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Identification of *Babesia* and *Theileria* species in small ruminants in Central Anatolia (Turkey) via reverse line blotting

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Abstract: This study was conducted between January 2006 and September 2008 to identify *Babesia* and *Theileria* species in sheep and goats in Central Anatolia via reverse line blotting (RLB). In total, 421 sheep and 152 goats were randomly selected from herds located in Kayseri, Yozgat, and Sivas provinces (all in central Turkey). The amplified hypervariable V4 region of the 18S rRNA gene of ovine piroplasm species, including *Theileria lestoquardi*, *T. ovis*, *Babesia ovis*, *B. motasi*, and *B. crassa*, was hybridized against species-specific probes. *Theileria ovis* and *B. ovis* were observed in the study animals. Among the 573 small ruminants included in the study, 182 (43.2%) of 421 sheep and 18 (11.8%) of 152 goats, (in total 200 [34.9%]) were catch-all positive based on RLB. Among the sheep, *T. ovis* was observed in 170 (40.4%), *B. ovis* in 5 (1.2%), and *B. ovis* + *T. ovis* in 7 (1.7%). *Theileria ovis*, *B. ovis*, and *B. ovis* + *T. ovis* were observed in 15 (9.9%), in 1 (0.7%), and in 2 (1.3%) of the examined goats, respectively. As a result, the molecular prevalence of *B. ovis* was as follows: 2.9% in sheep (12/421), 2% in goats (3/152), and 2.6% in the total study population (15/573), versus 42% in sheep (177/421), 11.2% in goats (17/152), and 33.9% in the total population (194/573) for *T. ovis*.

Key words: *Theileria* sp., *Babesia* sp., small ruminants, RLB, Central Anatolia

Orta Anadolu'da küçük ruminantlarda *Babesia* ve *Theileria* türlerinin reverse line blotting ile saptanması

Özet: Bu çalışma ile Ocak 2006-Eylül 2008 tarihleri arasında Orta Anadolu'da koyun ve keçilerde *Babesia* ve *Theileri*a türlerinin reverse line blotting ile saptanması amaçlanmıştır. Türkiye'nin merkezindeki Kayseri, Sivas ve Yozgat bölgelerindeki çiftliklerden rastgele olarak 421 koyun ve 152 keçi seçilmiştir. *Theileria lestoquardi*, *T. ovis*, *Babesia ovis*, *B. motasi* ve *B. crassa*'yı kapsayan küçük ruminant piroplasm türlerinin 18S rRNA geninin değişken V4 bölgesi tür spesifik problarla hibridize edilmiştir. Çalışmada *T. ovis* ve *B. ovis* türleri tespit edilmiştir. Dörtyüz yirmibir koyunun 182'si (% 43,2), 152 keçinin 18'i (% 11,8) ve toplamda 573 küçük ruminantın 200'ü (% 34,9) catch-all pozitif bulunmuştur. İncelenen koyunlardan 170'i (% 40,4) *T. ovis*, 5'i (% 1,2) *B. ovis* ve 7'si (% 1,7) *B. ovis* + *T. ovis* ile enfekte saptanmıştır. İncelenen keçilerin ise 15'i (% 9,9) *T. ovis*, 1'i (% 0,7) *B. ovis* ve 2'si (% 1,3) *B. ovis* + *T. ovis* ile enfekte bulunmuştur. Sonuç olarak; *B. ovis*'in moleküler prevalansı koyunlarda % 2,9 (12/421), keçilerde % 2 (3/152) ve toplamda % 2,6 (15/573); *T. ovis*'in moleküler prevalansı ise koyunlarda % 42 (177/421), keçilerde % 11,2 (17/152) ve toplamda % 33,9 (194/573) olarak tespit edilmiştir.

Anahtar sözcükler: *Theileria* sp., *Babesia* sp., küçük ruminant, RLB, Orta Anadolu

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Introduction

Piroplasmosis, caused by tick-borne hemoprotozoan parasites including *Theileria* spp. and *Babesia* spp., is a major problem in small ruminants. Theileriosis and babesiosis are parasitic diseases of economic importance and significantly affect the international trade of animals (1-4). *Theileria lestoquardi*, *T. ovis*, and *T. separata* are recognized as the species that cause ovine theileriosis (3), whereas ovine babesiosis is caused by *Babesia ovis*, *B. motasi*, and *B. crassa* (4,5).

Generally, the diagnosis of ovine and caprine piroplasmosis is based on morphological examination of blood smears and clinical symptoms. These methods are useful in acute cases, but are insufficient for carrier animals. However, as opposed to these conventional methods, molecular methods would allow direct, specific, and sensitive detection of parasites (6-8). PCR is the most commonly used molecular technique for detecting piroplasms, but at present it cannot be used to detect mixed infections. In order to overcome these limitations a reverse line blot (RLB) was developed to detect all the piroplasm species that infect sheep and goat populations. RLB has been proven to be a very valuable tool for identifying undocumented sequences and diversity (5,8-10). The small subunit ribosomal RNA gene (18S rRNA gene) has been successfully used to identify and classify several previously unknown *Theileria* and *Babesia* parasites (11-14).

It was reported that the prevalence of babesiosis in Central Anatolia ranged between 17.70% and 27.35% in sheep (15,16), and between 6.38% and 12.12% in goats (15,16) based on microscopic evaluation, versus between 70.9% and 91.02% in sheep (17,18), and 86.48% in goats (17) based on serological evaluation, and 2.6% in sheep and goats (19) based on RLB. It was also reported the prevalence of theileriosis in Central Anatolia ranged between 17.96% and 18.40% in sheep (15,20), and between 8.0% and 9.09% in goats (15,20) based on microscopic evaluation, versus 92.3% in sheep and goats (19) based on RLB.

The aim of the present study was to determine the molecular prevalence of subclinical piroplasmosis, and its reservoir potential for vector ticks in clinically healthy small ruminants in Central Anatolia (Turkey) using the RLB technique.

Materials and methods

Biological samples

The study was carried out between January 2006 and September 2008 in Kayseri, Yozgat, and Sivas provinces (central Turkey) (Figure). In all, 421 sheep and 152 goats were randomly selected from herds located in Kayseri, Yozgat, and Sivas. Blood samples were collected into tubes containing EDTA from 573 clinically healthy small ruminants and transported to the laboratory in a cold pack. They were stored at –20 °C until analysis. Data were analyzed and recorded.

DNA extraction and PCR

DNA extraction was carried out using Qiagen DNA extraction kits, according to the manufacturer's instructions. For amplification of *Theileria* spp. and *Babesia* spp. the usual primers ($RLBF_2$, 5`GAC ACA

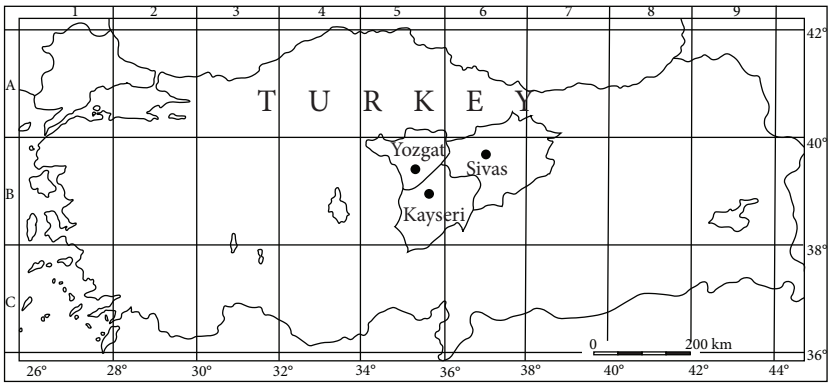

Figure. The locations surveyed in the current study.

GGG AGG TAG TGA CAA G`3 and $RLBR_2$, 5`TCT TCG ATC CCC TAA CTT TC`3) were used to amplify 460-520-bp fragments of the 18S ssu rRNA gene. PCR was performed in a thermocycler in a total reaction volume of 50 µL that contained 1 × PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 100 µM of dUTP, 1.25 U of Taq DNA polymerase (Fermentas), 1.25 U of Uracil DNA glycosylase (Fermentas), 50 pmol of each primer, and 5 µL of template DNA. The reaction was repeated for 40 cycles and PCR products were visualized via UV transillumination in 1.5% agarose gel, following electrophoresis and staining with ethidium bromide (9,21). *Babesia ovis* (Konya), *B. crassa* (Iran), *T. ovis* (Kayseri), and *T. lestoquardi* (Borstel, Germany) were used as positive controls. Sheep and goat DNA were used as the negative control.

RLB assay

The hypervariable V4 region of the 18S rRNA gene of the *Theileria* spp. and *Babesia* spp. was amplified using RLB-F_2 and RLB-R_2 primers for RLB assay. The oligonucleotide probes containing N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropyl phoshoramidite[TFA])-C_6 aminolinker were synthesized by Eurofins MWG Operon (Germany). The preparation, hybridization, and stripping of the RLB membrane were performed according to Georges et al. (9) and Gubbels et al. (21). The probes used in the present study are shown in Table 1.

Statistical analysis

Pearson's chi-square and Fisher's exact tests were used to statistically analyze the prevalence of *Theileria* and *Babesia* agents detected with RLB. Statistical calculations were performed using SPSS v.13.0 software.

Results

Among the 573 small ruminants, 182 (43.2%) of the 421 sheep and 18 (11.8%) of the 152 goats (in total: 200 [34.9%] of the 573 ruminants) were catch-all positive based on RLB. Among the sheep, *T. ovis* was detected in 170 (40.4%), *B. ovis* in 5 (1.2%), and *B. ovis* + *T. ovis* in 7 (1.7%). Among the goats, *T. ovis*, *B. ovis*, and *B. ovis* + *T. ovis* were detected in 15 (9.9%), in 1 (0.7%), and in 2 (1.3%) of the goats, respectively. As a result, the molecular prevalence of *B. ovis* was 2.9% in the sheep (12/421), 2% in the goats (3/152), and 2.6% in the total study population (15/573), versus 42% in the sheep (177/421), 11.2% in the goats (17/152), and 33.9% (194/573) in the total study population for *T. ovis*. These results are summarized in Table 2 and a statistically significant difference in the distribution of catch-all positivity was observed between the sheep and goats ($P < 0.001$). There was no statistically significant difference in the distribution of *T. ovis*, *B. ovis*, or *T. ovis* + *B. ovis* (mix) positivity ($P > 0.05$). A statistically significant difference in the distribution of *T. ovis* infection between the sheep and goats was

Table 1. Sequence of oligonucleotide probes hybridized onto the membrane.

Probes	**Sequence** (5'-3')	**Reference**
Catchall	TAATGGTTAATAGGA(AG)C(AG)GTTG	21
Theileria spp.	TGATGGGAATTTAAACC(CT)CTTCCA	5
Babesia spp.	CCT(GT)GGTAATGGTTAATAGGAA	8
T . ovis	TTTTGCTCCTTTACGAGTCTTTGC	5
B. ovis	GCGCGCGGCCTTTGCGTTACT	5
B. motasi	ATTGGAGTATTGCGCTTGCTTTTT	5
B. crassa	TTATGGCCCGTTGGCTTAT	8

* T: thymine; A: adenine; C: cytosine; G: guanine

observed (P < 0.001), but not in the distribution of *B. ovis* (P > 0.05) (Table 2).

The present study was carried out in Kayseri, Sivas, and Yozgat (central Turkey). The results are summarized by location in Table 3. The highest rate of *B. ovis* positivity was observed in the animals from Kayseri (1.4%), whereas positivity was not observed in any of the animals from the other districts. The highest rate of *T. ovis* positivity was observed in the animals from Yozgat (40%) and the lowest positivity rate was observed in the animals from Sivas (17.6%). In terms of *T. ovis* + *B. ovis* (mix) infection, the highest positivity was observed in the animals from Kayseri (2%), whereas positivity was not observed in any of the animals from the other districts.

Discussion

Blood smears and clinical symptoms are useful in acute cases of piroplasmosis, but are insufficient in subclinical cases. As opposed to these conventional methods, molecular methods would allow direct, specific, and sensitive detection of parasites (6-8). PCR is the most commonly used molecular

Table 2. Molecular prevalence of piroplasmosis by RLB according to the animal species in Central Anatolia.

Species	n	Catch-all (+)		*T. ovis*		*B. ovis*		*B. ovis* + *T. ovis*		Total			
										T. ovis		*B. ovis*	
		n	%	n	%	n	%	n	%	n	%	n	%
Sheep	421	182	43.2	170	40.4	5	1.2	7	1.7	177	42	12	2.9
Goat	152	18	11.8	15	9.9	1	0.7	2	1.3	17	11.2	3	2
Total	573	200	34.9	185	32.3	6	1.1	9	1.6	194	33.9	15	2.6
		χ^2 = 48.426 P < 0.001		*P = 0.140		*P = 0.436		*P = 0.189		χ^2 = 47.489 P < 0.001		*P = 0.769	

* Fisher's exact test

Table 3. Molecular prevalence of piroplasmosis by RLB according to the research centers in Central Anatolia.

Location	Animal	n	Catch-all		*B. ovis*		*T. ovis*		*B. ovis* + *T. ovis*	
			n	%	n	%	n	%	n	%
Kayseri	Sheep	343	155	45	5	1.5	143	42	7	2
	Goat	100	12	12	1	1	9	9	2	2
	Total	443	167	37.7	6	1.4	152	34.3	9	2
Yozgat	Sheep	45	18	40	-	-	18	40	-	-
	Goat	-	-	-	-	-	-	-	-	-
	Total	45	18	40	-	-	18	40	-	-
Sivas	Sheep	33	9	27.3	-	-	9	27.3	-	-
	Goat	52	6	11.5	-	-	6	11.5	-	-
	Total	85	15	17.6	-	-	15	17.6	-	-

technique, but is not capable of detecting mixed infections. Therefore, a more sensitive molecular technique, known as RLB, was developed to detect species-specific infections and mixed infections (5,8-10).

The RLB assay used in the present study is a molecular genetic diagnostic system that enables direct detection of small ruminant piroplasm species, based on the recognition of specific gene regions by oligonucleotide probes. A prerequisite for the development of such an assay is the presence of molecular marker sequences within the hypervariable V4 region of 18S rRNA genes that identify different piroplasm species (8).

The prevalence of *Babesia* and *Theileria* species in sheep in Spain based on RLB was reported to be 46.8% for *Theileria* sp. OT1, 46.6% for *Theileria* sp. OT3, 18% for *T. ovis*, 2.5% for *B. ovis*, and 2% for *B. motasi* (5). However, some phylogenetic studies on the detection of small ruminant piroplasms have been conducted (8,12,14,22,23). Along with the development of molecular techniques, some studies were carried out on sheep in Turkey that focused on the detection of blood parasites by PCR and RLB (11,19,20,22,24). A study about the prevalence of blood protozoan parasites by RLB in sheep in the Kayseri region was the first report in Turkey and it reported prevalence rates of 2.6% for *B. ovis* and 5.1% for *B. ovis* + *T. ovis* (mix) infection (19). Another study on the prevalence of blood parasites reported *B. ovis* prevalence rates of 4% in sheep and 3% in goats, versus 48% in sheep and 17% in goats for *T. ovis* (24). A study conducted in the East Anatolia region reported prevalence rates for *B. ovis*, *T. ovis*, *Theileria* sp. MK, and *Theileria* sp. OT3 of 5.43%, 34.56%, 1.3%, and 0.43%, respectively (22).

In the present study the prevalence of subclinical piroplasmosis in small ruminants was 34.9%. The prevalence of *T. ovis* ranged between 17.6% and 40% in Central Anatolia, whereas the prevalence of *B. ovis* was 1.4% in Kayseri province. The high molecular prevalence of *T. ovis* in clinically healthy small ruminants indicates that it is an important potential for vector ticks and acute clinical piroplasmosis infection, as previous studies based on microscopic evaluation (15,16,20) and serological evaluation (17,18) in Central Anatolia have reported.

In the present study we observed a significant difference in the prevalence of piroplasmic forms between the sheep and goats, whereas the distribution of the parasite species according to hosts did not differ. While there was a significant difference in the prevalence of *T. ovis* between the sheep and goats, the prevalence of *B. ovis* did not differ. It is thought that these differences may have been due to the epidemiology of the parasites, seasonal activity of vector ticks, infestation of the hosts with vector ticks, behavioral differences between the sheep and goats, and the available population of small ruminants in the study region.

In conclusion, the results of the present study indicate the presence of piroplasmosis in Central Anatolia. Additional molecular studies are essential for determining effective control strategies and developing vaccines against these protozoan parasites.

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