**Survey of Theileria, Babesia and Anaplasma Infections of Cattle and Ticks from Sivas Province of Turkey**

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**Summary:** This study was carried out to investigate the presence and distribution of Theileria, Babesia and Anaplasma species in cattle and their ticks from Sivas province. A total of 314 EDTA-blood samples and 610 ticks were analysed. A part of 18S and 16S rRNA genes of Theileria/Babesia and Anaplasma species were amplified from the genomic DNA samples and tick pools by reverse line blot (RLB) and polymerase chain reaction (PCR). A total of 14 probes (two catchall, two genera and ten species-specific) were bound on a membrane and then the PCR products were tested by reverse line blot (RLB) assay. The partial sequences of the 18S and 16S rRNA genes of representative positive samples were determined. According to the results of the blood and tick samples analysed by RLB and sequencing, *T. buffeli* (GenBank accession number: KJ183080), *A. centrale* (KJ183082), *A. marginale* (KJ183083), *A. bovis* (KJ183084), one Babesia genotype (*Babesia* sp. Sivas, KJ183081) and one Anaplasma genotype (Anaplasma sp. Sivas, KJ210855) were detected. *Babesia* sp. Sivas were found to be 99% identical with *B. occultans*, *Babesia* sp. Sivas were found to be 99% identical with *Anaplasma* sp. Clone 7 and *A. bovis*, respectively. Overall prevalences of *Theileria* and *Anaplasma* infections in cattle were found to be 5.10% and 11.15% by RLB, respectively. This study is the first molecular survey on species of *Theileria, Babesia* and *Anaplasma* in cattle and ticks from Sivas.

**Key words:** Anaplasma, Babesia, cattle, Theileria, tick, Sivas.

Sivas Yöresinde Sığır ve Kenelerde Theileria, Babesia ve Anaplasma Enfeksiyonlarının Araştırılması

**Özet:** Bu çalışmada, Sivas yöresinde sığır ve kenelerde Theileria, Babesia ve Anaplasma türlerinin varılığı ve yaygınlığını araştırılmıştır. **Örgütlendiren** toplanan 314 EDTA’lı kan ve 610 kene örnekleri analiz edilmiştir. **Kan ve keneler**den elde edilen genomic DNA’lardan polimeraz zincir reaksiyonu (PZR) ile *Theileria, Babesia* ve *Anaplasma* türlerinin 18S ve 16S rRNA parsiyel gen dizileri amplifiye edildi. Bir membran üzerine toplam 14 probe (iki cathall/genel, iki soygenel ve otuz dört tür específica) bağlanarak PZR reaksiyonu ve PCR testi yapıldı. Pozitif örneklerin 18S ve 16S rRNA gen dizileri membrana birikip belirlendi. RLB ve sekans analizi ile Sivas yöresinde sığır ve kenelerde *T. buffeli* (KJ183080), *A. centrale* (KJ183082), *A. marginale* (KJ183083), *A. bovis* (KJ183084) ve bir Babesia genotipi (Anaplasma sp. Sivas, KJ210855) tespit edildi. Babesia sp. Sivas izolatının, *B. occultans*, Babesia sp. Sivas, *Babesia* sp. Sivas ve *Anaplasma* sp. Sivas, *Anaplasma* sp. Sivas izolatının ise *Anaplasma* sp. Clone 7 ile %99 ve *A. bovis* ile %98 benzerliğe sahip olduğu belirlendi. Sığırarda *Theileria* ve *Anaplasma* enfeksiyonlarının genel prevalansı, RLB ile sırasıyla %5.10 ve %11.15 olarak bulundu. Bu çalışma Sivas yöresinde sığırlardan ve kenelerde *Theileria, Babesia* ve *Anaplasma* türlerinin bulunması ilki için sınırlar belirlendi.

**Anahtar kelimeler:** Anaplasma, Babesia, kene, sığır, Sivas, Theileria

**Introduction**

*Theileria, Babesia* and *Anaplasma* species are transmitted by ixodid ticks and infect domestic and wild animals throughout the world. The diseases cause important economic losses in livestock industry. Some of the species of these genera have zoonotic potential and known as emerging infectious diseases (Inci et al., 2013; Inci et al., 2016; Uilenberg, 1995; Uilenberg, 2001).

Genetically and pathogenetically different six *Theileria* species have been found in cattle. *Theileria anulata* and *T. parva* are the most common pathogen *Theileria* species of cattle. These two species cause lympho-proliferative disease with high mortality. *T. sergenti/buffeli/orientalia group, T. mutans, T. velfera and T. taurtragi* are known as lower pathogenic or apathogenic species (Uilenberg, 1995). *Babesia bovis*, *B. bigemina, B. divergens* and *B. major* are causative bovine babesiosis. Bovine babesiosis is an important livestock problem in tropical and subtropical regions (Uilenberg, 1995). Bovine anaplasmosis is caused by *Anaplasma marginale, A. centrale, A. phagocytophilum* and *A. bovis* (Inokuma, 2007). *A. marginale* is the most important species among these.
species and causes clinical infections characterized with anemia and jaundice. *A. centrale* causes mild infections in cattle (Dumler, 2001). *A. phagocytophilum* (compiled from previously known as *Ehrlichia phagocytophila*, *E. equi* and human granulocytic ehrlichiosis agent) is a causative agent of tick-borne fever (TBFI) in cattle. TBFI characterized by high fever, depression, decreased milk production and reduced fertility (Pusterla et al., 1997; Woldehiwet and Scott, 1993). *B. bovis* (previously *E. bovis*) infections have been reported mainly in Asian and African countries and much is not known about its epidemiology (Dumler et al., 2001). The infection is known asymptomatic but the pathogen can cause fever, anorexia, debility, incoordination, anemia, pale mucous membranes, weight loss, and enlargement of lymph nodes, rarely abortion and death in cattle (Donatien and Lestoquard, 1936; Kaufmann, 1996).

Molecular diagnostic methods such as polymerase chain reaction (PCR) and PCR-based reverse line blotting (RLB) have become widely used as sensitive and specific tools for detection and discrimination of tick-borne parasites in both their vectors and hosts (Aktas et al., 2006; Aktas and Ozubek, 2015; Altay et al., 2004; Merdivenci, 1969). Randomly selected 610 tick specimens were divided into 53 pools and used for total DNA extraction. The blood and tick samples were stored at 20°C until to use in DNA extraction.

### Microscopic examination

Thin blood smears of all sampled animals were prepared with EDTA-blood samples during the field study. Having been returned to the laboratory, the blood smears were fixed in methanol for 5 min and stained with 4% Giemsa stain for 30 min. The stained blood smears were examined under the stereo microscope (Estrada-Pena et al., 2004; Merdivenci, 1969). Randomly selected 610 tick specimens were divided into 53 pools and used for total DNA extraction. The blood and tick samples were stored at -20°C until to use in DNA extraction.

### Total DNA extraction from blood samples and tick pools

Total DNA extraction from EDTA-blood samples were performed using DNA extraction kit according to the

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**Table 1.** The blood samples and tick species collected from cattle in Sivas

| Location | n   | Hyalomma marginatum | Hyalomma excavatum | Rhipicephalus bursa | Rhipicephalus turanicus | Haemaphysalis sulcata | Boophilus annulus | Dermacentor marginatus | Total |
|----------|-----|---------------------|--------------------|--------------------|------------------------|----------------------|-------------------|----------------------|-------|
| Sivas    | 55  | 148                 | 13                 | 32                 | 293                    | 3                    | 146               | 2                    | 637   |
| Kangal   | 55  | 6                   | 76                 |                    |                        |                      |                   |                      |       |
| Koyulhisar | 52  | 171                 | 43                 | 1                  |                        |                      |                   |                      | 215   |
| Şarkışla | 50  | 108                 | 5                  | 73                 |                        |                      |                   |                      | 186   |
| Yıldızeli | 51  | 19                  | 2                  |                    |                        |                      |                   |                      | 21    |
| Zara     | 51  | 10                  | 54                 |                    |                        |                      |                   |                      | 64    |
| Total (%)| 31  | 461                 | 58                 | 168                | 366                    | 3 (0.25)             | 146               | 10 (0.82)            | 1212  |
|          | 4   | (38.04)             | (4.78)             | (13.86)            | (30.20)                | (3.42)               | (3.77)            | (8.29)               |       |
The hypervariable V4 region of 18S rRNA gene of *Theileria, Babesia* species and V1 region of 16S rRNA gene of *Anaplasma* species were sequenced. The PCR products were purified from 1.6% agarose gel after electrophoresis with a commercial kit (Wizard SV gel and PCR clean-up system, Promega, Madison, WI, USA). The purified PCR products were sequenced by a commercial company (Iontek, Istanbul, Turkey).

The partial sequences of 18S rRNA gene for *T. buffeli* and *Babesia* sp. and 16S rRNA gene for *A. centrale*, *A. marginale*, *A. bovis*, and *Anaplasma* sp. were determined. After the sequences were subject to BLAST similarity searches they were deposited in the EMBL/GenBank databases (GenBank database; National Center for Biotechnology Information, National Institute of Health).

**Results**
Prevalence of the tick-borne haemoparasites detected with the microscopic examination and RLB results of cattle from Sivas are given in Table 3. of 314 blood samples, 5.10% (16/314) were found as positive by microscopic examination, whereas 16.24% (51/314) were found as positive by reverse line blotting (Table 3).

Table 3. Microscopic examination and reverse line blotting results of cattle investigated for tick-borne haemoparasites (n: 314)

| Tick species          | Microscopic examination (%) | Total infection (%) | Single infection (%) | A. bovis | A. centrale |
|-----------------------|-----------------------------|---------------------|---------------------|----------|------------|
| Theileria spp.        |                             |                     |                     |          |            |
| T. buffeli            | 5 (1.59)                    | 16 (5.10)           | 15 (4.78)           | 1 (0.32) | 0          |
| Anaplasma spp.        |                             |                     |                     |          |            |
| A. centrale           | 11 (3.50)                   | 35 (11.15)          | 30 (9.55)           |          |            |
| A. marginale          |                             |                     |                     | 0        | 2 (0.64)   |
| A. bovis              |                             |                     |                     | 0        | 2 (0.64)   |
| Total                 | 16 (5.10)                   | 51 (16.24)          | 45 (14.33)          | 1 (0.32) | 2 (0.64)   |

Anaplasma spp. amplified by PCR were used in RLB and hybridised onto the membrane with specific oligonucleotide probes. All the PCR positive samples gave positive signals with their complementary probes. The reverse line blot assay revealed that T. buffeli, Babesia sp., A. centrale, A. marginale, A. bovis and Anaplasma sp. existed in the cattle and their tick (Table 3 and Table 4). Representative samples were chosen and sequenced. BLAST similarity searches showed that sequence of T. buffeli was determined in 45 (14.33%) cattle, whereas mixed infections were observed in 3 cattle (0.96%). Mixed infection of T. buffeli-A. bovis and A. marginale-A. centrale were detected in one (0.32%) and in two (0.64%) cattle, respectively.

Table 4. Tick polls used in reverse line blotting and results of reverse line blotting

| Tick species          | ntp  | np | Th. +B. positive | A. centrale | A. marginale |
|-----------------------|------|----|------------------|-------------|--------------|
| Hyalomma marginatum   | 211  | 20 | 5                | 1           | -            |
| Rhipicephalus turanicus| 209  | 14 | -                | 7           | 1            |
| Rhipicephalus bursa   | 86   | 8  | -                | -           | -            |
| Boophilus annulatus   | 77   | 6  | -                | -           | -            |
| Hyalomma excavatum    | 22   | 3  | 1                | -           | -            |
| Dermacentor marginatus| 3    | 1  | -                | -           | -            |
| Haemaphysalis sulcata | 2    | 1  | -                | -           | -            |
| Total                 | 610  | 53 | 6                | 8           | 1            | 5            |

ntp: ticks number used in pools, np: tick pool numbers
Theileria, Babesia and Anaplasma species have morphological and biological differences as well as their pathogenicity. Comparative studies conducted at the molecular level in recent years have shown that there are significant genetic differences between species and there are differences even among the isolates of the same species (Altay et al., 2007; Ciloglu et al., 2018; Dumler et al., 2001; Duzlu et al., 2011).

Reverse line blotting, in addition to allowing the identification of new species or genotypes, has high specificity and sensitivity (Altay et al., 2008a; Gubbels et al., 1999; Nagore et al., 2004; Oura et al., 2004). In this study, using RLB method; the presence of T. buffeli, A. centrale, A. marginale and A. bovis was detected in cattle and ticks from Central Anatolian region of Turkey. In addition, two new isolates, one of which was in Babesia genus and the other in Anaplasma genus, were found in the region. There are molecular-based studies showing the presence of new tick-borne parasite isolates in Turkey (Altay et al., 2008a; Ica et al., 2007b). In this study, two catchall (Theileria + Babesia spp. and Anaplasma + Ehrlichia spp.) and two strains (Theileria spp. and Babesia spp.) and 10 species specific probes (T. annulata, T. buffeli, B. bigemina, B. bovis, B. divergens, B. major, A. centrale, A. marginale, A. bovis and A. phagocytophilum) were used in RLB (Table 3). Six Babesia spp. probe positive and 2 Anaplasma + Ehrlichia spp. probe samples did not give signals to the species-specific probes in RLB. The Babesia spp. Sivas (KJ183081) was found 99% to be similar to the DNA sequences of Babesia spp. Kashi (AY726557) and Babesia sp. Kayseri (EF434766). The sequence of Anaplasma + Ehrlichia genus probe positive sample was similar 99% to Anaplasma sp. Clone A7 (AY851664) and 98% to A. bovis (JN558822). The genotypes detected in the study were named Babesia sp. Sivas and Anaplasma sp. Sivas and deposited in GenBank with the accession numbers; KJ183081 and KJ210855, respectively.

During the survey, 87 (27.70%) of the 314 cattle were found to be infested with Ixodid ticks. A total of 1212 ticks were collected from the cattle. Seven different tick species were identified from the cattle. The most prevalent tick species was H. marginatum (38.04%) followed by R. turanicus (30.20%), R. bursa (13.86%). The other tick species detected from the cattle were Boophilus annulatus (12.05%), Hyalomma excavatum (4.78%), Dermacentor marginatus (0.82%) and Haemaphysalis sulcata (0.25%) (Table 1).

Discussion and Conclusion

Theileriosis, babesiosis and anaplasmosis are among the most important tick-borne diseases of domesticated animals. These infections, which are common in tropical and subtropical regions including Turkey, cause significant economic losses in animal husbandry (Dumler et al., 2001; Ici et al., 2013; Kocan et al., 2000; Uilenberg, 2001). Theileria, Babesia and Anaplasma species have morphological and biological differences as well as their pathogenicity. Comparative studies conducted at the molecular level in recent years have shown that there are significant genetic differences between species and there are differences even among the isolates of the same species (Altay et al., 2007; Ciloglu et al., 2018; Dumler et al., 2001; Duzlu et al., 2011).

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There are many molecular-based studies aimed at detecting the prevalence and prevalence of theileriosis, babesiosis, anaplasmosis in cattle and ixodid ticks from Turkey (Aktas et., 2011; Altay et al., 2008a; 2014; Ica et al., 2007a; 2007b; Yildirim et al., 2013). However, there is not any molecular-based research on tick-borne parasites in cattle in Sivas. This study is the first investigation of the parasites in cattle and their ticks from Sivas in the Central Anatolia Region of Turkey using RLB and DNA sequencing. In this study, 314 cattle were examined with RLB, the prevalence of T. annulata, T. buffeli, B. bigemina, B. bovis, B. divergens, B. major, A. centrale, A. marginale, A. bovis and A. phagocytophilum were determined as 5.10%, 6.65%, 4.14%, and 0.32%, respectively. The results showed that tick-borne diseases prevalence is lower than in the other parts of Turkey. The main reason for this is that there are climatic and geographical differences in the region located in the continental climate zone.

It is important to diagnose disease in vector ticks, to understand their epidemiology and to develop appropriate control strategies. T. lestoquardi in H. anatolicum (Kirvar et al., 1998), T. annulata in Hyalomma sp. (d’Oliveira et al., 1997), B. bigemina and B. bovis in B. microphilus (Oliveira-Sequeira et al., 2005), T. ovis and B. ovis in R. bursa (Aktas et al., 2006; Altay et al., 2008c) were detected with PCR and DNA sequencing. In the study, H. marginatum, H. excavatum, R. bursa, R. turanicus, Hae. sulcata, B. annula-
 tus and D. marginatus were collected from cattle (Table 4). As a result of RLB and sequence analysis of the ticks pools, A. centrale, A. marginale and Anaplasma spp., in R. turanicus pools, Babesia spp. and Anaplasma spp., H. marginatum, Babesia spp. in H. excavatum pools were identified. The detection of pathogens in ticks does not prove that they are natural vectors, but they provide valuable contribution to the understanding of the diseases epidemiology.

As a result, in this study; T. buffeli, A. centrale, A. marginale, A. bovis and 2 new species or genotype (Babesia sp. Sivas and Anaplasma sp. Sivas) were determined by using molecular methods in cattle and ticks. The use of RLB method in such studies was found to be important in terms of revealing new species or genotypes. Further studies are needed on the identification of vector, host and pathogenesis of these genotypes.

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