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Research Article

Parasitological and Molecular Prevalence of Bovine *Theileria* and *Babesia* Species in the Vicinity of Kayseri

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Abstract: This study was carried out to detect and compare the prevalence of bovine *Theileria* and *Babesia* species in the vicinity of Kayseri by microscopic examinations (ME) and reverse line blotting (RLB). A total of 337 cattle usually grazed on pasture in 13 different regions of Kayseri were sampled randomly. Blood samples were collected into tubes containing EDTA from jugular veins. Thin blood smears were prepared from ear capillaries. On microscopic examination of smears, 51 (15.1%) were positive for piroplasms. In the RLB assay, 61 (18.1%) were positive for *T. annulata* and 3 (0.9%) for *T. buffeli/orientalis*. Two (0.6%) of the animals were infected with *B. bigemina* and also had a concurrent infection of *T. annulata*. No animals were positive for *B. bovis* or *B. divergens*. The differences between ME and RLB results were statistically significant ($P < 0.05$).

Key Words: *Theileria*, *Babesia*, cattle, RLB, blood smears

Kayseri Civarında Sığırlarda *Theileria* ve *Babesia* Türlerinin Parazitik ve Moleküler Prevalansı

Özet: Bu çalışma, Kayseri civarında sığırlarda görülen *Theileria* ve *Babesia* türlerinin mikroskobik muayene ve reverse line blotting (RLB) yöntemleri ile araştırılması amacıyla yapılmıştır. Kayseri'nin 13 farklı bölgesinden rastgele seçilen, meraya çıkan 337 sığırdan örnek alınmıştır. Kan örnekleri jugular venden EDTA'lı tüplere alınmıştır. Kulak uçlarından ince yayma frotiler yapılmıştır. Frotilerin mikroskobik muayenesinde 51 (% 15,1) örnek *Theileria* spp. ve *Babesia* spp. piroplasmları yönünden pozitif bulunmuştur. Reverse line blotting testi ile incelenen 337 örneğin 61 (% 18,1)'i *T. annulata*, 3 (% 0,9)'ü *T. buffeli/orientalis* yönünden pozitif bulunmuştur. İncelenen 2 (% 0,6) örnekte *B. bigemina* ve *T. annulata* miks olarak tespit edilmiştir. Örneklerde *B. bovis* ve *B. divergens* saptanamamıştır. Mikroskobik muayene ve RLB sonuçları arasındaki farklılık istatistiksel olarak anlamlı bulunmuştur ($P < 0,05$).

Anahtar Sözcükler: *Theileria*, *Babesia*, sığır, RLB, kan frotisi

Introduction

Tick-transmitted diseases such as babesiosis and theileriosis are economically important globally (1). *Theileria annulata* is the causative agent of tropical theileriosis. This disease is one of the most fatal types of theileriosis in Europe, North Africa and Asia as well as in Turkey (2-7). *Theileria buffeli/orientalis,* the agent of benign theileriosis, was also detected in Turkey (8).

Bovine babesiosis, caused by the tick-transmitted protozoan *Babesia bigemina, B. bovis* and *B. divergens*, is considered one of the most frequent and important tick-borne diseases of cattle worldwide (9). *Babesia bigemina, B. bovis, B. divergens* and *B. major* were detected in cattle in Turkey (10-13). The main vectors of *Theileria* and *Babesia* species such as *Hyalomma* spp., *Rhipicephalus* spp., *Boophilus* spp. and *Haemaphysalis* spp. have all been observed in Turkey (11,14).

If animals recover from infection, a long-lasting carrier status occurs in which low numbers of erythrocytes remain infected with *Theileria* and *Babesia* piroplasms. These carrier animals have an important role in the transmission of the infection by ticks (15,16).

Laboratory diagnosis of the disease is usually based on the light microscopic detection of the piroplasms in thin blood smears and on the presence of macroschizonts of *Theileria* in Giemsa-stained lymph node biopsy smears (17). However, microscopic detection of piroplasms in carrier animals is difficult because low numbers of

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parasites lead to a relatively high rate of false negative diagnosis. It is also difficult to differentiate species of parasites on the basis of morphology. This makes the diagnosis problematic in mixed infections. Mixed infections of tick-borne haemoparasites frequently occur; hence a multiple detection test such as reverse line blot hybridisation (RLB) is a valuable tool in the simultaneous detection of such infections (18). The RLB technique has been used in the simultaneous detection of *Theileria* and *Babesia* species in bovine blood (19).

In this study, we investigated the prevalence of *Theileria* and *Babesia* species in the carrier cattle in Kayseri, Turkey, by microscopic examination (ME) and RLB.

Materials and Methods

Animals and samples: A total of 337 cattle which usually grazed on pasture in 13 different regions of Kayseri were sampled randomly between September 2004- November 2005. Blood samples were collected from the jugular vein into tubes containing EDTA. Thin blood smears were prepared from ear capillaries.

Microscopic examinations (ME): The thin blood smears prepared from ear capillaries were fixed in methanol for 5 min and stained with 5% Giemsa solution for 30 min and the presence of *Theileria* and *Babesia* piroplasms was assessed microscopically. At least 50 microscopical areas were carefully examined for *Theileria* spp. and *Babesia* spp. piroplasms under the oil immersion lens. The presence of even a single piroplasm was considered positive.

DNA isolation and polymerase chain reaction (PCR) for the amplification of the 18S rRNA gene of *Theileria/Babesia*: DNA was isolated from 200 µl of fresh blood as described by Gubbels et al. (19). Blood samples were washed 3 times with 500 µl of lysis mixture (0.22% NaCl, 1 mM EDTA, 0.015% saponin) by centrifugation at 1000 x *g* for 5 min, resuspended in 100 µl of PCR mixture (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 0.05% Tween 20, 100 µg of proteinase K/ml), and incubated overnight at 56 ºC. Prior to PCR, samples were heated for 10 min at 100 ºC and centrifuged at maximum speed for 2 min. DNA samples were stored in -20 ºC until used in PCR.

The PCR for *Theileria* and *Babesia* was conducted as described by Gubbels et al. (19). For amplification of the *Theileria* and *Babesia* V4 region of the 18S rRNA gene, the forward primer used was RLB-F (5´-GAGGTAGTGACAAGAAATAACAATAA-3´) and the reverse primer was RLB-R (biotin-5´-TCTTCGATCCCCTAACTTTC-3´). Reaction conditions in a 50 µl volume were 1X PCR buffer (Fermentas, Lithuania), 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 200 µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP) (Fermentas, Lithuania), 100 mM (dTTP, dUTP) (Fermentas, Lithuania), 50 pmol of each primer (MWG Biotech, Germany), 1.25 U Taq polymerase (Fermentas, Lithuania), 1.25 U Uracyl DNA Glycosylase (Sigma-Aldrich, Germany) and 5 µl of DNA samples. The reactions were performed in an automated DNA thermal cycler (Thermo-Hybaid, Middlesex, UK) as described by Gubbels et al. (19).

Reverse line blotting: The procedure of RLB is described in detail in Gubbels et al. (19). Briefly, a Biodyne C blotting membrane (Pall, NY, USA) was activated at room temperature by incubation in 16% EDAC (Sigma-Aldrich, Germany), washed in distilled water and placed in a miniblotter (MN45, Immunetics, Cambridge, MA, USA). The specific oligonucleotide

Table 1. The 5´-3´sequence of oligonucleotide probes hybridised onto membrane.

Oligonucleotide probe	Sequence	References
Catch-all	TAATGGTTAATAGGA(AG)C(AG)GTTG	Gubbels et al. (1999)
Theileria annulata	CCTCTGGGGTCTGTGCA	Georges et al. (2001)
Theileria buffeli/orientalis	GGCTTATTTCGG(AT)TTGATTTT	Gubbels et al. (1999)
Babesia bovis	CAGGTTTCGCCTGTATAATTGAG	Georges et al. (2001)
Babesia bigemina	CGTTTTTTCCCTTTTGTTGG	Gubbels et al. (1999)
Babesia divergens	GTTAATATTGACTAATGTCGAG	Gubbels et al. (1999)

probes shown in Table 1 contained an N-terminal (TFA)-C6 aminolinker (MWG Biotech, Germany) and were diluted to give 100-400 pmol/150 µl in 500 mM NaHCO3 (pH 8.4). The dilution was inserted into the miniblotter slots and during an incubation of 1 min linking of oligonucleotides to the membrane occurred. After aspiration of solutions the membrane was incubated in 100 mM NaOH for 10 min, washed at 60 ºC for 5 min and then at 42 ºC for 5 min in 2X SSPE, 0.1% SDS. Subsequently, the membrane was placed perpendicular to its previous orientation in the miniblotter. Forty microlitres of PCR products were diluted with 2X SSPE, 0.1% SDS to a final volume of 150 µl heated to 95 ºC for 5 min and then cooled on ice. The denatured samples were inserted into the slots of the miniblotter for 60 min at 42 ºC, then aspirated and the membrane washed at 42 ºC for 10 min in 2X SSPE, 0.1% SDS. Subsequently, the membrane was treated at 42 ºC for 30 min with peroxidase labelled streptavidin diluted 1:4000 in 2X SSPE, 0.1% SDS, washed twice at 42 ºC for 10 min and twice at room temperature for 5 min in 2X SSPE, 0.1% SDS. Finally chemiluminescence detection was performed according to standard procedures (Amersham Biosciences, NJ, USA). After examination and recording the membranes they were stripped as described in Gubbels et al. (19) and reused.

Statistical analysis: A chi-squared test was used to evaluate the differences between different parameters (SPSS 10.0). $P < 0.05$ was considered statistically significant.

Results

Out of 337 smears examined microscopically, 51 (15.1%) were positive for *Theileria* or *Babesia* piroplasms. Of the 51 smears, one showed a mixed infection with *Theileria* spp. and *Babesia* spp.

In the RLB analysis, of the total of 337 animals studied, 61 (18.1%) were positive for *T. annulata*, 3 (0.9%) were positive for *T. buffeli/orientalis* and 2 (0.6%) samples reacted with 2 oligonucleotide probes (both *T. annulata* and *B. bigemina*). *Babesia bovis* and *B. divergens* were not found. PCR products were hybridised to the membrane and were shown to bind with specific oligonucleotide probes only (Figure). *Theileria annulata* was the predominant species detected. The prevalence of each of the haemoparasites at each location is given in Table 2. The highest prevalence was obtained in Yeşilhisar district with 55.2%. In 5 locations (Pınarbaşı, Bünyan, Develi, Özvatan and Erkilet) no *Theileria* and *Babesia* species were determined in ME and RLB. *Theileria buffeli/orientalis* was found in Sarız, Yahyalı and Felahiye districts for the first time. The prevalence of mixed infections was 1.5% and 4.7% in Yeşilhisar and Sarıoğlan, respectively. There were no observations of *Babesia* infections alone. *Babesia bigemina* was always found in combination with *T. annulata*.

The RLB technique for *Theileria* spp. and *Babesia* spp. was more sensitive for the detection of carrier animals than was ME (66 animals versus 51, respectively) ($P < 0.05$). Comparative results of ME and RLB are shown in Table 3.

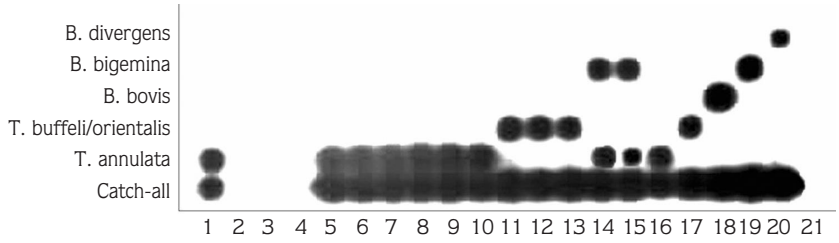

Figure. Reverse line blotting (RLB) of polymerase chain reaction (PCR) products obtained from field samples and controls. Lanes 1-15, field samples (lane 1, *T. annulata*; lanes 2-4, negative samples; lanes 5-10, *T. annulata*; lanes 11-13, *T. buffeli/orientalis*; lanes 14-15, *T. annulata* + *B. bigemina*); lanes 16-21, positive and negative controls (lane 16, *T. annulata*; lane 17, *T. buffeli/orientalis*; lane 18, *B. bovis*; lane 19, *B. bigemina*; lane 20, *B. divergens*; lane 21, negative control).

Table 2. Results of reverse line blotting showing the prevalence percentage of haemoparasites per location.

Location	*T. annulata*	*T. buffeli/orientalis*	*B. bigemina*	*B. bovis*	*B. divergens*	*T. annulata + B. bigemina*
Yeşilhisar (67)*	37 (55.2%)	0	0	0	0	1 (1.5%)
İncesu (34)	16 (47%)	0	0	0	0	0
Tomarza (23)	1 (4.3%)	0	0	0	0	0
Pınarbaşı (20)	0	0	0	0	0	0
Bünyan (30)	0	0	0	0	0	0
Develi (24)	0	0	0	0	0	0
Sarız (30)	0	1 (3.3%)	0	0	0	0
Özvatan (11)	0	0	0	0	0	0
Felahiye (9)	0	1 (11.1%)	0	0	0	0
Yahyalı (10)	4 (40%)	1 (10%)	0	0	0	0
Akkışla (19)	2 (10.5%)	0	0	0	0	0
Sarıoğlan (21)	0	0	0	0	0	1 (4.7%)
Erkilet (27)	0	0	0	0	0	0
Çiftlik (12)	1 (8.3%)	0	0	0	0	0
TOTAL (337)	61 (18.1%)	3 (0.9%)	0	0	0	2 (0.6%)

* Number of animals sampled

Table 3. Comparative results of microscopic examination (ME) and reverse line blotting (RLB).

	RLB (+)	RLB (-)
ME (+)	51	0
ME (-)	15	271
TOTAL	66	271

The haemoparasite prevalences in different age groups (0-1 year, >1-3 years, >3 years) were determined by RLB and are summarised in Table 4. There were no significant differences between age groups ($P > 0.05$).

Discussion

Tick-transmitted diseases such as babesiosis and theileriosis are economically important globally (1). Animals that have recovered from infection are carriers. These carrier animals have an important role in the transmission of the infection by ticks (15).

The RLB assay performed on blood samples was reproducible and proved very useful in detecting latent and mixed infections of haemoparasites. Previous reports (15,16) have clearly indicated the higher sensitivity and specificity of PCR-based techniques compared with ME in the diagnosis of *Theileria* and *Babesia* species. Another important advantage of the RLB assay is the identification of mixed infections (12,15,20).

In the present study, the sensitivity of the RLB assay was higher than that of ME (66 animals versus 51, respectively) ($P < 0.05$). Furthermore, 2 mixed infections with *T. annulata* and *B. bigemina* were determined using RLB.

The results of studies investigating the distribution of *Theileria* and *Babesia* species in Turkey based on ME and serological tests varied widely according to the different endemic regions (5,7,13,21-24). Recently, PCR-based assays were performed to diagnose the *Theileria* and *Babesia* species in Turkey. It has been reported that the prevalence of *T. annulata* was between 37.8% and 61.2% and of *T.buffeli/orientalis* was 7% by PCR in different parts of Turkey (25-27). İça (12) also reported that the prevalence of bovine *Babesia* species in Ankara was 10% by RLB.

Table 4. The haemoparasite prevalence in different age groups determined by reverse line blotting (RLB).

Age Groups	Number of Animals	RLB					*T. annulata* + *B. bigemina*
		Theileria annulata	*Theileria buffeli/orientalis*	*Babesia bigemina*	*Babesia bovis*	*Babesia divergens*	
0-1	114	15(13.2%)	0	0	0	0	0
>1-3	101	22(21.8%)	0	0	0	0	0
>3	122	24(19.7%)	3(2.5%)	0	0	0	2(1.6%)

Previous studies on the distribution of haemoparasites in Kayseri, where the present study was carried out, were based on ME of blood smears and serological tests (6,11). In this region, the prevalence of *T. annulata* with ME and indirect fluorescence antibody test (IFAT) has been reported as 59.29% and 60.66%, respectively (6). The seroprevalence of *B. bigemina, B. bovis* and *B. divergens* was 23.03%, 1.04% and 2.09%, respectively. Microscopic examinations revealed that only *B. bigemina* (6.8%) was present; on the other hand, *B. bovis* and *B. divergens* could not be detected (11). In the present study, the prevalence of *T. annulata* and *B. bigemina – T. annulata* mixed infections was 18.1% and 0.6% by RLB, respectively. The prevalence of *T. annulata* is not in agreement with a study carried out by İnci et al. (6) in Kayseri. In the present study, the prevalence of the disease has shown great differences among the towns in the region. The prevalence of the parasites was higher in Yeşilhisar, İncesu and Yahyalı districts than those determined for other locations in the region. We speculate that this might be due to differences in endemic features and the distribution of samples collected in the Kayseri region. However, *Theileria buffeli/orientalis* was determined for the first time in Sarız, Yahyalı and Felahiye districts by RLB. *Babesia major* probe was not used in this study.

The prevalence of haemoparasites was evaluated according to the age groups. The highest prevalences of *Theileria* spp. and *Babesia* spp. were seen in cattle over 3 years old. However, there were no significant differences between age groups ($P > 0.05$).

In conclusion, the RLB assay was more sensitive and specific for the diagnosis of *Theileria* and *Babesia* species than ME as demonstrated previously by others (12,19). Furthermore, the evidence demonstrating the existence of *T. buffeli/orientalis* in the region also suggests that other species of haemoparasites may be present in the region and this requires further investigation.

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