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A molecular survey of piroplasmida parasites from the hard ticks that infest the goats naturally in the mediterranean region of Turkiye

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Abstract

In this study, we conducted a survey to detect piroplasmid parasites in ticks from goats in Türkiye's Mediterranean region. A total of 127 tick pools, comprising 661 individual ticks representing *Rhipicephalus bursa*, *R. turanicus*, *R. sanguineus*, *Hyalomma marginatum*, and *Dermacentor raskamensis*, were screened for common caprine piroplasm parasites. DNA was extracted from each pool and subjected to PCR to amplify the 18S rRNA genes of *Babesia ovis*, *Babesia aktasi*, and *Theileria ovis*. Infection rates in tick pools were estimated using the Maximum Likelihood Estimation (MLE) method with 95% confidence intervals. The Maximum Likelihood Estimation (MLE) method was used to calculate infection rates in pooled ticks of varying sizes. Overall, the infection rate for piroplasmid parasites was estimated at 67.15 per 1,000 ticks. The rates varied greatly by species, from 0.03% in *D. raskamensis* to 71.70% in *R. bursa*. The most commonly detected parasite was *B. ovis*, followed by *T. ovis*. *Babesia aktasi* was not identified in any of the tick pools. A total of 27 out of 127 tick pools (21.25%) tested positive for *B. ovis*, with an MLE of 46.86% (95% CI: 31.83-66.79). In contrast, *T. ovis* was detected in 7 out of 127 pools (5.51%) with an MLE of 7.67% (95% CI: 2.87-16.84). No piroplasm parasites were detected in *R. sanguineus*, and co-infection with *B. ovis* and *T. ovis* was found in 3 out of 127 pools (2.36%), with an MLE of 4.58% (95% CI: 1.21-12.33).

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Introduction

Piroplasmid parasites, primarily represented by the genera *Babesia* and *Theileria*, are obligate intraerythrocytic protozoa transmitted by ixodid ticks and pose major challenges to animal health and livestock production worldwide (1-5). These parasites can cause a range of clinical manifestations, from subclinical to severe disease, characterized by anemia, fever, jaundice, weight loss, and death in some cases (6-11). Moreover, subclinical disease may reduce productivity and act as a reservoir for tick-borne transmission (12). *Babesia* and *Theileria* species are primarily transmitted by ixodid ticks and infect a wide range of domestic and wild animals, including goats (6-8,10). Molecular diagnostic methods have proven to be highly sensitive and specific tools for detecting and identifying piroplasmid parasites in both host and vector populations. These methods enable the detection of low-level parasitemia and mixed infections that may be ignored by traditional microscopic techniques (13,14). So, the molecular tools have facilitated the discovery of new *Theileria* and *Babesia* species (15-18). Molecular recognition targeting tick vectors provides valuable insights into parasite epidemiology, transmission dynamics, and infection risks in host populations (19,20). The coexistence of competent tick vectors and susceptible hosts creates a favorable environment for the maintenance and spread of piroplasmid parasite infections (21). Goats are extensively reared in the Mediterranean region of Türkiye, where the climate is highly conducive to the survival and proliferation of ixodid ticks. Several studies have investigated piroplasm parasites in small ruminants and their vectors in Türkiye (2,19); however, data on the distribution and frequency of these parasites in this region remain limited.

The present study aimed to detect piroplasmid parasites in ticks that infest goats in the Mediterranean region of Türkiye. These findings enhance our understanding of the local epidemiological parameters of *Theileria* and *Babesia* infections.

Materials and Methods

Study area, collection of ticks, and forming tick pools

Ethical statement: Ethical approval for this study was granted by the Animal Experiments Local Ethics Committee of the Elazığ Veterinary Control Institute (Document no. 2018/02). Study area: This molecular survey was conducted between May and September 2018 in domestic goats from the provinces of Antalya (36° 53′ N, 30° 42′ E) and Mersin (36° 47′ N, 34° 37′ E) in the Mediterranean region of Türkiye. The region is characterized by a Mediterranean climate, with hot and humid summers and mild, precipitation-rich winters, which may influence the ecology and seasonal activity patterns of vector species. During winter, goats are kept indoors in coastal villages; in spring, they migrate to graze in the Taurus Mountains until autumn. A total of 293 goats, randomly selected from 24 herds across 19 villages, were examined for tick infestation. Collection of ticks: A total of 1,091 adult ticks belonging to 3 genera and 5 species (*Rhipicephalus bursa*, *R. turanicus*, *R. sanguineus*, *Hyalomma marginatum*, *Dermacentor raskamensis*) were collected from 194 infested goats. Detailed information on tick load per goat, attachment sites, and tick species distribution has been previously published (22). Forming tick pools: From 1,091 ticks, 661 specimens were grouped into 127 pools (1-14 ticks per pool) based on species, geographic origin (province/district/village), gender, and engorgement level. PCR amplification may be inhibited in engorged adult ticks due to the higher host blood content, which can interfere with reaction efficiency. To reduce host DNA interference in PCR assays, ticks with low blood volume were selected.

DNA extraction

Before DNA extraction, the external surfaces of the ticks were disinfected using 70% ethanol, followed by three rinses with sterile water, and then dried on filter paper. The sterilized ticks were stored at -80°C overnight to facilitate tissue homogenization. For DNA isolation, the frozen ticks were homogenized in the same tubes using a sterile, disposable pestle with the aid of liquid nitrogen (20,23). DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's standard protocol. After extraction, the samples were stored at 4°C until processing for molecular analyses. DNA from *B. ovis* and *T. ovis-infected R. bursa* were used as positive controls.

Polymerase chain reaction and agarose gel electrophoresis

DNA samples from tick pools were first screened using a nested PCR targeting the 18S rRNA gene of all piroplasm species. First-round PCR was performed with primers Nbab1F and Nbab1R (24), followed by second-round PCR using RLB-F2 and RLB-R2 primers (25). The primers were obtained from BM Labosis (Ankara, Türkiye). Positive pools were subjected to species-specific PCRs targeting partial 18S rRNA regions of *B. ovis* (26), *B. aktasi* (27), and *T. ovis* (28). The primers (primer sequence 5'-3', product size (base pair) applied in the current study are presented in Table 1. PCR was performed in a final volume of 25 µL, comprising 12.5 µL of 2× PCR Master Mix (Thermo Scientific), 1 µL of each primer (10 pmol/µL), 2 µL of extracted DNA, and 8.5 µL of ultra-pure water. One µL of the amplicon from the first-round PCR was used as the template in the second-round PCR. The touchdown PCR procedure and thermal cycling conditions were carried out as previously reported (20). Briefly, the cycling conditions were denaturation at 94°C for 5 min, followed by 94°C for 20 s, 67°C for 20 s, and 72°C for 30 s. The annealing temperature was decreased by 2°C every second cycle to a "touchdown" temperature of 57 °C. Reactions were run on a thermal cycler (Labcycler Gradient, Göttingen, Germany). The amplified DNA fragments were electrophoresed on 1.5% agarose gels and subsequently visualized under ultraviolet illumination. DNA ladder (100 bp) served as a molecular weight reference. Each PCR assay included previously confirmed genomic DNA from *B. aktasi*, *B. ovis*, and *T. ovis* as positive controls, along with a negative control containing nuclease-free water.

Table 1: The primer (primer sequence 5'-3', amplicon size) applied in this study

Marker (*18S rRNA*)	Primer	Primer sequence 5'-3'	Amplicon size (bp)	Reference
Theileria/*Babesia* (First-round PCR)	Nbab1F Nbab1R	AAGCCATGCATGTCTAAGTATAAGCTTTT CCTCTCCTTCCTTTAAGTGATAAGGTTCAC	1600	24
Theileria/*Babesia* (Nested PCR)	RLBF2 RLBR2	GACACAGGGAGGTAGTGACAAG CTAAGAATTTCACCTCTGACAGT	360-430	25
Babesia ovis	Bbo-F Bbo-R	TGGGCAGGACCTTGGTTCTTCT CCGCGTAGCGCCGGCTAAATA	549	26
Babesia aktasi	Ba600F Ba1019R1	GAATCGACGTTCGTCGTTTA GTTTCAGCCTTGCGACCATACT	438	27
Theileria ovis	TSsr 170F TSsr 670R	TCGAGACCTTCGGGT TCCGGACATTGTAAAACAAA	520	28

Infection rates in pooled ticks

The Maximum Likelihood Estimation (MLE) approach was applied to estimate infection rates in pooled ticks with variable sizes, and corresponding 95% confidence intervals (CI; lower and upper bounds) were computed. The analyses were performed using the PooledInfRate software, implemented as a Microsoft Excel add-in, as described previously (2,20,29,30).

Results

Prevalence rate of caprine tick-borne piroplasmid parasites

A total of 127 tick pools, representing 661 individual ticks from 5 ixodid species, were analyzed by PCR. 37 pools (29.13%) tested positive for *Babesia ovis* and/or *Theileria ovis*. The most commonly detected parasite in the tick pools was *B. ovis*, followed by *T. ovis*. In contrast, no *B. aktasi* DNA was identified in any of the pooled ticks. Table 2 shows the number of examined tick pools and infection rates for piroplasmid parasites.

The MLE infection rate was 67.15% per 1,000 ticks. Infection rates varied by tick species, ranging from 0.03% in *D. raskamensis* to 71.70% in *R. bursa*. No infections were detected in *R. sanguineus*. Twenty-seven pools (21.25%) tested positive for *B. ovis*, corresponding to an MLE of 46.86%, with involvement of *R. bursa*, *R. turanicus*, and *H. marginatum*. *Theileria ovis* was detected in 7 pools (5.51%) from *R. bursa*, *R. turanicus*, and *D. raskamensis*, with an MLE of 7.67%. Single *B. ovis* infections were detected in *R. bursa* (24/85) with an MLE of 53.23%, in *R. turanicus* (2/17) at an MLE of 34.45%, and in *H. marginatum* (1/8) at an MLE of 58.25%. *Theileria ovis* infections were identified in *R. bursa* (5/85) with an MLE of 9.23%, in *R. turanicus* (1/17) at an MLE of 15.48%, and in *D. raskamensis* with an MLE of 0.03%. Small ruminant piroplasm DNA was not detected in *R. sanguineus*. The majority of tick pools were positive for a single pathogen (*B. ovis* or *T. ovis*). However, mixed infections with both parasites were observed in 3 pools (2.36%) from *R. bursa*, with an MLE of 4.58%.

Table 2: Infection rates of goats with piroplasmid parasites in ixodid ticks that infest the goats

Tick species	NET/NEP	NPP	MLE (%)	Single infection			*B. ovis with T. ovis*
				B. ovis	*B. aktasi*	*T. ovis*	
R. bursa	550/85	32 (37.64%)	71.70	24/85 (53.23) [a]	-	5/85 (9.23)	3/85 (5.50)
R. turanicus	62/17	3 (17.64%)	51.78	2/17 (34.45)	-	1/17 (15.48)	-
R. sanguineus	2/2	-	-	-	-	-	-
H. marginatum	16/8	1 (12.5%)	58.25	1/8 (58.25)	-	-	-
D. raskamensis	31/15	1 (6.66%)	0.03	-	-	1 (0.03)	-
Total	661/127	37 (29.13%)	67.15	27/127 (46.86)	-	7/127 (7.67)	3/127 (4.58)

NET; number of examined ticks, NAP; number of examined pools, NPP; number of positive pools; CI, Coinfidence Intervals; [a]The rate (…%) shows the MLE result.

Discussion

Piroplasms are apicomplexan parasites transmitted by hard ticks and distributed globally. They are responsible for piroplasmosis, including theileriosis and babesiosis in vertebrates, making them important from both medical and economic perspectives (31-33). This molecular recognition provides significant insights into the epidemiology of small ruminant babesiosis and theileriosis in Türkiye's Mediterranean region. The overall prevalence of piroplasmid parasites in hard ticks was 67.15% in the current study. In other regions, reported prevalence rates include 14% in Iran (34), 10% in the United Arab Emirates (35), 43% in Egypt (36), 3.49% in China (37), 5.4% in Palestine (38). Compared with these reports, the prevalence found in the present study is higher than that reported in other countries. It is well known that the distribution patterns of ixodid ticks and the transmission of tick-borne diseases are shaped by climatic factors such as rainfall, vegetation, altitude, and temperature, as well as by host availability (39). Pooling is an essential approach for screening large numbers of tick samples for pathogens, as it significantly reduces the resources and time required for molecular screening. However, this method has the drawback of reduced resolution, particularly when estimating individual infection rates. To balance efficiency and statistical accuracy, we limited each pool to 14 ticks. We also applied maximum likelihood estimation (MLE) to adjust for potential biases arising from differing pool sizes, thereby providing more accurate estimates of infection prevalence (2,20,29,30). In this study, the detection of piroplasm parasites (*B. ovis* and/or *T. ovis*) in tick pools underscores the considerable infection pressure on small ruminant populations in the sampled region, as evidenced by the number of infected ticks per 1,000 examined. The predominance of *R. bursa* as a carrier of both parasites in the current study is consistent with its recognised role as the principal vector of *B. ovis and T. ovis* (20, 40-43). Despite being lower than in *R. bursa*, the estimated piroplasm infection rate in *R. turanicus* is noteworthy, supporting previous findings that this tick species may serve as a potential vector for both parasites (44-46).

Babesia ovis is the most pathogenic piroplasmic parasite infecting small ruminants. Due to the presence of suitable climates and habitats in many regions of Türkiye, including the Mediterranean region, there is a broad diversity of tick species and piroplasm parasites in the country (19,20). In our previous tick survey (20), *T. ovis* was the most frequently detected pathogen, with an MLE of 1.38% (29 positive pools out of 310). This was followed by *B. ovis*, which was found in 20 out of 310 tick pools, corresponding to a MLE of 0.92%. The prevalence of *T. ovis* in hard ticks from Southern Iran (34) and Palestine (38) is comparable to our findings. In a recent study conducted in the United Arab Emirates (35), molecular analyses showed that *H. anatolicum* collected from goats were positive for *T. ovis* (10%). In this study, the detection of *B. ovis* and *T. ovis* in *R. bursa*, *R. turanicus*, and *H. marginatum* is consistent with previous studies from different regions of Tüekiye (20,40,44). However, this is the first time *T. ovis* has been detected in *D. raskamensis* in Türkiye. Interestingly, in a previous study conducted in the same region on sheep and goats, *B. aktasi* was identified as the most prevalent piroplasm species infecting goats (47). However, in the present tick survey from the same geographical area, *B. aktasi* DNA was not detected in any of the examined tick pools. This discrepancy suggests that the absence of *B. aktasi* in the tick populations may indicate that the primary vector(s) responsible for its transmission were not captured during our sampling.

Since certain tick species, such as *R. bursa*, can act as vectors for multiple piroplasm species simultaneously, mixed infections caused by piroplasm parasites are frequently detected in ticks. Small-ruminant Babesia and Theileria parasites often share tick vectors and vertebrate hosts, increasing the likelihood of simultaneous transmission. The transmission routes of *Babesia* and *Theileria* species differ markedly. *Theileria* species are transmitted exclusively via the transstadial route, whereas *Babesia* species can transmit via both transstadial and transovarian routes, highlighting fundamental differences in their epidemiology and vector-host interactions (1,3,21,48). Mixed infections can complicate disease diagnosis and management in affected animals, as they may result in concurrent or more severe clinical signs compared to single infections. In this study, the co-occurrence of *B. ovis* and *T. ovis* was detected in 3 of 127 pools, consistent with previous reports (2,19,20).

The detection of pathogens in ticks that have fed on hosts does not necessarily confirm the vector competence of the tick species (49-51). In this study, ticks screened for piroplasm parasites were collected from infested goats. Therefore, it is important to emphasize that the identification of parasite DNA in these feeding ticks may reflect the presence of pathogens in the host's bloodstream rather than active transmission or vector capacity. This constitutes a major limitation of the present study and should be considered when interpreting the results.

Conclusion

The current study provides molecular evidence of the presence of two significant piroplasms (*B. ovis*, *T. ovis*) in ticks reared on goats in Türkiye's Mediterranean region. *Babesia ovis* was predominantly identified in *Rhipicephalus* species, whereas it was found only sporadically in *H. marginatum*. *Theileria ovis* was also identified in the same tick species, and notably in *D. raskamensis*.

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Conflict of interest

There is no conflict of interest.

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دراسة مسحية جزيئية لطفيليات البيروبلازميدا من القراد الصلب الذي يصيب الماعز بشكل طبيعي في منطقة البحر الأبيض المتوسط في تركيا

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الخلاصة

في هذه الدراسة، أجرينا مسحا للكشف عن طفيليات البيروبلازميد في القراد من الماعز في منطقة البحر الأبيض المتوسط في تيشركي. تم فحص ما مجموعه ١٢٧ تجمعا" من القراد، تضم ٦٦١ قرادة فردية تمثل *Rhipicephalus bursa*، *R. Turanicus*، *R. Sanguineus*، *Hyalomma marginatum* و *Dermacentor raskamensis*، بحثا عن طفيليات الكابرين البيروبلازمية الشائعة. تم استخراج الحمض النووي من كل تجمع وعوملت بتفاعل البلمرة المتسلسل لتضخيم جينات 18S rRNA من *Babesia ovis*، *Babesia aktasi* و *Theileria ovis*. تم تقدير معدلات الإصابة في تجمعات القراد باستخدام طريقة تقدير الاحتمالية القصوى مع فترات ثقة بنسبة ٩٥٪. تم استخدام طريقة تقدير الاحتمالية القصوى لحساب معدلات الإصابة في القراد المجمع بأحجام مختلفة. بشكل عام، قدر معدل الإصابة بالطفيليات البيروبلازمية بـ ٦٧,١٥ لكل ١٠٠٠ علامة. تفاوتت المعدلات بشكل كبير حسب الأنواع، من ٠,٠٣ ٪ في *D. raskamensis* إلى ٧١,٧٠ ٪ في *R. bursa*. كان الطفيل الأكثر شيوعا هو *B. ovis*، يليه *T. ovis*. لم يتم التعرف على *Babesia aktasi* في أي من تجمعات القراد. ما مجموعه ٢٧ من أصل ١٢٧ تجمعات القراد (٢١,٢٥٪) اختبار إيجابي لـ *B. ovis*، مع MLE من ٤٦,٨٦ ٪ (٩٥ ٪ CI: ٣١,٨٣-٦٦,٧٩). في المقابل ، تم الكشف عن *T. ovis* في ٧ من أصل ١٢٧ تجمعا" (٥,٥١٪) مع MLE من ٧,٦٧ ٪ (٩٥ ٪ CI: ٢,٨٧-١٦,٨٤). لم يتم الكشف عن طفيليات البيروبلازم في *R. sanguineus*، وتم العثور على عدوى مشتركة مع *B. ovis* و *T. ovis* في ٣ من أصل ١٢٧ تجمعا" (٢,٣٦٪) ، مع MLE من ٤,٥٨ ٪ (٩٥ ٪ CI: ١,٢١-١٢,٣٣).