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Molecular Epidemiology of *Babesia* and *Theileria* Species in Sheep in Kars Region of Turkey

Kars Yöresindeki Koyunlarda Babesia ve Theileria Türlerinin Moleküler Epidemiyolojisi

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ABSTRACT

Objective: This study aimed to evaluate *Babesia* and *Theileria* species and vector ticks in sheep in the Eastern Anatolia Region of Turkey.
Methods: Blood samples were collected from 960 sheep, and ticks were collected from the same animals between January and December 2017. The reverse line blotting (RLB) method was used to analyze *Babesia* and *Theileria* piroplasm DNAs. Ticks and tick egg clusters were evaluated in terms of *Babesia* and *Theileria* species using the RLB technique.
Results: Microscopically, 3.96% (38/960) of *Theileria* spp. piroplasm forms were identified; however, no *Babesia* spp. piroplasm forms were identified. The distribution of *Babesia* and *Theileria* spp. by RLB was 35.52% (341/960). The species identified included *Theileria ovis* (24.79%, 238/960), *Theileria* sp. (6.15%, 59/960), *Theileria* sp. OT3 (4.27%, 41/960), and *Babesia ovis* (0.31%, 3/960). Tick infestation was found in 17.5% (168/960) of the sheep. *Dermacentor marginatus* (66.31%), *Haemaphysalis parva* (32.73%), *Hae. punctata* (0.21%), *Rhipicephalus bursa* (0.53%), and *Hyalomma marginatum* (0.11%) were identified in the infected sheep. No pathogenic species were found in the analysis of egg clusters or tick carcasses according to the RLB method.
Conclusion: *Theileria ovis* is the theileriosis agent in sheep in the study region. Species commonly detected in tick-infested sheep were *D. marginatus* and *Hae. parva*.
Keywords: *Babesia*, *Theileria*, reverse line blotting, sheep, Turkey

ÖZ

Amaç: Çalışmamızda, Kars yöresinde yetiştirilen koyunlarda *Babesia* ve *Theileria* türleri ile vektör kenelerin belirlenmesi amaçlanmıştır.
Yöntemler: Ocak-Aralık 2017 tarihleri arasında 960 koyundan kan örnekleri alınmış ve aynı hayvanların üzerinden kene toplanmıştır. *Babesia* ve *Theileria* piroplasm DNA'ları reverse line blotting (RLB) yöntemi ile incelenmiştir. Keneler ve kene yumurta kümeleri de *Babesia* ve *Theileria* türleri yönünden RLB tekniği ile değerlendirilmiştir.
Bulgular: Mikroskobik olarak %3,96 (38/960) oranında *Theileria* spp. piroplasm formları tespit edilmiş olup, *Babesia* spp. piroplasm formları saptanmamıştır. RLB ile *Babesia* ve *Theileria* türlerinin yaygınlığı %35,52 (341/960) olarak tespit edilmiş olup, başlıca *Theileria ovis* %24,79 (238/960), *Theileria* spp., %6,15 (59/960), *Theileria* sp. OT3 %4,27 (41/960) ve *Babesia ovis* %0,31 (3/960) türleri/genotipleri belirlenmiştir. Araştırmada kene enfestasyonlarının prevalansı %17,5 (168/960) olarak saptanmıştır. Enfeste koyunlarda *Dermacentor marginatus* %66,31, *Haemaphysalis parva* %32,73, *Haemaphysalis punctata* %0,21, *Rhipicephalus bursa* %0,53 ve *Hyalomma marginatum* %0,11 türleri identifiye edilmiştir. RLB yöntemi ile yumurta kümeleri veya kene karkaslarının analizinde patojenik türe rastlanmamıştır.
Sonuç: Bölgede koyunlarda theileriosis etkeni olarak *Theileria ovis*'in sorumlu tür olduğu tespit edilmiştir. Kene ile enfeste koyunlarda yaygın olarak belirlenen türler ise *D. marginatus* ve *Hae. parva*'dır.
Anahtar Kelimeler: *Babesia*, *Theileria*, reverse line blotting, koyun, Türkiye

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Tick-borne diseases caused by *Babesia* and *Theileria* are responsible for deaths and economic losses in sheep breeding (1).

Reverse line blotting (RLB) has been preferred as a diagnostic method due to the determination of multiple infectious agents simultaneously and used frequently in the diagnosis of parasitic diseases especially babesiosis and theileriosis. Hence, this method has been used successfully for the diagnosis of *Babesia* and *Theileria* species in parasitological researches (2,3).

There have been various studies on *Babesia* and *Theileria* infections and vectors in sheep in many countries and also different regions of Turkey (4-8). *Babesia ovis*, *B. crassa*, *B. motasi*, *T. ovis*, *T. uilenbergi*, *T. luwenshuni*, *T. separata*, *Theileria* sp. MK, *Theileria* sp. OT1 and *Theileria* sp. OT3 has been reported in sheep and goats of Turkey (1). However, there is no comprehensive research on *Babesia* and *Theileria* species and vector ticks that carry these blood parasites in sheep in the Kars province. Kars, located in the North-Eastern Anatolia Region of Turkey, is an area where pastured livestock breeding is carried out as well as intensive livestock breeding.

This study is aimed to determine *Babesia* and *Theileria* species in sheep by molecular methods, identify vector ticks, determine *Babesia* and *Theileria* species in ticks, and determine their molecular epidemiology in Kars.

METHODS

Ethics Committee Approval: For this study, Kafkas University Animal Experiments from the Local Ethics Committee (letter dated: 15.12.2016 and numbered: 2016-131) approval has been received.

Study Area

The study took place in the province of Kars located in the North-East Anatolia Region of Turkey and the Georgia-Armenia border (40°36'04.82''N, 43°05'50.83''E). Eight research foci (Akyaka, Arpacay, Digor, Kagızman, Kars City Center, Sarıkamıs, Selim, and Susuz) were visited every month (between January 2017 and December 2017). Ten blood samples and ticks were collected from each randomly selected farm (monthly 80, 960 in total, sheep breed 470 Akkaraman and 490 Morkaraman, age 210 of one-year-old, 244 of two years old and 506 of ≥ three-years-old). Blood smears were prepared for each collected blood sample. The sheep were also examined clinically in terms of theileriosis and babesiosis and as to whether they had any such infections previously. The foci/settlement with 96 different herds/sheepfold where the study was conducted are shown in Figure 1.

DNA Extraction from Blood Samples and Tick Identification and Egg Cluster Collection

Blood samples were collected from the *Vena jugularis* of sheep. DNA extraction from blood samples was performed using a commercial DNA isolation kit (Quick-DNATM Miniprep Kit, Zymo Research, USA) following the manufacturer's protocol. Genomic DNA concentration (ng/mL) was measured using a NanoDrop spectrophotometer (Biotek® Instruments, Inc. USA). The obtained genomic DNAs were stored at -20 °C until polymerase chain reaction (PCR) analysis.

Sheep were examined in terms of tick infestation, and nymphs or adult ticks from infected animals were collected. Engorged female ixodid ticks were ovulated at 28 °C and 85% relative humidity, and the nymphs were allowed to molt in the laboratory. Identification of ticks at the species level was performed under a stereomicroscope (9). Tick and egg clusters were stored at -20 °C until DNA extraction. DNA isolation was performed from engorged nymph-female-adult ticks and egg clusters using a commercial kit (Quick-DNATM Tissue/Insect Miniprep Kit, Zymo Research, USA).

PCR and Monitoring of Products

PCR of DNA samples used in the study was carried out using RLB-F2 and RLB-R2 primers that amplify the variable V4 region of the 18S SSU rRNA gene of *Babesia* and *Theileria* species. Accordingly, 111 PCR products were visualized by ultraviolet (UV) transillumination in a 1.5% agarose gel after electrophoresis and evaluated for the presence of specific bands. The remaining part of the PCR products (849) was stored at 4 °C for use in RLB testing.

A 25 µL solution (dH_2O 11.5 µL, 5x PCR Buffer 5 µL, 25 mM $MgCl_2$ 1.5 µL, 10 mM dNTPs 1.5 µL, 5 U/uL Taq 0.5 µL, reverse and forward primers (20 pmol/µL) 1.25 µL, and 2.5 µL template DNA) was used for a touchdown PCR using a thermal cycler (Biometra, Analytik Jena, USA). Positive and negative control DNA samples were used for each reaction. Touchdown PCR cycling parameters were applied according to Ozubek (8). Five microliters of PCR products were visualized by UV transillumination in a 1.5% agarose gel after electrophoresis and staining with ethidium bromide. Biotin-labeled PCR products (20 µL) were kept at 4 °C for RLB.

RLB

To detect *Babesia* and *Theileria* species, a RLB hybridization was performed on the PCR products, as described previously (10). Briefly, to 20 µL of the PCR products, 2X SSPE/0.1% SDS was added to a final volume of 150 µL and held in the Thermal Cycler at 99 °C for 10 min and denatured for RLB hybridization. A total of 16 different probes which are Catchall (*Babesia* + *Theileria*) and lineage-specific *Babesia* spp., and *Theileria* spp., probes, as well as some specific genotypes and probes which were identified for *Babesia* and *Theileria* species in sheep, were connected to a membrane (4,11-13). Primer and Probes were provided by the Humanizing Genomics Macrogen (Macrogen, Korea).

RLB probes and their nucleotide sequences and concentrations are provided in Table 1 (4,10-14).

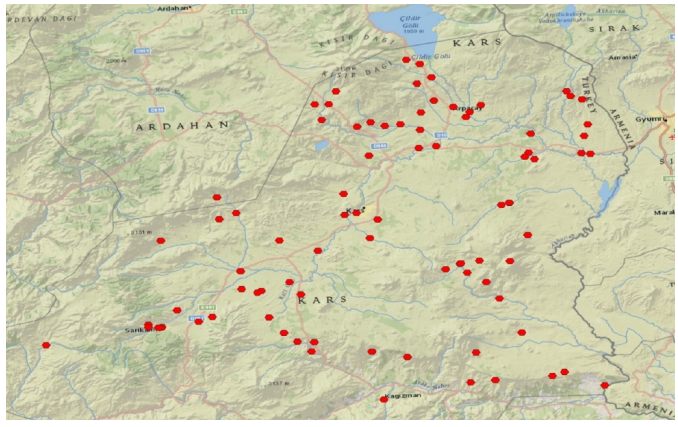

Figure 1. Foci/settlement where the study was conducted

The Biotin-C membrane was constructed as described previously (modifying the concentration and residence times and steps of some solutions) (10,11).

Sequence Analysis

In order to confirm the results obtained by PCR and RLB, a total of 4 isolates consist of *Babesia ovis*, *Theileria ovis*, and *Theileria* sp. and *Theileria* sp. OT3 genotypes were chosen for sequencing. DNA sequences obtained were evaluated with FinchTV chromatogram viewer is a popular desktop application that was developed by Geospiza, Inc., and compared for similarity to sequences deposited in GenBank. Isolates belonging to *Babesia* and *Theileria* species and genotypes were amplified with Nbab1F/Nbab1R primers targeting the *18S rRNA* gene region and then amplicons obtained with PCR were purified and bidirectionally sequenced with Nbab1F and Nbab1R (Macrogen, Turkey) (15). Sequence analyzes of tree species and one genotype determined in sheep were submitted to the Genbank database and an accession number was obtained.

Statistical Analysis

The results obtained from microscopic examination and RLB were compared by Mann-Whitney U tests for pairwise comparisons, and Kruskal-Wallis tests for multiple repetitive measurements (according to study site, month, season, breed, and age) using the SPSS 20.0 program. The incidence of *Babesia* and *Theileria* species was evaluated. Values of $p<0.05$ were considered to be statistically significant (16).

RESULTS

In the microscopic examination of the peripheral blood smears, *Theileria* spp. piroplasm forms were detected in all foci in 38 (3.96%) samples; however, *Babesia* spp. piroplasm forms were not found. Distribution of microscopic examination results by months, *Theileria* spp. in one of the two months with the highest positivity for piroplasm forms, 10% (8/80) of the sheep were found positive in March and 17.5% (14/80) of the sheep were positive in May ($p<0.05$). Considering the distribution by age groups, *Theileria* spp. microscopically, it was detected at a rate of 4.76% (10/210) in sheep up to one year old, and at least 1.64% (4/244) in two-year-olds ($p>0.05$).

The RLB image of *Babesia* and *Theileria* species/genotypes is shown in Figure 2. As seen in the figure, PCR products of *Babesia* and *Theileria* species were hybridized on the membrane to which species and specific probes were attached, and gave signals with their strain-specific probes.

Sixty-seven (60.4%) of 111 samples that were run out on agarose gel, were found positive for species of *Babesia* and *Theileria*. Also, 7 out of 51 samples that were negative on gel electrophoresis, were found positive with RLB. In total 60 samples were found positive that agarose gel electrophoresis and RLB.

It was determined that *Babesia* and *Theileria* species were prevalent in 35.52% (341/960) of the sheep. Single-species infections occurred at a rate of 93.55% (319/341) and mixed infections at 3.23% (11/341) (Table 2).

According to the distribution of RLB results by foci/settlement, (Table 3) the highest distribution of *Babesia* and *Theileria* spp. were determined in Kagızman (49.17%, 59/120) and the lowest in Arpacay (20.83%, 25/120). *Babesia ovis* was found at a very low level with only three positive samples (two of the positive animals were found in Akyaka and the other in Kagızman).

The distribution of RLB results by months is presented in Table 4. It was determined that the *Babesia* and *Theileria* spp. were seen mostly in May (72.5%, 58/80) and least in September (10.00%, 8/80). Comparing the distribution of RLB results by months, the difference was not statistically significant for *Babesia ovis* ($p>0.05$); however, it was significant for *Theileria* species and genotypes ($p<0.05$). *Theileria ovis* was seen every month of the year.

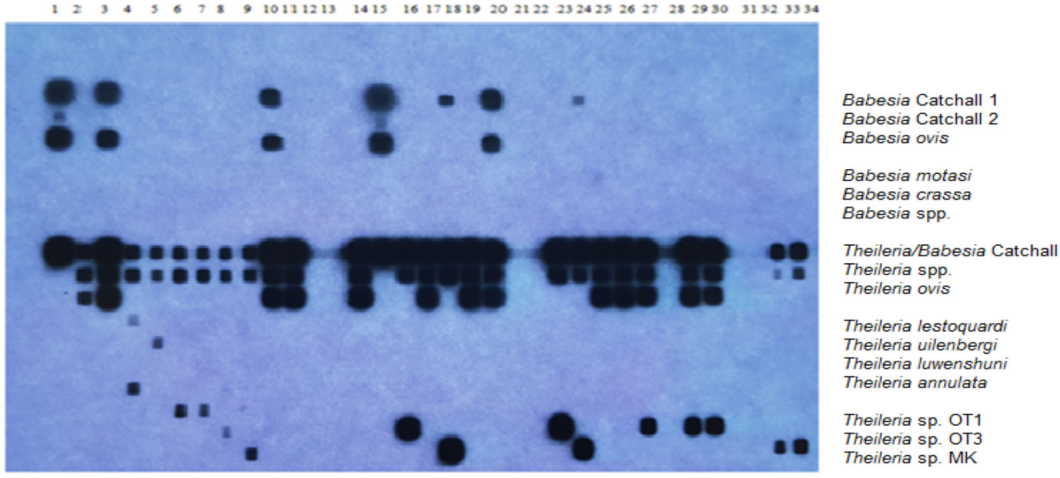

Figure 2. RLB image of *Babesia* and *Theileria* species/genotypes.
Probe rows from left to right, positive control from top to bottom, and samples 1-9 positive controls (1. *B. ovis*, 2. *T. ovis*, 3. *B. ovis+T. ovis*, 4. *T. lestoquardi/T. annulata*, 5. *T. uilenbergi*, 6. *T. luwenshuni*, 7. *Theileria* sp. OT1, 8. *Theileria* sp. OT3, 9. *Theileria* sp. MK, 10. positive control), 11,14-20,23-27,29,30,33,34 Field Samples (11,14,17,19,25,26 *T. ovis*, 15. *B. ovis*, 16,23. *Theileria* sp. OT3, 18,24,33,34. *Theileria* sp. 20. *B. ovis+T. ovis*, 27,29,30. *T. ovis+ Theileria* sp. OT3) 12,13,21,22,28, Buffer, 31,32. Negative control (Uninfected sheep blood DNA)
RLB: Reverse line blotting

Tick infestation was found at a rate of 17.5% (168/960) in sheep. In infected sheep, infestations with a single species were found at a rate of 13.75% (132/960), while infestations with more than one tick species (mixed) were detected at 3.75% (36/960). At least one, and at most sixty-six ticks were collected from tick-infested sheep. The distribution of tick infestation in sheep by months is detailed in Table 5.

A total of 944 ticks, one nymph, and larvae, were collected from the infested sheep. The prevalence of tick species detected in sheep in the study was determined as *Dermacentor marginatus* 66.31% (626/944), *Haemaphysalis parva* 32.73% (309/944), *Haemaphysalis punctata* 0.21% (2/944), *Rhipicephalus bursa* 0.53% (5/944), *Hyalomma marginatum* 0.11% (1/944), and *Haemaphysalis* sp. larvae 0.11% (1/944). The engorged nymph was made molt and identified as *R. bursa*. It was determined that the dominant genera have belonged to *Dermacentor* (66.31%) and *Haemaphysalis* (33.05%). *Dermacentor marginatus* was mostly seen in Kars City Center at a rate of 25.63% (242/944), while this species was never found in Kagızman. *Haemaphysalis parva* was mostly seen in Digor at a rate of 15.36% (145/944), and it was not detected in Kars City Center. *Haemaphysalis punctata* was found only in Sarıkamıs (0.21%, 2/944). *Rhipicephalus bursa* was found mostly in Kagızman (0.42%, 4/944) and least in Digor (0.11%, 1/944). *Hyalomma marginatum* was detected only in Akyaka (0.11%, 1/944). One *Haemaphysalis* sp. larva was found in Selim. When the distribution of tick species in sheep in the Kars Region was compared according to foci/settlement, the difference was statistically between *D. marginatus* and *Hae. parva* was significant for ($p<0.05$).

DNA extraction from adult ticks collected from sheep was carried out by creating tick pools of 1 to 20 according to the species. The collected engorged female ticks were left to lay eggs and a total of 65 egg clusters (56 *D. marginatus* and, 9 *Hae parva*) were obtained. No pathogenic species were found in the egg clusters.

The 1600 bp region of the *18S rRNA* gene of *Babesia* (*B. ovis*) and *Theileria* (*T. ovis*, *Theileria* sp., and *Theileria* sp. OT3) species or genotypes detected in the analysis of blood samples with RLB were sequenced and compared to other *Theileria* and *Babesia* sequences available in GenBank. *Theileria ovis*, *Theileria* sp. OT3 and *B. ovis* were found 100% identical with the sequences. Because *Theileria* sp. was found similar to *T. orientalis* (*T. orientalis/T. buffeli/T. sergenti*) at 99.05% and *T. sinensis* at 98.92%, it was judged as *Theileria* sp. Isolates obtained in the study were registered to the GenBank database with MN493109 (*Theileria* sp. OT3),

Table 1. Nucleotide sequences and concentrations for probes used in RLB

Probes	Sequence (5'-3')	pmol/150 µL	Reference
Theileria/Babesia catchall	AmMC6-TAATGGTTAATAGGA(AG)C(AG)GTTG	200	11
Theileria spp.	AmMC6-TGATGGGAATTTAAACC(CT)CTTCCA	200	4
T. ovis	AmMC6-TTTTGCTCCTTTACGAGTCTTTGC	400	4
T. lestoquardi	AmMC6-ATTGCTTGTGTCCCTCCG	400	12
T. uilenbergi	AmMC6-TGCATTTTCCGAGTGTTACT	400	12
T. luwenshuni	AmMC6-TCGGATGATACTTGTATTATC	400	12
T. annulata	AmMC6-CCTCTGGGGTCTGTGCA	400	10
Theileria sp. OT1	AmMC6-ATCTTCTTTTTGATGAGTTGGTGT	400	4
Theileria sp. OT3	AmMC6-ATTTTCTCTTTTTATATGAGTTTT	400	4
Theileria sp. MK	AmMC6-CATTGTTTCTTCTCATGTC	400	13
Babesia catchall 1	AmMC6-ATTAGAGTGTTTCAAGCAGAC	200	14
Babesia catchall 2	AmMC6-ACTAGAGTGTTTCAAACAGGC	200	14
Babesia spp.	AmMC6-CCT(GT)GGTAATGGTTAATAGGAA	400	12
B. ovis	AmMC6-GCGCGCGGCCTTTGCGTTTACT	400	4
B. motasi	AmMC6-ATTGGAGTATTGCGCTTGCTTTTT	400	4
B. crassa	AmMC6-TTATGGCCCGTTGGCTTAT	400	12

AmMC6: N-terminal N-[trifluoracetamidohexyl-cyanoethyl,N,N-diisopropylphosphoramidite (TFA)]-C6 amino linker, AmMC6 (6 Amino linker)

Table 2. Distribution of *Babesia* and *Theileria* infections according to RLB results (n=960)

RLB									
***Babesia* and *Theileria* species**									
Total Infection rate (%)				**Single-species Infection rate (%)**				**Mixed Infection rate (%)**	
B. ovis	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis*	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis* + *T. ovis*	*T. ovis* + *Theileria* sp. *OT3*
3 (0.31)	238 (24.79)	59 (6.15)	41 (4.27)	1 (0.10)	227 (23.65)	59 (6.15)	32 (3.33)	2 (0.21)	9 (0.94)

RLB: Reverse line blotting

Table 3. Distribution of *Babesia* spp. and *Theileria* spp. RLB results by foci/settlement

Foci/ settlement	Number of blood samples	RLB results									
		Total Number of infections (%)				Single-species Infections (%)				Mixed Infections (%)	
		B. ovis	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis*	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis* + *T. ovis*	*T. ovis* + *Theileria* sp. *OT3*
Akyaka	120	2 (1.67)	36 (30.00)	13 (10.83)	5 (4.17)	0 (0.00)	34 (28.33)	13 (10.83)	5 (4.17)	2 (1.67)	0 (0.00)
Arpacay	120	0 (0.00)	11 (9.17)	10 (8.33)	4 (3.33)	0 (0.00)	11 (9.17)	10 (8.33)	4 (3.33)	0 (0.00)	0 (0.00)
Digor	120	0 (0.00)	41 (34.17)	13 (10.83)	0 (0.00)	0 (0.00)	41 (34.17)	13 (10.83)	0 (0.00)	0 (0.00)	0 (0.00)
Kagızman	120	1 (0.83)	40 (33.33)	7 (5.83)	11 (9.17)	1 (0.83)	36 (30.00)	7 (5.83)	7 (5.83)	0 (0.00)	4 (3.33)
Kars City Center	120	0 (0.00)	24 (20.00)	4 (3.33)	5 (4.17)	0 (0.00)	24 (20.00)	4 (3.33)	5 (4.17)	0 (0.00)	0 (0.00)
Sarıkamıs	120	0 (0.00)	37 (30.83)	4 (3.33)	5 (4.17)	0 (0.00)	36 (30.00)	4 (3.33)	4 (3.33)	0 (0.00)	1 (0.83)
Selim	120	0 (0.00)	26 (21.67)	3 (2.50)	4 (3.33)	0 (0.00)	25 (20.83)	3 (2.50)	3 (2.50)	0 (0.00)	1 (0.83)
Susuz	120	0 (0.00)	23 (19.17)	5 (4.17)	7 (5.83)	0 (0.00)	20 (16.67)	5 (4.17)	4 (3.33)	0 (0.00)	3 (2.50)
Total (%)	**960**	**3 (0.31)**	**238 (24.79)**	**59 (6.15)**	**41 (4.27)**	**1 (0.10)**	**227 (23.65)**	**59 (6.15)**	**32 (3.33)**	**2 (0.21)**	**9 (0.94)**
p*		**0.169**	**0.000**	**0.047**	**0.058**	**0.429**	**0.000**	**0.017**	**0.361**	**0.051**	**0.034**

RLB: Reverse line blotting

MN493110 (*Theileria* sp.), MN493111 (*T. ovis*), and MN493112 (*B. ovis*) accession numbers.

DISCUSSION

In this study carried out on sheep in the Kars Region, *Theileria* spp. piroplasm forms were detected in 38 (3.96%) of 960 sheep by microscopic examination, but *Babesia* spp. were not found, and the rate determined in this study was found to be similar with some studies (2,5,8).

In a study on sheep and goats in the Elazıg Region, *Theileria* spp. piroplasm forms were identified by microscopic examination in 15.78% of animals younger than one year old and 31.81% of animals older than one (17). In this study, the results are not similar to other previous studies in that *Theileria* spp. were found to be higher in animals up to the age of one (4.76%) compared to other groups (1.64-4.74%) ($p>0.05$). This is due to the unequal distribution between age groups when sampling.

In recent years, there have been many studies using molecular methods on babesiosis and theileriosis in sheep in various countries of the world and in Turkey (5,6,18,19). In the present study, according to the RLB method, *B. ovis*, *T. ovis*, *Theileria* sp., *Theileria* sp., OT3, a total of three species and one genotype were identified. When the studies on the prevalence of *Babesia* and *Theileria* species in sheep were examined, it was determined that the infection rate differed in different regions of Turkey with the results obtained from this study. The prevalence of *Babesia ovis* (0.31%) was determined at a very low rate since the sampled animals did not show clinical signs and were randomly selected animals. However, it has been reported that blood parasites are common in every region of Turkey. Both in this study and in other studies, *Theileria* species were found to be more common than *Babesia* species (6,8).

The higher rate of the detected species in Kagızman and Digor can be explained by the fact that the province of Kars where the study was conducted has geographic differences within itself as these settlements are lower in altitude and there are also valley settlements.

To date, no research has been found that examines the distribution of *Babesia* and *Theileria* species according to months and seasons. As seen in previous studies on tick-borne diseases, it is seen in the months and seasons when ticks are active (9).

It has been reported that *Babesia* and *Theileria* species can be found in hosts other than their natural hosts (7). In Turkey and various locations of the world, *T. orientalis* (*T. orientalis/T. buffeli/T. sergenti*) and *T. sinensis* groups of species, which have benign effects, have been reported (20,21) in cattle. Additional studies of this species in other regions of Turkey and different gene regions (such as ITS 1, 2, and 5.8S, Cytochrome b, heat shock protein 70) are needed to clarify this subject.

There are numerous studies revealing tick species in Turkey, in which the data for Eastern Anatolia Region are presented (22-24). Also, a total of 17 tick species including 16 from the Ixodidae family and one from the Argasidae family (25,26) have been described in sheep and goats. In Kars, *D. marginatus* was found at a high rate of 66.31% in sheep, followed by *Hae. parva* at a rate of 32.73%. According to these results, compared to other studies, *D. marginatus* and *Hae. parva* species showed a higher prevalence in the Kars province. *Dermacentor marginatus* is a tick

Table 4. Distribution of RLB results of *Babesia* spp. and *Theileria* spp. species by months

Month	Number of blood samples	RLB results									
		Total Number of infections (%)				Single-species Infections (%)				Mixed Infections (%)	
		B. ovis	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis*	*T. ovis*	*Theileria* sp.	*Theileria* sp. *OT3*	*B. ovis* + *T. ovis*	*T. ovis* + *Theileria* sp. *OT3*
January	80	0 (0.00)	6 (7.50)	8 (10.00)	9 (11.25)	0 (0.00)	6 (7.50)	8 (10.00)	9 (11.25)	0 (0.00)	0 (0.00)
February	80	0 (0.00)	29 (36.25)	2 (2.50)	6 (7.50)	0 (0.00)	29 (36.25)	2 (2.50)	6 (7.50)	0 (0.00)	0 (0.00)
March	80	0 (0.00)	40 (50.00)	8 (10.00)	6 (7.50)	0 (0.00)	36 (45.00)	8 (10.00)	2 (2.50)	0 (0.00)	4 (5.00)
April	80	0 (0.00)	18 (22.50)	2 (2.50)	2 (2.50)	0 (0.00)	17 (21.25)	2 (2.50)	1 (1.25)	0 (0.00)	1 (1.25)
May	80	0 (0.00)	47 (58.75)	4 (5.00)	7 (8.75)	0 (0.00)	45 (56.25)	4 (5.00)	5 (6.25)	0 (0.00)	2 (2.50)
June	80	0 (0.00)	16 (20.00)	10 (12.50)	1 (1.25)	0 (0.00)	16 (20.00)	10 (12.50)	1 (1.25)	0 (0.00)	0 (0.00)
July	80	2 (2.50)	8 (10.00)	4 (5.00)	7 (8.75)	0 (0.00)	5 (6.25)	4 (5.00)	6 (7.50)	2 (2.50)	1 (1.25)
August	80	1 (1.25)	6 (7.50)	14 (17.50)	0 (0.00)	1 (1.25)	6 (7.50)	14 (17.50)	0 (0.00)	0 (0.00)	0 (0.00)
September	80	0 (0.00)	8 (10.00)	0 (0.00)	0 (0.00)	0 (0.00)	8 (10.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
October	80	0 (0.00)	27 (33.75)	1 (1.25)	1 (1.25)	0 (0.00)	27 (33.75)	1 (1.25)	1 (1.25)	0 (0.00)	0 (0.00)
November	80	0 (0.00)	16 (20.00)	0 (0.00)	0 (0.00)	0 (0.00)	16 (20.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
December	80	0 (0.00)	17 (21.25)	6 (7.50)	2 (2.50)	0 (0.00)	16 (20.00)	6 (7.50)	1 (1.25)	0 (0.00)	1 (1.25)
Total (%)	**960**	**3 (0.31)**	**238 (24.79)**	**59 (6.15)**	**41 (4.27)**	**1 (0.10)**	**227 (23.65)**	**59 (6.15)**	**32 (3.33)**	**2 (0.21)**	**9 (0.94)**
p*		**0.107**	**0.000**	**0.000**	**0.000**	**0.443**	**0.000**	**0.000**	**0.000**	**0.024**	**0.026**

RLB: Reverse line blotting

species that is mostly reported in forest areas in cold and high-altitude northern regions and is seen on sea coasts up to 800-1.000 meters altitude. Therefore, it is more common in Kars compared to the mentioned provinces (27). The altitude of the Kars Region is as high as 1.800 meters and the higher rate of *D. marginatus* species detected is in line with the literature. Also, in a study conducted on tick infestations in cattle in the Kars Region (28), *D. marginatus* species was found to be widespread, indicating that the prevalence of *D. marginatus* was higher in both cattle and sheep in this region. Tick species in the *Haemaphysalis* genus are found in Turkey where vegetation is rich, with large forest areas, a soft continental climate, and a high altitude (29). Our findings are in line with other studies. *Rhipicephalus bursa* was seen in places with a Mediterranean climate, covered with maquis vegetation, not too hot in summer, short and moderate winters, and rugged terrain (30). In the present study, *R. bursa* (0.53%) and *H. marginatum* (0.11%) in sheep in the Kars Region were found to be less prevalent than those reported in other studies.

According to the collection foci/settlement, the highest rate of *D. marginatus* was 25.63% (242/944) in Kars City Center, whereas this species was not found in Kagızman. This can be explained by the fact that the center of Kars includes a high plateau and altitude. It was thought that the lower rate in Kagızman may be due to the lower altitude and location in the valley. It has been reported that the highest rate of *Hae. parva* in Digor (15.36%), *Hae. punctata* in Sarıkamıs (1.67%) *R. bursa* in Kagızman (44.44%) and *H. marginatum* in Akyaka (0.43%). These results support that the distribution of tick species may differ according to foci/settlement which is related to the distribution of vector ticks.

In the present study, the presence of *Babesia* and *Theileria* species in ticks collected from sheep was also investigated. In this study, as in other studies (5,6,31), no pathogenic species were found in the analysis of egg clusters or tick carcasses with the RLB method. This is due to the fact that there are no pathogenic species in the detected tick species.

Table 5. Distribution of tick infestation in sheep by months

Months	NSE	NSI	IR (%)	NTC	ID	ANT
January	80	1	1.25	1	1	0.01
February	80	1	1.25	1	1	0.01
March	80	0	0.0	0	0	0
April	80	26	32.5	239	9.2	0.33
May	80	13	16.25	55	4.23	0.16
June	80	4	5.0	4	1.25	0.05
July	80	0	0.0	0	0	0
August	80	21	26.25	55	2.62	0.26
September	80	11	13.75	71	6.45	0.14
October	80	36	45.0	199	5.53	0.45
November	80	35	43.75	225	6.43	0.44
December	80	20	25.0	94	4.7	0.25
Total	**960**	**168**	**17.5**	**944**	**5.62**	**0.98**

NSE: The number of sheep examined, NSI: The number of infested sheep, IR: Infestation rate, NTC: The number of ticks collected, ID: Infestation density, ANT: The average number of ticks

CONCLUSION

This study covers the entire Kars Region. The research was carried out on randomly selected sheep that did not show clinical signs, so *B. ovis,* which is a pathogenic species for sheep, and *R. bursa* species tick, which carries this infection, was found at a very low rate. It had been determined that *T. ovis* was the responsible species for theileriosis agent in sheep in the region. Species commonly detected in tick-infested sheep were *D. marginatus* and *Hae. parva*. The data obtained from the present study will help in determining the species and ecological characteristics of the ticks in the region, as well as in creating risk maps of these tick-borne diseases in the future, and determining prevention and control strategies. Additional studies are needed to detect the pathogens in vector ticks.

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*Ethics

Ethics Committee Approval: For this study, Kafkas University Animal Experiments from the Local Ethics Committee (letter dated: 15.12.2016 and numbered: 2016-131) approval has been received.

Informed Consent: In the study, with the permission of the animal owners, samples were collected and used in the study.

Peer-review: Externally and internally peer-reviewed.

*Authorship Contributions

Concept: N.A., Z.V., M.Ö.A., Design: N.A., Z.V., M.Ö.A., Data Collection or Processing: N.A., Z.V., M.Ö.A., Analysis or Interpretation: N.A., Z.V., M.Ö.A., Literature Search: N.A., Z.V., M.Ö.A., Writing:. N.A., Z.V., M.Ö.A.

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