



Short communication

A survey of ixodid ticks feeding on cattle and prevalence of tick-borne pathogens in the Black Sea region of Turkey[☆]Munir Aktas^{a,*}, Kursat Altay^b, Sezayi Ozubek^a, Nazir Dumanli^a^a Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, 23119 Elazig, Turkey^b Department of Parasitology, Faculty of Veterinary Medicine, University of Cumhuriyet, Sivas, Turkey

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ABSTRACT

The study reports the frequency of infestation and the prevalence of tick-borne pathogens in feeding adult ticks detached from cattle in two climatic zones of the Black Sea region of Turkey. A total of 2160 adult ticks were collected during 2007–2008. Of these, 1062 were randomly selected, divided into 224 pools, and tested for the presence of bovine *Theileria*, *Babesia*, and *Anaplasma* species. Eleven tick species were recognized on cattle in the study. *Hyalomma marginatum* was widely distributed in the semi-arid bioclimatic zone, but few specimens were collected in the humid bioclimatic zone. The most prevalent tick species in the humid climatic zone was *Ixodes ricinus*. Infection rates were calculated as the maximum likelihood estimation with 95% confidence intervals (CI). Overall, 4% (CI 2.87–5.44) of 224 tick pools were found to be positive for the pathogens by Reverse line blot. Maximum likelihood estimation of the infection rate varied among tick species, ranging from 2.68% (CI 0.16–12.68) in *Haemaphysalis sulcata* to 10.49% (CI 4.07–23.66) in *Rhipicephalus bursa*. The most prevalent tick-borne pathogen was *Anaplasma phagocytophilum* at 6.78% (CI 3.41–12.18) followed by *A. centrale* (6.56%, CI 0.42–31.47), *Anaplasma/Ehrlichia* spp. (3.61%, CI 1.99–6.06), *Babesia* spp. (3.33%, CI 1.65–6.03), and *T. buffeli/orientalis* (2.71%, CI 0.73–7.18). Sequencing results indicated that *Babesia* spp. shared 99% to 100% similarity with the unnamed *Babesia* sp. Kashi 1 and 2, *Babesia* sp. Kayseri 1 and *Babesia* sp. CS58. *Anaplasma/Ehrlichia* spp. were 98% and 100% identical to *Ehrlichia canis* and *Ehrlichia* sp. Omatjenne strain, respectively.

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1. Introduction

Ixodid ticks are vectors of species of *Theileria*, *Babesia*, and *Anaplasma* that infect domestic livestock and wild animals in most regions of the world, causing diseases of veterinary, medical, and economic importance (de La Fuente et al., 2008). For the development and

implementation of control strategies, it is important to know the epidemiology of these pathogens in the target geographical region.

Although there have been a number of studies of the prevalence of tick-borne pathogens in vertebrate hosts and tick vectors in Turkey (Tonbak et al., 2006; Aktas et al., 2009, 2011), little information is available about the frequency of ixodid tick species and the prevalence of tick-borne diseases in most areas of the country. The aim of this study was to determine the frequency of tick infestation and the prevalence of *Theileria*, *Babesia*, and *Anaplasma* species in adult ticks detached from cattle in the Black Sea region of Turkey.

[☆] Note: Nucleotide sequence data reported in this paper are available in GenBank, EMBL 21 and DDBJ databases under accession numbers from JF923655 to JF923658.

* Corresponding author. Tel.: +90 424 237 0000; fax: +90 424 238 8173. E-mail address: maktas@firat.edu.tr (M. Aktas).

2. Materials and methods

The study was conducted in an area comprising the provinces of Giresun, Trabzon, Rize, Tokat, Amasya, and Gümüşhane in the Black Sea region of Turkey. This area covers two climatic zones: a typical humid climate in the coastal region with frequent rainfall and mild temperatures (Giresun, Trabzon, Rize) and a continental semi-arid cold bioclimatic zone in the interior (Tokat, Amasya, and Gümüşhane) with warmer and colder temperatures than the coastal region. Ixodid ticks were collected from 389 cattle, 173 from the coastal region and 216 from the semi-arid cold zone. Cattle were randomly selected from 74 farms (23 from humid zone, 51 from semi-arid cold zone) between May and September in 2007 and 2008. Cattle were kept under traditional communal grazing management and were either tethered or grazed on communal pastures during the day and tied up around homesteads at night. The manually removed ticks were placed in bottles with moistened cotton wool and placed into an incubator at 28 °C and relative humidity of 85% for 4 days prior to DNA extraction.

A total of 2160 ticks were collected from infested cattle. Of these, 1062 were randomly selected and analyzed for the presence of tick-borne pathogens (*Theileria*, *Babesia*, and *Anaplasma* species). The ticks were disinfected in 70% ethanol for 10 min, rinsed with sterilized distilled water, and dried on filter paper. They were divided into 224 pools comprising 2–14 tick specimens according to the source species and bioclimatic zones. The pooled ticks were stored in tubes and frozen at –80 °C for later DNA extraction. Detailed information on the material used in this study is presented in Table 1.

The frozen tick pools were crushed using sterile metal rods in liquid nitrogen in 1.5 ml Eppendorf tubes. Total DNA was extracted using the RTA (AbsoGene, Gebze, Turkey) DNA isolation kit following the manufacturer's recommendations. The PCR and reverse line blot (RLB) assays were applied, as described previously (Aktas et al., 2011). The biotinylated *Theileria/Babesia* and *Anaplasma/Ehrlichia* PCR products were hybridized with genus- and species-specific probes for bovine *Theileria/Babesia* (catchall – *Theileria/Babesia*, *Theileria* spp., *T. buffeli/orientalis*, *T. annulata*, *Babesia* spp., *B. bigemina*, *B. bovis*, *B. divergens* and *B. major*) and *Anaplasma* (catchall – *Anaplasma/Ehrlichia*, *A. marginale*, *A. centrale*, *A. bovis* and four variants of *A. phagocytophilum*) (Bekker et al., 2002; Matjila et al., 2008).

For sequencing, the PCR products found positive with catchall and genus-specific probes were purified from agarose gel using a commercial PCR purification kit (Wizard SV gel and PCR clean-up system Promega, Madison, WI, USA). The sequences were compared with those of reference strains from the NCBI database (<http://blast.ncbi.nlm.nih.gov/>).

Infection rates in tick pools were calculated using maximum likelihood estimation (MLE) methods with 95% confidence intervals (CI) for unequal pool sizes and expressed as MLE of infection rate per 100 ticks. Calculation of MLE for different pool sizes requires numerical iterations and computer implementation. We used Pooled-InfRate estimation software (version 4.0) as an Add-In to Microsoft Excel (Biggerstaff, 2009). In addition, a Pearson

chi-square test was used and *P* value of ≤ 0.05 was considered statistically significant.

3. Results

In the present study, 34% (133/389) of cattle examined for tick infestations were carrying at least one tick species. Of these, 54% (117/216) were inspected at semi-arid bioclimatic zone and 9% (16/173) at humid zone. A significantly higher proportion of the cattle infested with ixodid ticks were inspected in semi-arid bioclimatic zone than in humid zone ($P < 0.001$). The mean rate of infestation of cattle was 16.2, with the number of ticks per animal ranging from 0 to 123.

Occurrence and abundance of identified tick species, the number of collected ticks, the number of analyzed and pooled ticks, and infection rates for *Theileria/Babesia* and *Anaplasma/Ehrlichia* for each bioclimatic area are shown in Table 1. Of the 2160 ticks collected, 1644 (76%) were taken from the semi-arid cold zone with the remaining 516 (24%) from the humid zone. Eleven tick species were identified, and most of the infested cattle were infested with more than one species. Cattle in the semi-arid cold zone were infested with 8 species, whereas 5 species were identified in the humid zone (Table 1).

A total of 224 pools representing 1062 ticks belonging to 11 species were selected for RLB analysis using a panel of probes for bovine tick-borne pathogens. Of these 224 tick pools, 36 (16.07%) were found to be positive for *Theileria/Babesia* and *Anaplasma/Ehrlichia* pathogens and MLE of the infection rate was calculated as 4% (CI 2.87–5.44) per 100 ticks (Table 1). MLE of the infection rate varied among tick species, ranging from 2.10% (CI 0.56–5.58) in *Rhipicephalus bursa* to 6.53% (CI 2.47–13.74) in *Hyalomma excavatum* in semi-arid bioclimatic zone. Two tick species, *Rh. bursa* and *Ixodes ricinus*, were infected with the pathogens at 10.49% (CI 4.07–23.66) and 4.10% (CI 1.94–7.66) from humid bioclimatic zone, respectively. No pathogen was detected in *Hyalomma detritum*, *Rhipicephalus sanguineus*, *Haemaphysalis punctata*, or *Ixodes hexagonus*. The most prevalent tick-borne pathogen was *A. phagocytophilum* at 6.78% (CI 3.41–12.18) followed by *A. centrale* (6.56%, CI 0.42–31.47), *Anaplasma/Ehrlichia* spp. (3.61%, CI 1.99–6.06), *Babesia* spp. (3.33%, CI 1.65–6.03), and *T. buffeli/orientalis* (2.71%, CI 0.73–7.18). Most tick pools were infected with a single pathogen. However, two pools derived from *Rh. bursa* displayed mixed infections with *Anaplasma/Ehrlichia* spp. and *Babesia* spp. (Table 1). All *A. phagocytophilum*, *T. buffeli/orientalis*, and *A. centrale* positive samples also showed positive signals to the catchall and genus-specific probes. *Theileria* and *Babesia* species were significantly more prevalent in the semi-arid cold area ($P < 0.05$), while *Anaplasma/Ehrlichia* species showed similar prevalence in the two zones ($P > 0.05$).

Ten PCR products from six *Hy. marginatum*, two *Hy. excavatum*, one *Rh. bursa*, and one *Rh. turanicus* showed no reaction to the *Theileria* and *Babesia* species-specific probes present in RLB (*T. annulata*, *T. buffeli/orientalis*, *B. bigemina*, *B. bovis*, *B. divergens*, *B. major*) but did hybridize catchall – *Theileria/Babesia* and *Babesia* genus-specific probes. For detection at the species level, these

Table 1
Distribution of ixodid tick species collected from cattle and infection rates of tick-borne pathogens in two different climatic zones in Black Sea region of Turkey.

Climatic zone	Identified tick-borne pathogens byPCR and RLB										
	Tick species	NCT/NAT/NAP	NPP	MLE (%)	95% CI	Single infection					Mixed infection
<i>T. buffeli/ orientalis</i>						<i>Babesia</i> spp.	<i>Anaplasma/ Ehrlichia</i>	<i>A. centrale</i>	<i>A. phagocytophilum</i>	<i>Babesia</i> spp. + <i>Anaplasma/ Ehrlichia</i>	
Semi-arid bioclimatic zone (Tokat, Amasya, Gumushane)	<i>Hy. marginatum</i>	974/232/46	7 (15.21%)	3.19	1.42–6.24	–	6/46 (3.47%) CI 1.44–7.11	1/46 (2.08%) CI 0.12–9.81	–	–	–
	<i>Hy. excavatum</i>	90/90/15	5 (33.33%)	6.32	2.47–13.74	1/15 (1.11%) ^a CI 0.06–5.33	2/15 (2.29%) CI 0.42–7.39	2/15 (2.29%) CI 0.42–7.39	–	–	–
	<i>Hy. Detritum</i>	10/10/2	–	–	–	–	–	–	–	–	–
	<i>Rh. bursa</i>	311/145/40	3 (7.5%)	2.10	0.56–5.58	–	–	1/40 (1%) CI 0.06–4.77	–	–	2/40 (4.55%) CI 0.83–14.24
	<i>Rh. turanicus</i>	39/39/8	2 (25%)	5.41	1.01–17.15	–	1/8 (6.56%) (CI 0.42–31.47)	–	1/8 (6.56%) CI 0.42–31.47	–	–
	<i>Rh. sanguineus</i>	10/10/2	–	–	–	–	–	–	–	–	–
	<i>Rh. (Boophilus) annulatus</i>	173/173/38	6 (15.78%)	3.60	1.51–7.29	2/38 (8.93%) CI 1.69–27.64	–	4/38 (4.90%) CI 1.65–11.34	–	–	–
	<i>Hae. sulcata</i>	37/37/7	1 (14.28%)	2.68	0.16–12.68	–	–	–	–	1/7(3.32%) CI 0.20–15.69	–
Subtotal		1644/736/158	24 (15.18%)	3.57	2.36–5.19	–	–	–	–	–	–
Humid bioclimatic zone	<i>Hy. Marginatum</i>	115/44/9	–	–	–	–	–	–	–	–	–
	<i>Rh. bursa</i>	82/42/7	4 (57.14%)	10.49	4.07–23.66	–	–	4/7 (18.29%) CI 6.61–42.50	–	–	–
	<i>Hae. Punctata</i>	20/20/4	–	–	–	–	–	–	–	–	–
	<i>I. ricinus</i>	289/210/44	8 (18.18%)	4.10	1.94–7.66	–	–	–	–	8/44 (7.64%) CI 3.68–14.15	–
	<i>I. hexoganus</i>	10/10/2	–	–	–	–	–	–	–	–	–
Subtotal		516/326/66	12 (18.18%)	5.22	2.90–8.71	–	–	–	–	–	–
Total		2160/1062/224	36 (16.07%)	4.00	2.87–5.44	3/224 (2.71%) CI 0.73–7.18	9/224 (3.33%) CI 1.65–6.03	12/224 (3.61%) CI 1.99–6.06	1/224 (6.56%) CI 0.42–31.47	9/224 (6.78%) CI 3.41–12.18	2/224 (4.55%) CI 0.83–14.24

NCT, number of collected tick; NAT, number of analyzed tick; NAP, number of analyzed pool; NPP, number of positive pool; MLE, Maximum Likelihood Estimation; CI, confidence Intervals.

^a The rate (. . .%) shows the MLE result.

amplicons were sequenced, and a BLAST search performed with the 18S rRNA gene sequences showed one amplicon to be 100% identical to the sequence of *B. ovis* (accession no. EF092454). The remaining sequences clearly differed from all known *Babesia* species infective for cattle, but shared from 99 to 100% similarity with unnamed *Babesia* isolates, *Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2, *Babesia* sp. Kayseri 1, *Babesia* sp. CS58, (accession nos. AY726556, AY726557, EF434786, EU622824, respectively) and *B. occultans* (accession nos. EU376017, HQ331478).

The 14 pools comprising seven *Rh. bursa*, four *Rh. (Boophilus) annulatus*, two *Hy. excavatum*, and one *Hy. marginatum* gave positive signals to the *Anaplasma/Ehrlichia* spp. probe but did not show a signal to the cattle *Anaplasma* species-specific probes (*A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis*). Four representative specimens randomly selected from these samples were sequenced. A BLAST search conducted with the 16S rRNA gene sequences from these samples showed three to be 98% identical to the sequence of *Ehrlichia canis* (accession no. AY621071), which causes canine ehrlichiosis. Sequences of the fourth sample revealed a 100% identity with *Ehrlichia* sp. Omatjenne strain (accession no. U54806) previously identified in cattle in the same region.

Gene sequences determined in this study were deposited in GenBank under accession numbers (JF923655 for *Babesia* spp., JF923656 for *Babesia ovis*, JF923657 for *Ehrlichia* sp. Omatjenne strain, JF923658 for *Ehrlichia* spp.).

4. Discussion

In the present study, a survey was conducted to identify the ixodid tick species parasitizing cattle in the Black Sea region of Turkey. We recorded the prevalence of the tick-borne pathogens *T. buffeli/orientalis*, *Babesia* spp., *Anaplasma/Ehrlichia* spp., *A. centrale*, and *A. phagocytophilum* in ticks feeding on cattle. Cattle in the surveyed region were infested with 11 tick species that coincided with previous reports (Tonbak et al., 2006; Aktas et al., 2009), with the exception of *I. hexogonus*, a rare species previously found only in the Marmara region of Turkey (Aydin and Bakirci, 2007). Here, we report for the first time the occurrence of *I. hexogonus* in cattle feeding ticks in the Black Sea region of Turkey. *Hyalomma anatolicum*, previously widely described in cattle in some areas of Turkey (Aktas et al., 2004) was not found in our samples. *Hy. marginatum* and *I. ricinus* were the main species found from the semi-arid cold and humid bioclimatic regions, respectively. The findings confirm with previous reports from these regions (Tonbak et al., 2006).

Among the tick species examined for pathogens, seven were infected with one or more of the pathogens targeted by our protocols (Table 1). RLB indicated the presence of five tick-borne pathogens with an overall prevalence of 4% (CI 2.87–5.44). Among the pathogens detected in ticks, *T. buffeli/orientalis*, *A. centrale*, and *A. phagocytophilum* were identified by RLB. These pathogens were previously reported in a wide range of healthy cattle in some parts of Turkey including the study area (Aktas et al., 2009, 2011; Gokce et al., 2008). However, *T. buffeli/orientalis* and

A. centrale, benign parasites of cattle have not been previously reported in ticks in Turkey.

In serological studies of cattle in the same region, serum antibodies to *B. bigemina*, *B. Bovis*, and *B. divergens* were detected in 62%, 44%, and 75% of samples, respectively (Dincer et al., 1991). In the present study, *B. bigemina*, *B. Bovis*, and *B. divergens* were not detected, although *I. ricinus*, *Rh. (Boophilus) annulatus*, and *Rh. bursa*, the vector ticks of these species, were among those analyzed. PCR products showed a signal to catchall *Theileria/Babesia* and *Babesia* spp. probes in 10 samples, without showing a signal to any of the species-specific probes tested. This could indicate that there were not sufficient amplicons in the samples to give a species-specific signal, but could also signify the presence of a novel species or a variant of a known species. This is supported by the number of new pathogens and genotypes that have recently been identified (Bekker et al., 2002; Altay et al., 2007).

The sequencing indicated that one amplicon detected in *Rh. bursa* was 100% identical to the sequence of *B. ovis* (accession no. EF092454), the cause of ovine babesiosis in small ruminants. The other sequences shared from 99% to 100% similarity with the unnamed *Babesia* sp. Kashi 1 and Kashi 2 isolated from *Hy. anatolicum* (Luo et al., 2005), *Babesia* sp. Kayseri 1 isolated from *Hy. marginatum* (Ica et al., 2007), and *Babesia* sp. CS58, isolated from cattle in the same region (Altay et al., 2008). These sequences also shared 99% identity with *B. occultans*, a species that was first described in 1981 in South Africa as a bovine parasite (Gray and de Vos, 1981) and recently identified in *Hy. marginatum* (Ros-García et al., 2011) and sable antelope (Oosthuizen et al., 2008). The finding provided by the 18S rRNA gene sequence analysis in this study supported the presence of *B. occultans* in Turkey, but the 346 nucleotides were not adequate for comparison and definite identification. Further molecular and phylogenetic studies regarding the isolate will contribute greater insight into bovine piroplasm distribution and phylogenetic diversity. Additional studies are also needed to clarify the tick vector and the pathogenesis of the parasite in cattle in Turkey.

In view of the frequency of *Anaplasma* infections detected in the present study, it can be assumed that the risk of multiple pathogens for humans and animals is relatively high in the surveyed area. Among PCR products reacting with *Anaplasma/Ehrlichia* spp. probes only, four representative PCR products from these samples were sequenced to identify the pathogenic organisms. Three were 98% identical to the sequence of *E. canis* (accession no. AY621071), which causes canine ehrlichiosis. The other revealed a 100% identity with the sequence of *Ehrlichia* sp. Omatjenne strain (accession no. U54806) isolated from *H. truncatum* and goats (Bekker et al., 2002). The latter pathogen has been detected in cattle (Aktas et al., 2011), but *E. canis* has not been previously reported. These results show that more species-specific probes, particularly in studies of ticks, should be added on to the membrane to enhance pathogen detection prior to setup of the RLB assay.

There have been no reports of clinical cases associated with pathogenic *Anaplasma* species in Turkey. Recently, *A. phagocytophilum* and *A. marginale* have been reported

in ruminants (Gokce et al., 2008; Aktas et al., 2011), and *I. ricinus* (Aktas et al., 2009) in the Black Sea region of Turkey. This indicates that bovine anaplasmosis caused by *A. phagocytophilum* and *A. marginale* is present and prevalent in ruminants and ticks in the region. These pathogens can have a substantial impact on livestock production and predispose animals to other bacterial and viral infections (Larsen et al., 1994). *A. phagocytophilum* also infects humans, causing granulocytic anaplasmosis. It is possible that people exposed to *Ixodes* bites may become infected with *A. phagocytophilum*. The detection of *A. phagocytophilum* in domestic ruminants and ticks raises the question of whether human granulocytic anaplasmosis occurs in Turkey, although no clinical cases caused by *A. phagocytophilum* have been reported.

Anaplasma species may be transmitted mechanically by biting flies or blood-contaminated fomites and transstadially by ixodid ticks (Kocan et al., 2004). The present study showed that 8 of 44 (18%) *I. ricinus* were infected with *A. phagocytophilum*. This result was expected, as *I. ricinus* is considered the chief vector for *A. phagocytophilum* in Europe (Stuen, 2003). It has also been speculated that other ticks may play an important role in the transmission of this agent (Bown et al., 2003). In this study, one out of 7 (2.68%, CI 0.16–12.68) *Hae. sulcata* was infected with *A. phagocytophilum*. However, more epidemiological studies are required to identify the vectors and reservoirs of the agent. Additional studies are also needed to clarify the pathogenesis of *A. phagocytophilum* in both humans and domestic animals in Turkey.

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