and phylogeographic patterns among isolates from different geographic regions. Thus, molecular approaches based on SSU 18S rRNA gene sequencing are particularly appropriate not only for accurate detection but also for investigating sequence homology, phylogenetic relationships, and population structure of *Babesia* species.

In Turkey, ovine babesiosis has been reported mainly through microscopic and limited serological surveys, and available data indicate that the disease represents a substantial constraint to sheep production (Ceylan et al., 2021). However, despite the presence of suitable tick vectors and intensive sheep husbandry in eastern Anatolia, particularly in Van Province, comprehensive molecular studies addressing species identification, genetic diversity, and phylogenetic relationships of *Babesia* in sheep remain scarce. Region-specific molecular data are lacking, and information on the genetic characteristics of *Babesia* in clinically healthy or subclinically infected animals is particularly limited. This lack of molecular and phylogenetic evidence restricts accurate epidemiological assessment and hampers the development of effective surveillance and control strategies. To date, no detailed molecular and phylogenetic characterization of *B. ovis* isolates from Van Province has been available, representing a critical gap in understanding the regional epidemiology and genetic structure of ovine babesiosis in eastern Turkey.

Therefore, the present study aimed to investigate *Babesia* infection in sheep in Van Province,

Turkey, and to provide molecular characterization and phylogenetic analysis of the detected isolates. By generating region-specific molecular data, this study sought to clarify the epidemiological status and genetic diversity of *Babesia* species in eastern Turkey and to contribute to a better understanding of the molecular epidemiology of ovine babesiosis.

MATERIALS AND METHODS

Study area

This study was conducted in Van Province, located in eastern Turkey (38°29′39″ N, 43°22′48″ E), between May and October 2021 (Figure 1). Van Province represents one of the major sheep-rearing regions of eastern Anatolia, characterized by extensive grazing systems and ecological conditions favorable for ixodid tick vectors.

Sampling strategy

A total of 155 sheep were sampled from nine farms across the study area. Specifically, 81 sheep were collected from three farms in Özalp, 10 sheep from one farm in Tuşba, 26 sheep from one farm in Gevaş, and 38 sheep from four farms in Gürpınar. The sampled population comprised 66 clinically suspected animals showing signs compatible with babesiosis and 89 apparently healthy animals.

Sample collection

Blood samples were collected aseptically from the sub-tail or auricular veins using sterile techniques. Samples intended for molecular analysis were collected into EDTA-containing tubes and transported to the laboratory under appropriate conditions until further processing.

Microscopic examination

Thin blood smears were prepared immediately after sampling, fixed in absolute methanol, and stained with Giemsa according to standard procedures. Smears were examined under a light microscope at 100× magnification. Piroplasms were identified based on their morphological characteristics. A sample was considered microscopically positive when at

Figure 1. Geographic location of Van Province in eastern Turkey and the districts where blood samples were collected from sheep between May and October 2021.

least one piroplasm was observed in the examined fields.

To minimize observer bias, each smear was examined independently by at least two experienced investigators, and discrepant results were re-evaluated jointly to reach a consensus. For each slide, multiple microscopic fields were systematically screened before a sample was recorded as negative.

DNA extraction

Genomic DNA was extracted from whole blood samples using the Hydra Genomic DNA Isolation Kit (Hydra, Turkey), following the manufacturer's protocol. DNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific™, USA). Extracted DNA samples were stored at −20 °C until PCR analysis.

All extractions were performed under sterile conditions to avoid cross-contamination, and negative extraction controls (reagent blanks) were included. Only DNA samples with acceptable purity ratios (A260/A280) were used for downstream PCR analyses.

Molecular detection of *Babesia ovis*

Molecular detection of *Babesia ovis* was performed by amplifying a 549-bp fragment of the small subunit ribosomal RNA (SSU 18S rRNA) gene using species-specific primers described by Aktas et al. (2005):

Forward primer (Bbo-F): 5′-TGGGCAGGACCTTGGTTCTTCT-3′

Reverse primer (Bbo-R): 5′-CCGCGTAGCGCCGGCTAAATA-3′

PCR reactions were performed in a total volume of 50 µl containing 2.5 µl of 10× PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.5 µl of 25 mM dNTPs, 0.5 µl of each primer, 0.2 µl of Taq DNA polymerase (5 U/µl), 5 µl of template DNA, and nuclease-free water to complete the final volume.

Thermal cycling conditions consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min.

PCR products were analyzed by electrophoresis on 1.5% agarose gels at 90 V for approximately 60 min. A 100-bp DNA ladder was used as a molecular size marker. Appropriate positive and negative controls were included in each PCR run. Only samples yielding a clear band of the expected size were considered PCR-positive.

Sequence and phylogenetic analysis

Among the PCR-positive samples, four representative amplicons were selected for sequencing. Purified PCR products were sequenced bidirectionally at a commercial facility (Hibrigen, Van, Turkey).

The obtained SSU 18S rRNA gene sequences were edited and aligned using the ClustalW algorithm implemented in MEGA version 7. Sequence identity was assessed by comparison with reference sequences available in GenBank. The sequences generated in this study were deposited in GenBank under accession numbers ON137543.1, ON139020.1, ON139021.1, and ON139024.1.

Phylogenetic relationships were inferred using the Neighbor-Joining method with 1,000 bootstrap replicates in MEGA 7. *Plasmodium vivax* (GenBank accession no. XR003001206) was used as the outgroup. Bootstrap values were used to assess tree robustness.

RESULTS

Microscopic examination results

Microscopic examination of Giemsa-stained peripheral blood smears revealed *Babesia* spp. piroplasms in 7 of 155 sheep, corresponding to an infection rate of 4.51%. All microscopically positive samples were obtained from clinically suspected animals, whereas no piroplasms were detected in apparently healthy sheep (Table 1).

The district-wise distribution of microscopically positive cases is presented in Table 2.

Table 1. Infection rates of *Babesia* spp. in sheep detected by microscopic examination and PCR.

Method	Target	Positive (n)	Negative (n)	Positive (%)	Negative (%)
Microscopic examination (ME)	*Babesia* spp.	7	148	4.51	95.49
PCR	*Babesia ovis*	21	134	13.54	86.46

Table 2. Comparison of *Babesia* spp. infection rates in sheep among districts of Van Province based on microscopic examination and PCR results.

District	ME (*Babesia* spp.) Positive (n)	ME Negative (n)	ME Positive (%)	PCR (*B. ovis*) Positive (n)	PCR Negative (n)	PCR Positive (%)
Tuşba	0	9	0.00	1	8	11.11
Özalp	3	78	3.70	10	71	12.35
Gevaş	2	24	7.69	3	23	11.54
Gürpınar	2	37	5.13	7	32	17.95
Total	**7**	**148**	**4.51**	**21**	**134**	**13.54**

Molecular identification results

PCR amplification targeting the SSU 18S rRNA gene of *Babesia ovis* produced the expected 549-bp amplicon in 21 of 155 blood samples (13.54%) (Figure 2). All microscopically positive samples (n = 7) were also PCR-positive. Additionally, 14 samples that were negative by microscopic examination were positive by PCR. No amplification was observed in negative control reactions.

Sequence homology and phylogenetic analysis

Four representative PCR-positive amplicons were sequenced and deposited in GenBank under accession numbers ON137543.1, ON139020.1, ON139021.1, and ON139024.1. Sequence analysis demonstrated 100% nucleotide identity with previously reported *Babesia ovis* isolates available in GenBank. Phylogenetic analysis showed that all isolates obtained in this study clustered within the *B. ovis* clade together with reference sequences retrieved from GenBank (MT114713.1, MN611761.1, MN309738.1, MH194407.1, and MH208613.1) (Figure 3). No distinct subclades were observed among the Van Province isolates.

DISCUSSION

Babesiosis caused by *Babesia ovis* is an economically important tick-borne disease affecting sheep in endemic regions (Ceylan et al., 2021; Schnittger et al., 2022). Accurate detection and molecular characterization of circulating isolates are essential for understanding the epidemiology and genetic structure of this pathogen.

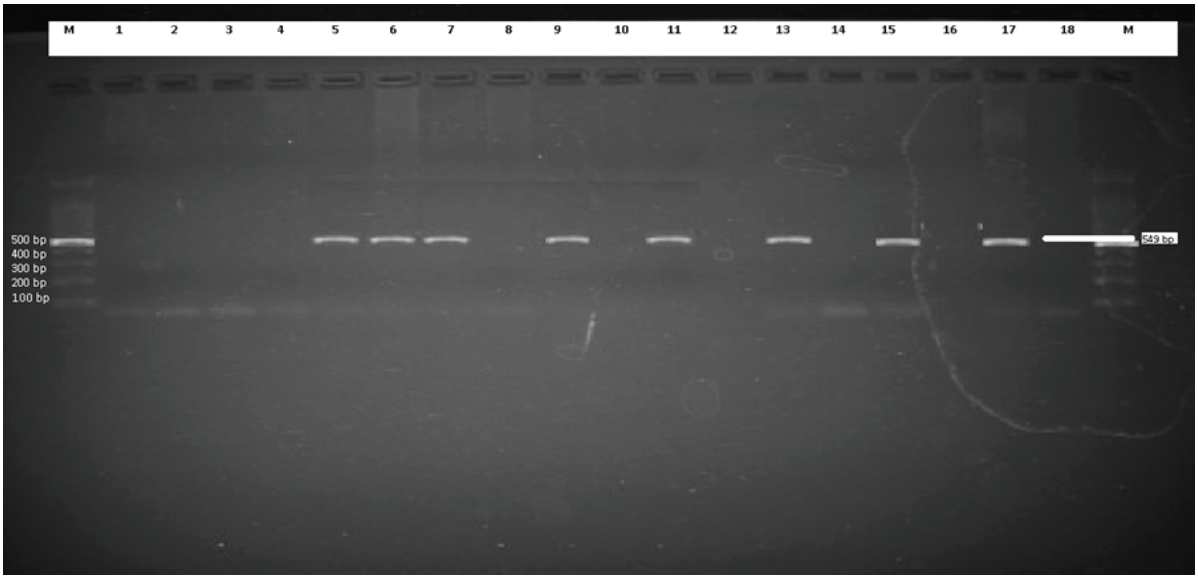

Figure 2. Agarose gel electrophoresis of PCR products obtained using *Babesia ovis*-specific primers (Bbo-F/Bbo-R) targeting the SSU 18S rRNA gene. Lanes M: 100 bp **DNA** ladder; lanes 1–18: sheep blood samples. A specific band at 549 bp indicates PCR-positive samples.

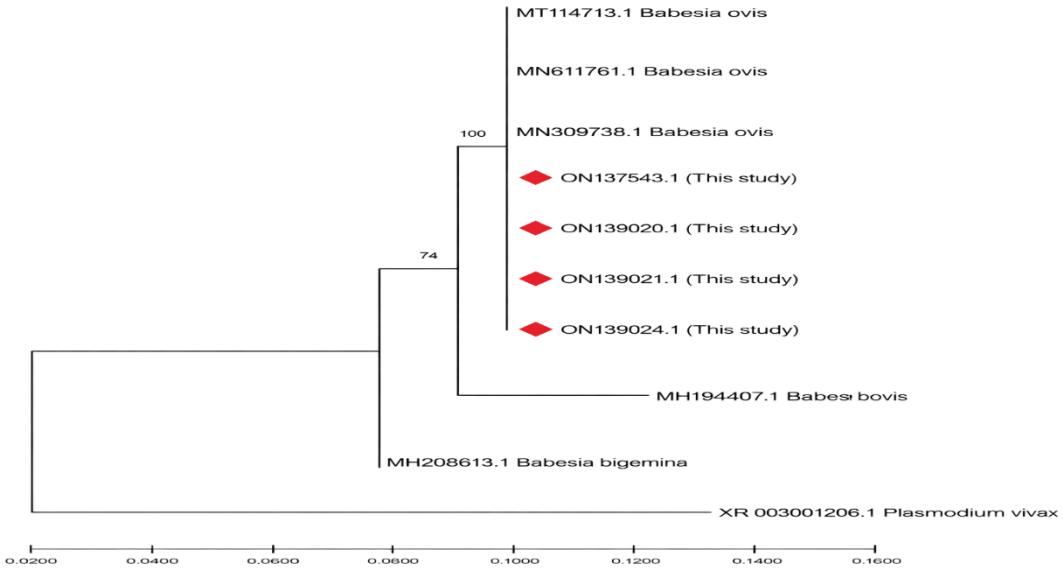

Figure 3. Phylogenetic tree based on SSU 18S rRNA gene sequences of *Babesia ovis* isolates obtained in this study and reference sequences from GenBank (MT114713.1, MN611761.1, MN309738.1, MH194407.1, and MH208613.1). *Plasmodium vivax* (XR003001206.1) was used as the outgroup. The tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates.

In the present study, the infection rate of *B. ovis* was determined as 4.51% by microscopic examination and 13.54% by PCR. All microscopically positive samples were confirmed by PCR, whereas 14 additional samples that were negative by microscopy were detected by molecular analysis. These findings indicate that a considerable proportion of infections may remain undetected when relying solely on light microscopy, particularly in animals with low parasitemia or subclinical infections.

The molecular infection rate observed in this study is comparable to those reported in other regions of Turkey (Bilgiç et al., 2017; Aydın and Dumanlı, 2019; Ceylan and Sevinç, 2020; Ceylan et al., 2021), supporting the continued circulation of *B. ovis* in sheep populations. Although nationwide surveillance data remain limited, available studies consistently demonstrate that *B. ovis* represents a persistent parasitic threat to small ruminant production in Turkey.

The SSU 18S rRNA gene was selected as a molecular marker due to its widespread use in species identification and phylogenetic analysis of tick-borne protozoa (Habibi et al., 2004; Aktaş et al., 2005; Niu et al., 2009; Altay et al., 2012; Tian et al., 2013; Hakimi et al., 2019). This locus contains conserved and variable regions that enable reliable amplification and comparative sequence analysis across geographic regions. The sequences obtained in this study exhibited 100% nucleotide identity with previously reported *B. ovis* isolates, indicating strong genetic conservation within the analyzed SSU 18S rRNA fragment.

Phylogenetic analysis demonstrated that the Van Province isolates clustered within the established *B. ovis* clade together with reference sequences retrieved from GenBank. No distinct subclades were observed among local isolates, suggesting limited genetic divergence within this genomic region. Similar clustering patterns have been reported in previous phylogenetic studies conducted in Turkey and other endemic areas.

Although comprehensive genomic data for *B. ovis* remain limited (Yamagishi et al., 2023), region-specific molecular studies provide valuable baseline information on genetic identity and phylogenetic relationships. In this context, the present study contributes molecular evidence from Van Province and supports the placement of local isolates within the broader national and global genetic framework of *B. ovis*.

Overall, the findings highlight the importance of integrating molecular detection with phylogenetic analysis to better understand the epidemiology and genetic characteristics of ovine babesiosis in endemic regions.

ACKNOWLEDGMENTS

This study was supported by the Scientific Research Projects Department of Van Yüzüncü Yıl University (Project No. TYL-2022-9822). The authors gratefully acknowledge the financial support provided by Van Yüzüncü Yıl University.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

FINANCIAL SUPPORT

This study has been supported by Van Yüzüncü Yıl University Scientific Research Projects Department with TYL-2022-9822 project number.

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