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Serological and Molecular Survey of *Babesia ovis* in Healthy Sheep in Türkiye

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Abstract: Babesiosis, caused by *Babesia ovis*, is a major seasonal issue in sheep, particularly in countries like Türkiye with high *Rhipicephalus bursa* tick populations. Previous studies employing various methods such as microscopy, serology, or molecular techniques have reported different epidemiological data concerning ovine babesiosis. Addressing this knowledge gap, our study employed a combined nested PCR (nPCR)/indirect ELISA (iELISA) approach, analyzing blood samples collected from 414 sheep between April and July 2023 using both techniques. nPCR amplified the *18S ribosomal RNA* gene of *B. ovis* and determined a molecular prevalence of 1.9%. Conversely, serological testing using iELISA targeted the BoSA1 antigen and revealed a significantly higher positivity rate of 59.9% for anti-*B. ovis* antibodies. The temporary presence of *Babesia* after recovery reduces nPCR sensitivity, resulting in lower molecular prevalence. However, even if *Babesia* is not present in the host, anti-*B. ovis* antibodies remain in the serum for a long time and can be detected serologically. Our study underscores the necessity of concurrently employing molecular and serological methods for an accurate assessment of *B. ovis* prevalence. It highlights the importance of comprehensive epidemiological approaches for effective disease management in sheep populations.

Keywords: *Babesia ovis*; ELISA; epidemiology; PCR; sheep

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1. Introduction

Babesiosis is a protozoan disease caused by *Babesia* species and is commonly found in both domestic and wild animals, especially in tropical and subtropical regions, and it has zoonotic potential [1]. In sheep and goats, babesiosis is often attributed to *Babesia ovis*, *B. motasi*, and *B. crassa* species. *Babesia taylori* and *B. foliata* were identified in sheep and goats in India years ago, but sufficient information about these two species has not been available since then [2]. In the past 20 years, new species or genotypes of *Babesia* affecting sheep and goats have been identified, including *Babesia* sp. Xinjiang [3], *Babesia aktasi* [4], and *Babesia motasi*-like (*B. motasi* Lintan, *B. motasi* Tianzhu, *B. motasi* Hebei) [5]. Additionally, based on morphological, serological, pathogenic, genetic, and virulent characteristics, *B. motasi* has been further classified into two subgroups: *B. motasi* Lintanensis and *B. motasi* Hebeinensis [6,7]. *Babesia ovis* causes clinical symptoms characterized by fever, hemolytic anemia, and hemoglobinuria, ultimately resulting in death, and it has a significant economic impact, particularly on sheep farming [5]. The main vector of *B. ovis* is *Rhipicephalus bursa*, and although there is no case registration system for ovine babesiosis, it is known from the field studies that this disease is a serious problem for the sheep breeding industry in Türkiye [8]. *Babesia ovis* has also been reported in unusual hosts other than sheep and goats, but its epidemiological significance is not yet fully understood [2,9,10].

While there have been molecular reports indicating the presence of *B. ovis* in goats, the extent of its clinical significance in actually causing infection is still a matter of debate. Despite the detection of the pathogen at the molecular level, there is ongoing discussion and uncertainty regarding whether its presence correlates directly with clinical symptoms or if other factors are at play. Further research and investigation are needed to fully elucidate the role of *B. ovis* in goat health and its potential impact on veterinary medicine and livestock management. Previous reports have demonstrated the presence of *B. ovis* in sheep in Eastern Europe, former Soviet Union countries, Spain, Italy, Portugal, Türkiye, Syria, Israel, Jordan, Iran, Iraq, Nigeria, Ghana, and Somalia, as well as in Central and South America [9]. New epidemiological and clinical data have confirmed its importance as a tick-borne disease in sheep in Bosnia and Herzegovina [11], Türkiye [12], Egypt [13], Palestine [14], Iran [15], Pakistan [16], and Iraq [17]. Additionally, molecular evidence of *B. ovis* infection in healthy small ruminants has been recorded in European countries such as Greece [18], Spain [19], Italy [20], and Portugal [21]. In Africa, recent molecular studies have confirmed the presence of *B. ovis* in Nigeria [22], Uganda [23], Algeria [24], and Tunisia [25]. There is a microscopic study reporting the presence of *B. ovis* in sheep in Cuba, but since then, these data have not been molecularly supported [26]. *Babesia motasi*, transmitted by ticks of the *Haemaphysalis* spp., exhibits varying levels of pathogenicity and is highly pathogenic in the Mediterranean Basin but less so in Northern Europe [8]. In Iran, *B. crassa*, which typically shows low pathogenicity in goats, has been documented; however, the specific vector responsible for transmitting this parasite remains unidentified [2]. Moreover, isolated instances of human babesiosis induced by *B. motasi* and *B. crassa* have been recently documented in Asia [27,28]. *Babesia* sp. Xinjiang, the first *Babesia* species causing ovine babesiosis to have its full genome analyzed, is prevalent among sheep in China [29]. It is transmitted by ticks of *Hyalomma anatolicum* and *Haemaphysalis qinghaiensis* species and exhibits low pathogenicity [2]. A recently discovered species, *B. aktasi*, has been identified as common among local breed goats in the Mediterranean region of Türkiye [6,30]. While it causes moderate infections in local breed goats, its pathogenicity in various goat breeds and potential hosts, as well as information regarding relevant vector ticks and natural reservoirs, remains unknown. Imidocarb dipropionate is commonly utilized as an anti-babesial medication for treating animals afflicted with the disease. Its efficacy has been demonstrated both therapeutically and prophylactically in the management of ovine babesiosis, as outlined in the research by Sevinc et al. in 2007 [31].

Babesiosis is traditionally diagnosed through the microscopic examination of parasites in blood smears. However, this method lacks the sensitivity to detect parasites during the subclinical or chronic stages of infection [10,32]. Molecular approaches based on nucleic acids, such as Polymerase Chain Reaction (PCR) analyses, offer superior sensitivity and specificity compared to current diagnostic tests [10,21,32]. On the other hand, serological tests, including the Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA), have the capability to detect antibodies in carrier animals [12,32]. ELISA facilitates the detection of antibodies specific to the parasite, which can indicate exposure or past infection, while PCR enables the direct detection of parasite DNA, providing evidence of current infection [33,34]. Several studies have been conducted in Türkiye to determine the prevalence of *B. ovis* using microscopic, serological, and molecular methods. According to the results of these studies, the prevalence of *B. ovis* ranges from 0.5% to 22.2% by microscopic methods, 30.5% to 80.2% by serological methods, and 2.6% to 21.4% by molecular methods [8]. The literature acknowledges that the combination of ELISA and PCR is a potent approach with notable sensitivity and accuracy in conducting babesiosis epidemiological studies [35–38]. Therefore, this study aims to investigate the prevalence of *B. ovis* in sheep in Türkiye using a combination of ELISA and PCR methods, hypothesizing that this approach will provide a more accurate estimation of *B. ovis* prevalence compared to individual methods alone.

2. Results

The prevalence of *B. ovis* infection in sheep was assessed using nested PCR (nPCR) and indirect ELISA (iELISA). In the nPCR assay, *B. ovis* infection was detected in 8 out of 414 (1.9%) blood samples examined. The positive rates of nPCR were 5% in Bismil, 2.8% in Çınar, and 1.5% in Sur, with Bağlar and Çermik districts showing no positive samples. Indirect ELISA revealed that 248 out of 414 sheep (59.9%) exhibited a reaction to *B. ovis*. The highest serological positivity rate was observed in Çınar (90.1%) and the lowest was in Çermik (18.2%) (Table 1). All samples that tested positive with nPCR were also positive with iELISA, and a statistically significant difference in positivity rates between the two tests was found (Figure 1a). Furthermore, when investigating the prevalence of *B. ovis* considering the ages of the animals, no significant association was found between *B. ovis* positivity and the age of the animals (Table 2) (Figure 1b).

Table 1. The frequency of *B. ovis* detected by nPCR and iELISA from samples at locations.

		nPCR		iELISA	
Province	**n**	**+**	**−**	**+**	**−**
Bağlar	65	-	65	17 (26.1%)	48
Bismil	40	2 (5%)	38	18 (45%)	22
Çermik	33	-	33	6 (18.2%)	27
Çınar	142	4 (2.8%)	138	128 (90.1%)	14
Sur	134	2 (1.5%)	132	79 (58.9%)	55
Total	**414**	**8 (1.9%)**	**406**	**248 (59.9%)**	**166**

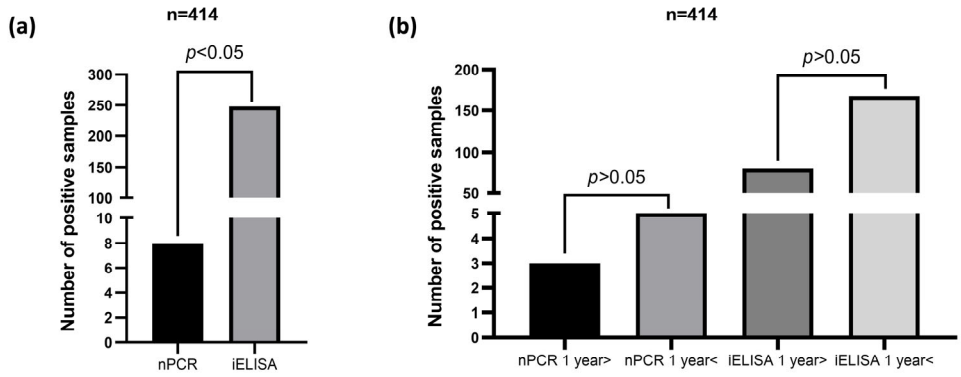

Figure 1. (**a**) Comparison of positive samples obtained by nPCR and iELISA. (**b**) Comparison of age-related positive status obtained by nPCR and iELISA.

Table 2. The relationship between age and positivity obtained through nPCR and iELISA.

		nPCR		iELISA	
	n	**+**	**−**	**+**	**−**
1 year >	139	3 (2.2%)	139	80 (57.5%)	59
1 year <	275	5 (1.8%)	270	168 (61.1%)	107
Total	**414**	**8 (1.9%)**	**406**	**248 (59.9%)**	**166**

3. Discussion

Babesiosis caused by *B. ovis* is a hemolytic disease transmitted to sheep by ticks, leading to significant economic losses in the small ruminant industry. *Babesia ovis* has a widespread geographical distribution and is commonly found in different regions [2,8]. The combination of molecular and serological assays, such as the PCR and ELISA, offers a robust approach to the epidemiological study of *Babesia* infections [35–38]. In this study,

blood samples collected from 414 sheep in 12 foci from five different districts of Diyarbakır were examined using nPCR and iELISA, with positivity rates of 1.9% and 59.9%, respectively. In various geographical locations, a combination of PCR and ELISA has been used to determine the prevalence of *Theileria* and *Babesia* species.

In a study conducted on horses in Mexico, it was observed that *B. caballi* and *T. equi* were determined to be 55.7% and 68.4% positive, respectively, using ELISA, and 7.8% and 78.8% positive, respectively, using nPCR. The higher detection of *B. caballi* with ELISA was attributed to factors such as fluctuations in parasitemia levels, some samples possibly falling below the nPCR detection levels, the persistence of specific antibodies after clearance of the agent, and occasional clustering of the parasite in capillary endothelial cells [39]. In a similar study conducted on horses in Venezuela, *B. caballi* was reported to be 23.2% positive with ELISA and 4.4% positive with PCR. Interestingly, *T. equi* was found to be 14% positive with ELISA and 61.8% positive with PCR in the same study. This situation was suggested to be due to early infection in some animals without serum antibodies and lower levels of protective antibodies, as well as the rare elimination of the parasite from the host and its tendency to remain detectable at observable levels throughout the infection stages (despite fluctuating parasitemia levels) [40]. In a study conducted on cattle in Egypt, the prevalence of *B. bigemina* was reported to be 5.3% with nPCR and 10.6% with ELISA, while for *B. bovis*, these rates were 4% and 9.3%, respectively [37]. In a similar study conducted in Indonesia, the nPCR and ELISA results for *B. bovis* were reported to be 50.7% and 69.8%, respectively, and for *B. bigemina*, they were 19.1% and 27.5%, respectively [38]. In a study conducted on buffaloes in Thailand, nPCR, ELISA, and IFAT techniques were used, with reported prevalence rates for *B. bovis* ranging from 11.2% to 14.7% with nPCR, 3.6% to 5.9% with ELISA, and 16.8% with IFAT, and for *B. bigemina*, they ranged from 3.6% to 5.9% with nPCR, 5.6% with ELISA, and 21.7% with IFAT [36]. In a study conducted on cattle in Syria using the same techniques, the prevalence rates for *B. bovis* were reported to range from 9.2% to 15.5% with nPCR, from 15.5% to 18.3% with ELISA, and from 18.8% to 21.7% with IFAT, while for *B. bigemina*, they ranged from 15.5% to 18.8% with nPCR, from 15.5% to 18.8% with ELISA, and from 18.8% to 21.7% with IFAT [35].

Studies generally indicate that serological prevalence tends to be higher than molecular prevalence. *Theileria* species are known to persist within the host for prolonged periods compared to *Babesia* species [41,42]. This prolonged persistence of *Theileria* within the host can result in elevated molecular prevalence among hosts that have recovered from theileriosis. However, unlike *Theileria*, *Babesia* species do not exhibit prolonged persistence within the host. This distinction results in significant differences between the prevalence obtained by serological methods based on antibody detection and the molecular prevalence. The findings of this study aligned with expectations, revealing a significantly higher serological prevalence compared to molecular prevalence. The indiscriminate use of imidocarb dipropionate in the treatment of babesiosis may be a contributing factor to the low molecular prevalence. It is known that the annual consumption of this drug is very high in Türkiye, and it is known to be applied intensively to sheep showing symptoms such as high fever and hemoglobinuria, especially during the seasons when ticks are seeking hosts [43]. This widespread use of imidocarb dipropionate might lead to the suppression of parasite DNA in treated animals, resulting in lower molecular prevalence compared to serological prevalence. Studies in the literature have shown that imidocarb dipropionate is effective in acute babesiosis in lambs and leads to parasite elimination shortly after treatment [31]. Similarly, it has been observed that lambs infected with *B. ovis* were found to be negative by microscopy after treatment with imidocarb dipropionate [44]. Another study conducted on horses experimentally infected with *Theileria equi* showed that the pathogens could not be detected by molecular methods two weeks after treatment, but serological reactions continued until the 33rd week [45]. These findings suggest that serological tests may remain sensitive for a longer period after treatment and

may yield higher prevalences compared to molecular tests. In addition, the high seroprevalence value determined in this study can be attributed to the long-term persistence of anti-*B.ovis* antibodies in the sera of sheep treated or recovered from the disease.

Babesia ovis Secreted Antigen 1 (BoSA1) is an immunoreactive protein secreted by *B. ovis*. It has been reported that this protein has strong antigenic structures capable of detecting anti-*B. ovis* antibodies and that the natural BoSA1 protein is abundantly present in the cytoplasm of infected erythrocytes [46]. It has been stated that the rBoSA1-based indirect ELISA technique holds strong serodiagnostic significance in detecting anti-*B. ovis* antibodies from the 7th and 8th days of experimental infections, and positivity has been detected in all sera collected throughout the sample. In naturally infected sheep, approximately 70% of serum samples taken before treatment during the infection period were found to be seropositive. However, particularly between days 20 and 30 after treatment, strong positivity was detected in all animals (100%) [46]. It has been shown by the sandwich ELISA method that this antigen can be detected from the 7th day after experimental infection with *B. ovis* carried out with *R. bursa* ticks [47]. In a comprehensive study using this protein, 4115 sheep were examined with iELISA, and the serological prevalence of *B. ovis* was determined to be 29.9% [12]. Additionally, no serological cross-reactivity has been observed among various piroplasm species including *Babesia bovis*, *B. bigemina*, *B. caballi*, *B. canis*, *B. gibsoni*, *Theileria equi*, and *T. annulata* [47]. However, it is not yet known whether this protein exhibits a cross-reaction with *Theileria* and *Babesia* species found in sheep.

Another possible reason for the difference between serological and molecular prevalences could be attributed to the fluctuating course of *Babesia* parasites in their hosts. For example, antibody responses of cattle experimentally infected with *B. bovis* were monitored regularly for a year after infection, and the presence of antibodies was detected during this period. However, molecular tests conducted during the same period showed fluctuations in parasite presence. These fluctuations may reflect the active multiplication and control processes of parasites at different stages in the host organism [48]. Therefore, while the antibody responses detected by serological tests remain stable, fluctuations in parasite presence observed by molecular tests indicate the dynamic course of infection and host organism responses. Hence, the combination of serological and molecular tests allows for a more comprehensive and accurate assessment of *Babesia* infections.

The substantial serological prevalence rate of 59.9% detected in serum samples from sheep in Diyarbakır, Türkiye, prompts a deeper examination of the epidemiological landscape of ovine babesiosis in the region. Despite the absence of clinical cases among the sampled clinically healthy sheep, this high seroprevalence rate raises important questions regarding the potential for disease transmission and the effectiveness of current control measures. Although the detection of the enzootic situation was not one of the primary goals of this study, the differences in *B. ovis* seroprevalence values in Diyarbakır districts support the findings of this study, reporting an unstable endemic situation in Türkiye. For instance, the marked disparity in seroprevalence rates between Çınar (90.1%) and Bağlar (26.7%) underscores the complex interplay of factors influencing disease transmission. In light of these findings, effective control practices must be tailored to the specific epidemiological context of each region. This necessitates a holistic approach encompassing diagnostic seroprevalence, vector control measures, and targeted vaccination strategies [49]. Furthermore, research efforts should focus on elucidating the genetic diversity of *Babesia* species circulating in Turkish sheep populations and evaluating the efficacy of control measures in mitigating disease transmission. By addressing these key areas, we can enhance our understanding of ovine babesiosis epidemiology in Türkiye and develop more effective strategies for disease management and control.

4. Materials and Methods

4.1. Study Area and Blood Sample Collection

Field studies were conducted on 414 sheep in 12 randomly selected farms in the districts of Bağlar, Bismil, Çermik, Çınar, and Sur, all located within the province of Diyarbakır, between April and July 2023 (Figure 2). Blood samples were collected from apparently healthy sheep that had not previously received babesiosis treatment.

Figure 2. Map of Türkiye, showing the sampling districts.

Diyarbakır is characterized by a harsh continental climate, with extremely hot summers; July temperatures typically exceed 35 °C on average. Due to significant differences between winter and summer temperatures in Diyarbakır, the degree of continentally in this region is exceptionally high. The annual average temperature in Diyarbakır generally ranges around 17–18 °C. The annual average precipitation is approximately 350–400 mm [50].

The age of the sampled animals, along with information regarding the sampling site and date, was recorded according to the relevant protocol. Blood samples collected in both EDTA and serum tubes were transported in a thermos maintaining a temperature of +4 °C to the Department of Parasitology, Faculty of Veterinary Medicine, Firat University. Blood samples in EDTA tubes were stored at −20 °C until DNA extraction was performed. Serum samples from the serum tubes were centrifuged at 3000 rpm for 15 min, and the resulting serum was transferred to Eppendorf tubes and stored at −20 °C. The distribution of the samples among the sampling sites is provided in Table 1.

4.2. Genomic DNA Extraction and Nested PCR (nPCR)

Genomic DNA extraction was performed from 200 µL of EDTA-anticoagulated blood using the PureLink™ Genomic DNA Mini Kit (Invitrogen Corporation, Carlsbad, United

States) following the manufacturer's instructions. The concentration of DNA was measured using a spectrophotometer (NanoDrop® ND-2000 UV/Vis Spectrophotometer, Thermo Fisher Scientific Inc., Wilmington, DE, USA).

To verify the DNA extraction, genomic DNA obtained was amplified using OaITGAM F (5′-TGGATGGACTGGTAGACTTG-3′) and OaITGAM R (5′GGGTCACGACATTCATACAC-3′) primers targeting the *ovine integrin alpha M* gene [51]. The cycling conditions involved an initial phase at 94 °C for 4 min, followed by 35 cycles consisting of a 30 s step at 94 °C and by annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and concluding with a final extension step at 72 °C for 5 min. The validated genomic DNA samples were initially amplified for approximately 1600 bp in length in a region of the *18S ribosomal RNA (rRNA)* gene of *Theileria/Babesia* species using Nbab1F (5′-AAGCCATGCATGTCTAAGTATAAGCTTTT3′) and Nbab1R (5′-CCTCTCCTTCCTTTAAGTGATAAGGTTCAC-3′) primers [52]. Subsequently, nested PCR was performed targeting a region of approximately 549 bp in length within the *18S rRNA* gene of *B. ovis* using PCR products obtained from the initial amplification and BboF (5′-TGGGCAGGACCTTGGTTCTTCT-3′) and BboR (5′-CCGCGTAGCGCCGGCTAAATA-3′) primers [53] (Figure 3). The first PCR started with denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Subsequently, the nested PCR was conducted for 35 cycles with denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min.

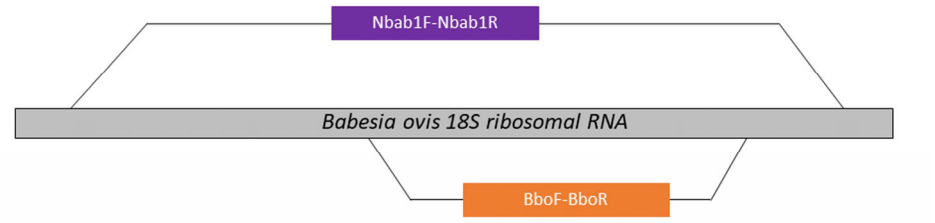

Figure 3. Schematic representation of the region amplified by the primers used in this study.

4.3. Indirect ELISA (iELISA)

The recombinant BoSA1 antigen was coated onto ELISA microplates overnight at 4 °C at a concentration of 2 μg/mL in a coating buffer (carbonate–bicarbonate buffer, pH: 9.6) [46]. Following this, the microplates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 5 min and subsequently blocked with 3% skim milk in PBS for 1 h at 37 °C. After blocking, the plates were washed again as described above. Serum samples were diluted 1:100 in 3% skim milk solution, with 50 μL added per well. The plates were then incubated for 1 h at 37 °C, followed by another round of washing. Next, a secondary antibody, anti-sheep IgG HRP conjugated (Sigma-A3415) at a dilution of 1:1000, was added to individual wells, and the plates were incubated for 1 h at 37 °C. The reaction was developed using the 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA, USA), and the plates were read at an optical density (OD) of 450 nm (ELx800 Absorbance Reader, BioTek Inc., VT, USA). The cutoff value was determined by calculating the average value plus three times the standard deviation of the OD measurements obtained from negative sheep sera.

4.4. Statistical Analysis

The comparison of nPCR and iELISA results and the evaluation of the positivity rates in the age were conducted using the Pearson ChiSquare ($\chi 2$) test. p values ≤ 0.05 were evaluated to be statistically significant. The SPSS 22.00 package program was utilized for performing these tests.

5. Conclusions

In conclusion, employing a PCR/ELISA combination represents a robust approach for epidemiological investigations into ovine babesiosis. By integrating serological and molecular assays, a more nuanced and accurate depiction of *Babesia* infection dynamics emerges. Serological assays capture stable antibody responses over time, offering insight into past and persistent infections, while PCR identifies current parasite presence, which can fluctuate due to various factors. This integrated methodology thus affords a comprehensive understanding of ovine babesiosis epidemiology, transcending the limitations of individual testing methods. By accurately assessing the prevalence and distribution of *Babesia* infections in ovine populations, informed decisions can be made regarding treatment protocols, vector control measures, and vaccination strategies. Additionally, understanding the interplay between seropositivity and parasite presence can aid in identifying potential reservoirs of infection and assessing transmission dynamics. Ultimately, embracing the PCR/ELISA combination represents a crucial step forward in advancing our understanding of ovine babesiosis and facilitating more targeted and effective interventions to mitigate its impact on animal health and welfare. Particularly, prevalence studies targeting parasite DNA may not accurately reflect the reality due to the indiscriminate use of imidocarb dipropionate. Combining serological and molecular tests provides a more comprehensive approach for the accurate assessment of *Babesia* infection, considering stable antibody responses and fluctuating parasite presence in host organisms. Embracing this integrated approach promises to advance our understanding of ovine babesiosis epidemiology and inform more effective strategies for disease management and control.

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Institutional Review Board Statement: This study was conducted according to the regulations of animal welfare issued by the Turkish legislation for the protection of animals (Animal Experiment Ethic Committee, protocol No. 28/09/2023-572385).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available in a publicly accessible repository.

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