Molecular detection of *Theileria* and *Babesia* infections in cattle

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**Abstract**

This study was carried out to determine the presence and distribution of tick-borne haemoprotozoan parasites (*Theileria* and *Babesia*) in apparently healthy cattle in the East Black Sea Region of Turkey. A total of 389 blood samples were collected from the animals of various ages in six provinces in the region. Prevalence of infection was determined by reverse line blot (RLB) assay. The hypervariable V4 region of the 18S ribosomal RNA (rRNA) gene was amplified with a set of primers for members of the genera *Theileria* and *Babesia*. Amplified PCR products were hybridized onto a membrane to which generic- and species-specific oligonucleotide probes were covalently linked. RLB hybridization identified infection in 16.19% of the samples. Blood smears were also examined microscopically for *Theileria* and/or *Babesia* spp. and 5.14% were positive. All samples shown to be positive by microscopy also tested positive with RLB assay. Two *Theileria* (*T. annulata* and *T. buffeli/orientalis*) and three *Babesia* (*B. bigemina*, *B. major* and *Babesia* sp.) species or genotypes were identified in the region. *Babesia* sp. genotype shared 99% similarity with the previously reported sequences of *Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2 and *Babesia* sp. Kayseri 1. The most frequently found species was *T. buffeli/orientalis*, present in 11.56% of the samples. *T. annulata* was identified in five samples (1.28%). *Babesia* infections were less frequently detected: *B. bigemina* was found in three samples (0.77%), *B. major* in two samples (0.51%) and *Babesia* sp. in five samples (1.28%). A single animal infected with *T. buffeli/orientalis* was also infected with *B. bigemina*. © 2008 Elsevier B.V. All rights reserved.

**Keywords:** *Theileria*; *Babesia*; PCR; Reverse line blot; Cattle; Turkey

1. **Introduction**

*Theileria* and *Babesia* species are tick-borne haemoproteozoan parasites of vertebrates that have a major impact on livestock production, mainly cattle and small ruminants, in tropical and subtropical areas (Mehlhorn and Schein, 1984). *Theileria annulata* and *Theileria parva* cause lymphoproliferative disease with high morbidity and mortality, whereas *Theileria buffeli/orientalis* causes mild or asymptomatic disease in cattle. Bovine babesiosis is caused by *Babesia bigemina*, *Babesia bovis*, *Babesia divergens* and *Babesia major*. *Babesia* species have the potential for wide distribution wherever their tick vectors are encountered. Two species, *B. bovis* and *B. bigemina*, have a considerable impact on cattle health and productivity in tropical and subtropical countries (Uilenberg, 1995).

Techniques for detection of these haemoparasites have been developed separately for use in each species. The traditional method of identifying the agents in infected animals is by microscopic examination of blood smears stained with Giemsa. This technique is usually adequate for detection of acute infections, but not for detection of carrier animals, where parasitaemias may be

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low (Friedhoff and Bose, 1994). Serological methods are employed in diagnosing subclinical infections in epidemiological studies, but false-positive and false-negative results, due to cross-reactions or weakening of specific immune responses, are common (Passos et al., 1998). Therefore, a sensitive and highly specific method for the diagnosis of piroplasms is required. Recently, species-specific polymerase chain reaction (PCR) and PCR-based reverse line blot (RLB) hybridization methods have been developed for the detection and identification of Theileria and Babesia species (Figueroa et al., 1992; Calder et al., 1996; Gubbels et al., 1999; Georges et al., 2001; Aktas et al., 2005; García-Sanmartín et al., 2006; Altay et al., 2007a; M’ghirbi et al., 2008).

The main tick-borne haemoparasitic diseases occurring in cattle throughout Turkey are theileriosis and babesiosis. Bovine theileriosis has been investigated using molecular techniques, and the presence of T. annulata and T. buffeli/orientalis has been reported in the some part of the country (Aktas et al., 2002, 2006; Dumanli et al., 2005). B. bovis, B. bigemina and B. divergens have been detected by microscopy and serological tests (Aktas et al., 2001). However, these methods are less sensitive and specific in the detection of carrier animals and do not generally distinguish between current infections and previous exposures. Identification of carrier animals is important for the assessment of infection risk. They serve as reservoirs of infection for ticks and cause natural transmission of the disease (Calder et al., 1996). PCR-based techniques provide an alternative method for the direct detection of piroplasms in carrier animals. In the present study, a molecular survey of Theileria and Babesia species, based on PCR amplification and RLB hybridization, was conducted in cattle in the East Black Sea Region of Turkey. The results of RLB were compared to those of examination of thin blood smears.

2. Materials and methods

2.1. Study area and collection of samples

The study was conducted on cattle in the provinces of Tokat, Amasya, Gumushane, Giresun, Trabzon and Rize located in the East Black Sea Region of Turkey (Fig. 1). This area has varying weather conditions. Unlike the dry and hot East and Central Anatolia regions, the East Black Sea region covers two different climatic zones: an Atlantic climate in the coastal region with frequent rainfall and mild temperatures. The main tick species here are Ixodes ricinus, Haemaphysalis punctata, Haemaphysalis sulcata, Dermacentor marginatus, Rhipicephalus bursa and a Continental Mediterranean climate in the interior regions, with warmer summers and colder winters. The main tick species here are Hyalomma marginatum marginatum, Boophilus annulatus, Rhipicephalus spp.

Blood samples from 389 cattle randomly selected from 74 farms, also randomly selected, were taken in EDTA containing tubes. A thin blood smear from each sample was prepared and numbered in the field by the same person. The blood samples were stored at −20 °C until DNA extraction. The age of animals ranged between 1 and 7 years, and all were clinically healthy.

2.2. Microscopic examination

In the laboratory, the blood smears were fixed in methanol for 5 min and stained for 30 min in Giemsa

![Fig. 1. Map of the Turkish provinces, showing the locations surveyed in the current study.](image-url)
stain diluted with 5% buffer. Slides were examined for intra-erythrocytic forms of *Theileria* and *Babesia* spp. piroplasms at 100× objective magnification. Approximately 20 000 erythrocytes per slide were examined for the calculation of percentage of infected erythrocytes. The smears were recorded as negative for piroplasms if no parasites were detected in 50 oil-immersion fields.

### 2.3. DNA extraction

DNA extraction was performed as described by d’Oliveira et al. (1995). Briefly, 125 μl of blood was added to 250 μl of lysis solution (0.32 M sucrose, 0.01 M Tris, 0.005 M MgCl₂, 1% Triton X-100, pH 7.5). The mixture was centrifuged at 11 600 g for 1 min. The pellet was washed three times by centrifugation with 250 μl lysis buffer. The supernatants were discarded, and the final pellets were resuspended in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8), 0.1% Triton X-100, pH 8.3). Proteinase K (50 μg/ml) was added to the pellet suspension, and the mixture was incubated at 56 °C for 1 h. Finally, the samples were heated at 100 °C for 10 min.

### 2.4. 18S rRNA gene amplification and RLB hybridization

For the amplification of *Theileria* and *Babesia* species, one set of primers was used to amplify an approximately 390–430 bp fragment of the hypervariable V4 region of the 18S rRNA gene. The forward [RLB-F2 (5’-GACACAGGGAGTGACACAG-3’)] and the reverse [RLB-R2 (Biotin-5’-CTAAGAATTTCACCTCTGACAGT-3’)] primers were as described by Georges et al. (2001). The PCR volume and reaction conditions applied were similar to those described by Altay et al. (2007a). The primers and oligonucleotide probes (catchall *Theileria/Babesia, Theileria* spp., *T. buffeliorientalis, T. annulata, Babesia* spp., *B. bigemina, B. bovis, B. divergens, B. major*), containing an N-trifluoroaceticidohexyl-cyanoethy, N,N-diisopropyl phosphoramidite (TFA)-C6 aminolinker were synthesised by Isogen, Maarssen, Netherlands. Preparation, hybridization and stripping of RLB membrane were performed as described by Altay et al. (2007a).

### 2.5. Sequencing and phylogenetic analysis

To confirm RLB results, representative PCR products were chosen randomly for sequencing. Generated DNA fragment of approximately 390 and 430 bp of *Theileria* and *Babesia* were extracted from 1.5% agarose gel using a commercial kit (Wizard SV gel and PCR clean-up system, Promega, Madison, WI, USA). The purified PCR products were sequenced and submitted to GenBank. Each construct was sequenced at least three times and subjected to BLAST similarity searches. A phylogenetic tree was created from the sequences of the 18S rRNA genes of cattle *Babesia* species identified in this study and those available from GenBank, using the neighbour-joining method in MEGA version 3.1 (Kumar et al., 2004). The nucleotide sequences used in this study are available in GenBank under the following accession numbers: AY726556 for *Babesia* sp. Kashi 1; EF434786 for *Babesia* sp. Kayseri 1; AY726557 for *Babesia* sp. Kashi 2; AY596729 for *B. orientalis*; AY603400, AY603401 and AY081192 for *B. ovata*; DQ785311 and EF612434 for *B. bigemina*; AY648886 and AY603339 for *B. major*; AY789076 and AY572456 for *B. divergens*; AF316893 for *Plasmodium vivax*.

### 2.6. Statistical analysis

A χ²-squared test was used to evaluate the differences among various parameters. *P* < 0.05 was accepted to be statistically significant.

### 3. Results

#### 3.1. Specificity of the RLB assay

Primers RLB-F2 and RLB-R2 amplified bands of ~390 and ~430 bp corresponding to the hypervariable V4 region of the 18S rRNA gene of *Theileria* and *Babesia* species. PCR performed on uninfected control cattle DNA and a water control did not yield detectable product on agarose gel (data not shown). All PCR positive samples showed positive reactions with their corresponding specific probes. However, some samples gave positive signals to catchall and *Babesia* genus-specific probes, but did not show any signal to the species-specific probes tested (Fig. 2). This situation indicated the presence of a novel *Babesia* genotype.

#### 3.2. Sequencing and phylogenetic analysis

Two *Theileria* and three *Babesia* sequences were identified. The partial sequences of the 18S rRNA genes for *T. buffeliorientalis, B. bigemina, T. annulata, Babesia* sp. CS58 and *B. major* were deposited in the EMBL/GenBank databases under accession numbers from EU622821 to EU622825, respectively. *Theileria* sequences shared 99% identity with the recently
reported sequences for the 18S rRNA gene of *T. buffeli* (*orientalis*) (EU407247) and *T. annulata* (AY508473). From the three *Babesia* sequences, two of which were most closely related to the *B. bigemina* and *B. major*, and they shared 99% identity to recently reported sequences for *B. bigemina* (EF612434) and *B. major* (AY603339). The third sequence differed clearly from all known *Babesia* species infective for cattle, but shared 99% similarity with the unnamed *Babesia* isolates (*Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2 and *Babesia* sp. Kayseri 1) and *B. orientalis*.

The constructed phylogenetic tree revealed that *Babesia* species infective to cattle split into five monophyletic clades. *Babesia* sp. CS58 identified in this study demonstrated a close relationship and was included in the clade with unnamed *Babesia* isolates (*Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2 and *Babesia* sp. Kayseri 1) and *B. orientalis* (Fig. 3).

### 3.3. Prevalence of piroplasm infections in cattle

Thin blood smears revealed parasitaemia in infected cattle ranging from 0.01% to 0.1%. Piroplasms, detected inside erythrocytes, were pleomorphic and ring-or pear-shaped. Prevalence of piroplasms detected by microscopy and RLB from samples at locations in the Black Sea Region of Turkey are presented in Table 1. Of the 389 blood samples examined, microscopy revealed 20 (5.14%) positive for piroplasms, whereas 63 (16.19%) of DNA amplified products hybridized with the probes for catchall, and genera- and species-specific probes. These results demonstrated that RLB showed a significantly higher rate of detection of *Theileria* and *Babesia* infections (*P* < 0.01) than did microscopic examination. In the RLB and microscopy analysis, the highest number of positive samples and the highest carriers of piroplasms were obtained from the province of Tokat with 31.08% and 9.45%.

Prevalence of each piroplasm species identified in cattle is shown in Table 2. *Theileria* spp. prevalence was 12.85% (50/389), and prevalence of *Babesia* spp. was 2.57% (10/389). The most frequently found species was *T. buffeli* (*orientalis*), present in 11.56% of the samples. *T. annulata* was identified in five samples (1.28%). *Babesia* infections were less frequently detected: *Babesia* sp. were found in five samples (1.28%); *B. bigemina* in three samples (0.77%) and *B. major* in two samples (0.51%), one animal infected with *T. buffeli* (*orientalis*) was also infected with *B. bigemina* (Fig. 2, lane 8).

### 4. Discussion

Cattle with subclinical theileriosis and babesiosis become chronic carriers of the piroplasm and, hence, sources of infection for tick vectors. Therefore, latent infections are important in the epidemiology of the diseases. The diagnoses of piroplasm infections are based on clinical findings and microscopic examination of Giemsa-stained blood smears. However, this method is not sensitive enough or sufficiently specific to detect chronic carriers, particularly when mixed infections occur. Molecular techniques enable sensitive and specific detection of the parasites. The RLB method is an effective and practical tool, since it is able to detect extremely low parasitaemia levels and simultaneously identify *Theileria* and *Babesia* species using specific oligonucleotide probes (Gubbels et al., 1999; Altay
et al., 2007a). In this study, a molecular survey based on PCR amplification and RLB hybridization was performed for detection of bovine *Theileria* and *Babesia* species. By this method, 63 of 389 (16.19%) samples examined showed a positive signal to one or more species-specific probes as well as to the corresponding genus-specific probes. It was reported that the oligonucleotide probes used in this study reacted with their corresponding species and did not cross-react, with the exception of the *T. lestoquardi*-specific probe which cross-reacted with *T. annulata* (Nagore et al., 2004; Altay et al., 2007a). *T. lestoquardi* infects sheep and goats and has not been reported in Turkey (Altay et al., 2007a,b).

The survey identified different two *Theileria* (*T. annulata, T. buffelliorientalis*) and three *Babesia* (*B. bigemina, B. major, and a new Babesia genotype*) species and genotypes infecting cattle. The survey revealed that the most frequently found species was *T. buffelliorientalis*, present in 11.56% of the samples. We also found that *T. annulata* was present in the same area, but the prevalence of this species was lower (1.28%).

These results are not in agreement with previous studies carried out in Eastern Turkey (Dumanli et al., 2005; Aktas et al., 2006). The lower prevalence of *T. annulata* compared to *T. buffelliorientalis* was related to the geographic distribution of the tick vectors associated with these species.
B. bigemina presence of Babesia spp. were present in cattle in Turkey. The parasite has been reported previously (Dincer et al., 1991). However, B. major was detected for the first time in Turkey and therefore contributed greater insight into bovine piroplasm distribution and phylogenetic diversity. B. bovis and B. divergens were not detected in the cattle examined, although I. ricinus, B. annulatus and R. bursa, the vector ticks of these species, were identified among the tick collected (unpublished data). The absence of these species in RLB can possibly be explained by fluctuations in low level parasitaemia.

In conclusion, this study has revealed two Theileria (T. annulata and T. buffeli) and three Babesia (B. bigemina, B. major and Babesia sp.) infecting cattle. The RLB performed has revealed a novel bovine Babesia genotype. The assay provided more accurate data on prevalence of infection and allowed direct identification of species.

Acknowledgement

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References


Table 2
Distribution and frequency of bovine Theileria and Babesia species detected by RLB (n = 389).

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<td>63 (16.19%)</td>
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with each species. In the present study, Hyalomma anatolicum anatolicum, the main vector tick of T. annulata in Turkey, was not found on cattle, whereas Haemaphysalis spp. were the dominant tick species (unpublished data).

The prevalence of Babesia infection was lower than that of Theileria infection. In a previous serological study in the same region, serum antibodies against B. bigemina, B. bovis and B. divergens were detected in 62%, 44% and 75% of the samples, respectively (Dincer et al., 1991). In the present study, the lower prevalence of Babesia species detected among carrier cattle as compared to carriers of Theileria species could be explained by the fluctuations in parasitaemia that occur in the chronic phase of infection by Babesia species (Calder et al., 1996; Gubbels et al., 1999). This situation could also be explained by the low number of intraerythrocytic piroplasms circulating in the bloodstream of Babesia carriers (Homer et al., 2000).

By sequencing the 18S rRNA gene of the Babesia isolates identified in this study, a phylogenetic tree was created (Fig. 2). It showed that the Babesia sp. CS58 isolate was in the clade with the unidentified Babesia isolates from China (Kashi 1 and Kashi 2) and Turkey (Kayseri 1) as well as with B. orientalis. Sequence comparisons (357 nucleotides) of Babesia sp. CS58 revealed that the isolate differed clearly from all known Babesia species infective for cattle but shared 99% similarity with the Babesia sp. Kashi 1 and Kashi 2 isolated from H. anatolicum anatolicum (Luo et al., 2005) and with Babesia sp. Kayseri 1, isolated from H. marginatum marginatum (Ica et al., 2007). These results demonstrated that B. bigemina, B. major, and unnamed Babesia sp. were present in cattle in Turkey. The presence of B. bigemina was expected, since the parasite has been reported previously (Dincer et al., 1991). However, B. major was detected for the first time in Turkey and therefore contributed greater insight into bovine piroplasm distribution and phylogenetic diversity.


