



# Bovine anaplasmosis in Turkey: First laboratory confirmed clinical cases caused by *Anaplasma phagocytophilum*



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## ABSTRACT

*Anaplasma* species are obligate intracellular rickettsial pathogens that affect the health of humans and other animals. Clinical cases of anaplasmosis caused by *Anaplasma phagocytophilum* were evaluated, and the frequency of bovine *Anaplasma* species was determined in cattle. Blood samples and thin blood smears were collected from 10 cattle exhibiting clinical signs of tick-borne fever. In addition, blood samples were collected from 123 apparently healthy cattle from the same area. DNA was screened by reverse line blot assay for the presence of the hypervariable V1 region of the 16S rRNA gene of *Anaplasma/Ehrlichia* species. Intracytoplasmic inclusion bodies of *A. phagocytophilum* were observed in neutrophils of 6 sick animals. Parasitemia ranged from 0.2 to 1.6% in individual slides. Reverse line blot showed 45.1% (60/133) of the sampled cattle to be positive for one or more of five *Anaplasma* species. The frequency of single infections was 20.3% (27/133), while mixed infections were found in 24.8% (33/133) of samples with six different combinations of species and a maximum of four pathogens detected. *A. phagocytophilum* was the most prevalent (41/133, 30.8%) followed by *Anaplasma marginale* (25/133, 18.8%), *Anaplasma centrale* (24/133, 18%), *Ehrlichia* sp. strain Omatjenne (18/133, 13.5%) and *Anaplasma bovis* (1/133, 0.7%). This is the first report of *A. bovis* in a cow from Turkey. This is also the first report of clinical cases caused by *A. phagocytophilum* in cattle from the country. Therefore, *A. phagocytophilum* should be taken into account as differential diagnosis in cases of high fever and anorexia in pastured animals.

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## 1. Introduction

Bovine anaplasmosis is a tick-borne rickettsial disease caused by *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Anaplasma bovis*, and *Anaplasma centrale* (Dumler et al., 2001). *A. phagocytophilum* is the causative agent of human granulocytic anaplasmosis and of tick-borne fever in a wide range of hosts, including cattle (Woldehiwet, 2010). The presence of the pathogen is closely related to the occurrence of *Ixodes ricinus*, the most abundant species of tick in Europe (Woldehiwet, 2010). *A. phagocytophilum* causes febrile disease in cattle varying from undetectable clinical signs to serious complications, including death (Pusterla and Braun, 1997; Stuen et al., 2005). The disease is characterized by high fever, cough, abortion, decreased milk production, and anorexia (Woldehiwet, 2010). In cattle, the incubation period after experimental inoculation was reported to be 4–9 days with fever lasting 1–13 days (Pusterla et al., 1997).

*A. marginale* is the most widely distributed of the mentioned species worldwide, and causes disease characterized by progressive anemia, weakness, fever, anorexia, decreased milk yield, jaundice, abortion, and sometimes death, while *A. centrale* infection results in mild anemia in most cases (Dumler et al., 2001; Kocan et al., 2010). *Anaplasma bovis* is more closely related to *A. phagocytophilum* than to *A. marginale* or *A. centrale* (Dumler et al., 2001). Cattle infections with *A. bovis* have been reported mainly in Asia and Africa, and little is known about its epidemiology (Dumler et al., 2001). *A. bovis* infection can lead to anorexia, fever, incoordination, pale mucous membranes, and enlargement of lymph nodes in cattle (Kaufmann, 1996).

Tick-borne hemoprotozoan parasites in domestic ruminants and ticks have been documented in Turkey (Aktas et al., 2005, 2007 Altay et al., 2007, 2008), but there is a paucity of information about the presence of rickettsiae in the country. In recent molecular studies, *A. phagocytophilum*, *A. marginale*, *A. centrale*, and the *Ehrlichia* sp. strain Omatjenne have been detected in some areas of Turkey (Gokce et al., 2008; Aktas et al., 2009, 2010, 2011, 2012). However, to date, no laboratory confirmed clinical case of natural infection by *A. phagocytophilum* has been reported in the country. We performed a PCR-based RLB survey on symptomatic and

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asymptomatic cattle in the Bartın province of the western Black Sea region of Turkey. The aims of the study were to evaluate clinical cases of anaplasmosis, and to determine the distribution and frequency of bovine *Anaplasma* spp. This is the first report of *A. bovis* in cattle from Turkey. It is also the first report of clinical cases associated with *A. phagocytophilum* in cattle from Turkey.

## 2. Materials and methods

### 2.1. Study area, blood sampling and microscopic examination

This study was conducted in Bartın province, a coastal area 25–300 m above sea level, in the west Black Sea region of Turkey (41°53'N, 32°45'E). Forest vegetation generally consists of coniferous trees. It has a humid oceanic climate with mean annual rainfall of 1000 mm and mean annual temperature of 13.4 °C. Cattle in the area are maintained under small-scale family farming systems. During the day, the cattle graze on communal pastures, generally 7–9 h per day. In the evening the cows are milked and held overnight in enclosures. In May/June 2014, 4 heifers, 2 female calves, and 4 lactating cows (crossbreeds of Jersey, Simmental, and brown Swiss) with symptoms typical of tick-borne disease, including high fever, anorexia, cough, and decreased milk yield, were subjected to a routine physical examination by a private veterinary clinician, and clinical signs were recorded. Blood samples were drawn from the jugular vein into anticoagulant (K3-EDTA) and stored at 4 °C until DNA extraction. Thin blood smears were prepared from the EDTA-treated samples. The blood samples and smears were transported on ice to the Department of Parasitology, Firat University. As the animals were not checked for the presence of ixodid tick infestations, no data regarding tick species on the cattle were available. However, *I. ricinus* was the most prevalent tick species previously reported feeding on cattle near the study site (Aktas et al., 2012). The cattle were treated with an intramuscular injection of long-acting oxytetracycline at a dose of 20 mg/kg body weight, with some animals also receiving supportive therapy (iron supplements, dextrose, vitamins) (Table 1).

Thin blood smears were air dried, fixed in absolute methanol for 5 min, and stained with 10% Giemsa stain for 30 min. Samples were examined for intracytoplasmic *Anaplasma* spp. inclusion bodies under oil immersion (1000×). In each blood smear, 20 fields (minimum of 5000 red blood cells) were screened for the presence of *A. marginale* and *A. centrale* in the erythrocytes. A minimum of 500 neutrophils were also screened in each smear, and the number of cells containing *Anaplasma* basophilic morulae was recorded

and the parasitemia was calculated as the percent of infected neutrophils (Stuen et al., 2011). Counts were made without knowledge of the molecular results.

### 2.2. DNA isolation

Genomic DNA was extracted from 200 µL of EDTA anti-coagulated blood with a QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µL elution buffer and stored at –80 °C for later analysis. Negative control purifications using sterilized de-ionized water were performed to monitor cross-contamination. DNA concentrations (ng/µL) and purity ( $A_{260nm}/A_{280nm}$ ) were determined by spectrophotometry (NanoDrop<sup>®</sup> ND-2000 UV/vis Spectrophotometer, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA).

In addition, DNA samples previously isolated (May/June 2013) from 123 cattle in the same province, with no clinical symptoms, were included. All further analysis was conducted on samples from both symptomatic and asymptomatic cattle.

### 2.3. PCR amplification

To improve on the quality of the amplification, a nested PCR assay was performed using two universal primer pairs. The primers EC12A (5'-TGATCCTGGCTCAGAACGAACG-3') and EC9 (5'-TACCTTGTTACGACTT-3') were used for initial amplification of an approximately 1450 bp fragment of the 16S rRNA gene in *Anaplasma* and *Ehrlichia* spp. (Chen et al., 1994). The PCR reactions were performed in PCR Sprint (Thermo Electron Corporation, USA) as previously described (Aktas et al., 2010). The nested amplification performed using the RLB primers 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYTACG-3') and BGA1B-new (Biotin-5'-CGGGATCCCAGTTTGCCGGGACTTYTCT-3') produced 492–498 bp corresponding to the hypervariable V1 region of *Anaplasma* and *Ehrlichia* spp. (Schouls et al., 1999; Bekker et al., 2002). For nested PCR amplification, 1 µL of first round PCR product was used as the template. The PCR was performed in a total reaction volume of 25 µL containing 2.5 µL of 10× PCR buffer [100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100], 250 µM of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega, Madison, WI, USA), and 10 pmol of each primer. A touchdown PCR program was performed: two cycles of 20 s at 94 °C, 30 s at 67 °C, and 30 s at 72 °C, followed by a succession of two-cycles with conditions identical to the previous cycles with the annealing temperature reduced by 2 °C until it reached 57 °C. A further

**Table 1**  
Summary of clinical and laboratory findings in cattle exhibiting clinical signs of anaplasmosis.

Animal ID	Age	Fever (°C)	Main clinical signs	Laboratory findings			Outcome <sup>a</sup>
				Blood smear	Infected leukocytes (%)	RLB	
brt18	2	40.5	Depression, nasal discharge, anorexia	Positive	0.2	Aph + Am + Ac	Recovery
brt45	2	41.5	Anorexia, coughing, diarrhea	Positive	1.2	Aph	Recovery
brt46	1	40.9	Recumbency, diarrhea, nasal and eye discharge	Positive	0.8	Aph + Am + Ac + Emo	Recovery
brt42	2	41	Weakness, anorexia, swelling of the hind limbs	No data	–	Aph + Am + Ac + Emo	Recovery
brt3	6	39	Weakness, decrease of milk production, anorexia	Negative	–	Aph	Recovery
brt17	4	39.8	Weakness, decrease of milk production, anorexia	Positive	1.6	Aph + Am + Ac + Emo	Recovery
brt4	1	40.5	Weakness, coughing, anorexia	Positive	0.4	Aph + Am + Ac + Emo	Recovery
brt41	4	40	Weakness, decrease of milk production, coughing, anorexia	No data	–	Aph + Am + Ac + Emo	Recovery
brt40	2	41.2	Anorexia, coughing, nasal and eye discharge	Positive	0.6	Aph + Am + Ac	Recovery
brt39	3	41.2	Decrease of milk production, coughing, anorexia, nasal discharge	Negative	–	Aph + Am + Ac	Recovery

Abbreviations: RLB – reverse line blotting; Aph – *Anaplasma phagocytophilum*; Am – *Anaplasma marginale*; Ac – *Anaplasma centrale*; Emo – *Ehrlichia* sp. strain Omatjenne.  
<sup>a</sup> Oxytetracycline (20 mg/kg) used for treatment.

40 cycles of 45 s at 94, 57, and 72 °C followed, with a final extension at 72 °C for 10 min. DNA from *A. marginale*, *A. phagocytophilum*, *A. centrale*, and *Ehrlichia* sp. strain Omatjenne positive by RLB and DNA sequencing (GenBank accession nos. GU201518, FJ172530, GU223364, GU201519, respectively) obtained in previous studies (Aktas et al., 2010, 2011) were used as positive controls in the PCR and for the subsequent RLB hybridization. In addition, the A/E-probe encoding the *Anaplasma* and *Ehrlichia* probe sequences, provided by a TBD-RLB Kit (Isogen Life Science, Maarsse, Netherland), was also used as positive control. A positive control could not be used for *A. bovis*, because DNA from this agent was not available. Sterilized de-ionized water was used as negative control. In order to minimize risk of contamination, DNA extractions, PCR preparation, PCR amplification, and agarose gel electrophoresis were performed in separate rooms.

#### 2.4. Reverse line blotting (RLB)

The RLB membrane was prepared as described previously (Aydin et al., 2013). Briefly, to 20 µL of the PCR product, 2× SSPE/0.1% SDS was added to a final volume of 150 µL and held in a Thermal Cycler at 99 °C for 10 min and denatured for RLB hybridization. The biotinylated *Anaplasma/Ehrlichia* nested PCR products were hybridized with catch-all and species-specific oligonucleotide probes linked to an RLB membrane (Table 2). The primers and oligonucleotide probes were provided by the Midland Certified Reagent Company (Texas, USA).

#### 2.5. DNA sequencing

One sample obtained from a symptomatic animal (brt45) tested positive with microscopy and RLB for *A. phagocytophilum* was selected for 16S rRNA gene sequencing. The 16S rRNA gene was amplified using 20 pmol of primers EC12A and EC9 to produce a PCR amplicon of approximately 1450 bp. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to a commercial laboratory (İontek, Turkey) for sequencing. The product was separated in an automated DNA genetic analyzer (ABI 310 Prism; PerkinElmer Corporation, Foster City, CA). Each construct was sequenced at least three times. Sequencing results were evaluated with Chromas-Lite ([www.technelysium.com.au](http://www.technelysium.com.au)). Sequence homology searches were made by BLASTn analysis of GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), and multiple alignments were performed with ClustalW2 ([www.ebi.ac.uk](http://www.ebi.ac.uk)). Primers were deleted from DNA sequences prior to BLAST comparison.

### 3. Results

#### 3.1. Clinical signs and direct evidence of infectious agent

The most prevalent clinical signs exhibited were anorexia and hyperthermia (>39 °C). Decreased milk yield was observed in lactating cows. Some animals experienced diarrhea and cough (Table 1). No hematological profiles or biochemical parameters for the animals with symptoms of tick-borne fever were available; therefore, calculation of correlation with clinical signs was not possible. Of the 10 Giemsa-stained blood smears obtained from clinical cases, eight were evaluated and assessed for the presence of *Anaplasma* inclusion bodies. No *A. marginale* or *A. centrale* were detected by microscopy, whereas intracytoplasmic inclusion bodies compatible with *A. phagocytophilum* were observed in granulocytic neutrophils of six animals (Fig. 1). Microscopic findings revealed parasitemia of 0.2–1.6%. Clinical and microscopic findings were confirmed by RLB positive signals for *A. phagocytophilum*. All animals received treatment and recovered within 5–8 days.

#### 3.2. Frequency of tick-borne *Anaplasma* species in cattle

Frequency of *Anaplasma* or *Ehrlichia* species in single and mixed infections is shown in Table 3. Of the 133 PCR products, 60 (45.1%) hybridized to the *Anaplasma/Ehrlichia* catch-all probe, and all exhibited positive reactions with their corresponding specific probes. The frequency of single infections was 20.3% (27/133), while mixed infections were found in 33 (24.8%) samples with six species combinations and a maximum of four pathogens detected in seven animals, five of which were symptomatic (Table 3). A total of 30.8% of the studied animals were positive for *A. phagocytophilum*, indicating a considerable number of animals exposed to anaplasmosis. The second most commonly found pathogen was *A. marginale* at 18.8%. Other tick-borne pathogens identified in the blood samples included *A. centrale* (18%), *Ehrlichia* sp. strain Omatjenne (13.5%), and *A. bovis* (0.7%).

#### 3.3. DNA sequencing

A nearly full-length sequence (1404 bp) of the 16S rRNA gene of *A. phagocytophilum* was obtained and deposited in the EMBL/GenBank database under accession number KP745629. A BLAST search revealed a nucleotide identity of 99.86% with the 16S rRNA gene of *A. phagocytophilum* Webster strain (Accession no. U02521).

**Table 2**  
*Anaplasma* specific oligonucleotide probe sequences used in this study.

Oligonucleotide probe	Sequence (5'–3')	Reference
<i>Anaplasma/Ehrlichia</i>	TTA TCG CTA TTA GAT GAG CC	Schouls et al., 1999
<i>Ehrlichia/Anaplasma</i> catch-all	GGG GGA AAG ATT TAT CGC TA	Bekker et al., 2002
<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG	Bekker et al., 2002
<i>Anaplasma centrale</i>	TCG AAC GGA CCA TAC GC	Bekker et al., 2002
<i>Anaplasma bovis</i>	GTA GCT TGC TAT GAG AAC A	Bekker et al., 2002
<i>Ehrlichia</i> sp. strain Omatjenne	CGG GTT TTT ATC ATA GCT TGC	Bekker et al., 2002
<i>Anaplasma phagocytophilum</i> 1	TTG CTA TAA AGA ATA ATT AGT GG	Schouls et al., 1999
<i>Anaplasma phagocytophilum</i> 3	TTG CTA TGA AGA ATA ATT AGT GG	Schouls et al., 1999
<i>Anaplasma phagocytophilum</i> 5	TTG CTA TAA AGA ATA GTT AGT GG	Schouls et al., 1999
<i>Anaplasma phagocytophilum</i> 7	TTG CTA TAG AGA ATA GTT AGT GG	Schouls et al., 1999
<i>Anaplasma phagocytophilum</i> A-HGE	GCT ATA AAG AAT AGT TAG TGG	Schouls et al., 1999
<i>Anaplasma phagocytophilum</i> A-D-HGE	GCT ATG AAG AAT AGT TAG TG	Schouls et al., 1999

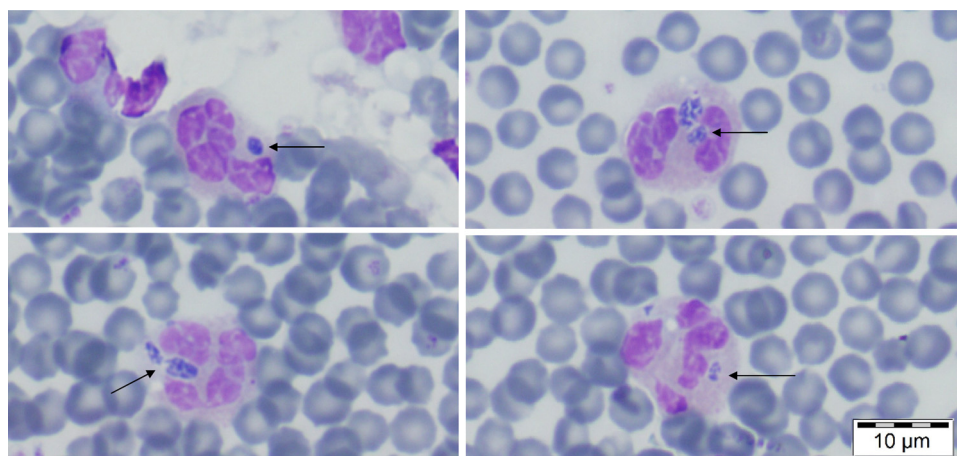


Fig. 1. Blood smears showing inclusion bodies (morulae next to the nucleus; arrows) of *Anaplasma phagocytophilum* in leukocytes from naturally infected cattle.

Table 3

Number of cattle infected with single or multiple *Anaplasma* sp. detected by the RLB (n = 133)

Nature of infection	<i>A. phagocytophilum</i>	<i>A. marginale</i>	<i>A. centrale</i>	<i>E. sp. Omatjenne</i>	<i>A. bovis</i>	No. positive samples (%)
Single infection	+	–	–	–	–	24 (18%)
	–	+	–	–	–	1 (0.7%)
	–	–	–	+	–	2 (1.5%)
Subtotal						27 (20.3%)
Mixed infection	+	–	–	–	+	1 (0.7%)
	+	+	+	–	–	1 (0.7%)
	–	+	+	+	–	1 (0.7%)
	+	+	+	+	–	7 (5.3%)
	+	–	–	+	–	8 (6%)
	–	+	+	–	–	15 (11.3%)
Subtotal						33 (24.8%)
Total	41 (30.8%)	25 (18.8%)	24 (18%)	18 (13.5%)	1 (0.7%)	60 (45.1%)

#### 4. Discussion

The clinical findings of fever, anorexia, and decreased milk yield from the diseased animals pointed to a tick-borne disease. These clinical signs were consistent with previous reports that anaplasmosis in sheep and cattle normally produced mild to moderate symptoms that are seldom fatal unless complicated by other infections (Pusterla and Braun, 1997; Stuen, 2007; Laloy et al., 2009; Guyot et al., 2011). Intracytoplasmic inclusion bodies of *A. phagocytophilum* were observed in leukocytes of the diseased cattle (Fig. 1). These clinical and microscopic findings were confirmed by PCR-based RLB hybridization and DNA sequencing. Although tick-borne fever caused by *A. phagocytophilum* is widespread in Europe (Hofmann-Lehmann et al., 2004; Laloy et al., 2009; Guyot et al., 2011; Ceci et al., 2014), the disease has not been considered a major pathogen in Turkey. Here, for the first time, we described clinical disease in cattle. A possible reason that the disease has not been previously diagnosed may be the lack of awareness and experience of veterinary practitioners. Since global sale and transport of cattle, especially from Europe, is common practice, importation of cattle infected with *A. phagocytophilum* may play a role.

Diagnosis of bovine anaplasmosis is usually based on clinical findings and detection of inclusion bodies in Giemsa-stained thin blood smears. However, morulae were not observed in the two clinical cases that were RLB positive for *A. phagocytophilum*. This finding is supported by a previous report of *Anaplasma* DNA detected as early as five days before inclusions were observed by blood smear microscopy (Stuen et al., 2011). We found parasitemia

of 0.2–1.6% in animals, consistent with the view that the percent of infected leukocytes varies markedly (Pusterla et al., 1997). In addition, a recent study found the infection rate to be significantly different on days 3, 6, 7, and 8 in experimentally infected sheep (Stuen et al., 2011). Therefore, molecular diagnostic tools represent a more sensitive method, and the time frame for detection of *A. phagocytophilum* is longer when using molecular methods than with thin blood smears (Pusterla et al., 1997; Nieder et al., 2012).

The results of this survey indicated the presence of *A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis*, and *Ehrlichia* sp. strain Omatjenne circulating in the sampled cattle in the west Black Sea region of Turkey. Most of these pathogens were anticipated, since they have been previously reported in cattle and ticks in areas of Turkey (Gokce et al., 2008; Aktas et al., 2010, 2011; Aktas, 2014). However, this is the first time that *A. bovis* has been detected in cattle from Turkey, and this gives greater insight into distribution and phylogenetic diversity of bovine anaplasmosis. High prevalence of *Anaplasma* infections (45.1%) was observed here compared to the findings of studies conducted in other provinces of Turkey (Gokce et al., 2008; Aktas et al., 2011). Seasonal, geographic, and climatic differences might have contributed. Among the detected pathogens, *A. phagocytophilum* was the most frequently found, present in 30.8% of the samples (41/133). The abundance of *I. ricinus*, a vector of *A. phagocytophilum*, might explain the higher positive rate of *A. phagocytophilum* in the area.

*A. marginale* and *A. centrale* were found in 18.8% and 18% of the cattle examined, respectively, thus confirming the presence of these species in the study area, as reported elsewhere in Turkey (Aktas et al., 2011, 2012). Although *A. bovis* has been reported

mainly in Asian and African countries (Ooshiro et al., 2008; Liu et al., 2012), recent studies have indicated that the pathogen is present in cattle in continental Europe (Georges et al., 2001; Ceci et al., 2014). *A. bovis* is usually associated with subclinical infection in cattle, but symptoms including fever, anorexia, and depression can be present (Wanduragala and Ristic, 1993). In the present study, RLB results revealed co-infection of *A. bovis* and *A. phagocytophilum* in a sample obtained from an apparently healthy cow. Thus, it is difficult to evaluate the pathogenesis of *A. bovis* in this study. More epidemiological studies are required to determine the pathogenicity, vectors, and reservoir animals of the agent.

It is well known that RLB is useful in detecting mixed infections. Our results revealed that 33 animals (24.8%) presented co-infections with species of *Anaplasma*/*Ehrlichia* in six combinations (Table 3). The frequent detection of co-infections, also reported by other (Georges et al., 2001; de la Fuente et al., 2005; Ceci et al., 2014), is an interesting finding in tick-borne diseases and requires further studies to better understand relationships between pathogens and the host immune system.

In this study, 13.5% of samples were positive for *Ehrlichia* species (*Ehrlichia* sp. strain Omatjenne). The pathogen was initially isolated from sheep in the Omatjenne region of Namibia (Allsopp et al., 1997). Recent studies detected this *Ehrlichia* species in cattle (Aktas et al., 2011; Mtshali et al., 2013) and in African buffalo (Eygelaa et al., 2015). Although there is still little known about the pathogenicity of *Ehrlichia* sp. strain Omatjenne in ruminants, the results suggest that cattle may be a natural host of the pathogen and could play an important role in its epidemiology.

The presence and frequency of bovine *Anaplasma* species in the west Black Sea region of Turkey were determined using the RLB hybridization assay. Four *Anaplasma* (*A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis*) and one *Ehrlichia* (*Ehrlichia* sp. strain Omatjenne species) infecting cattle were identified. *A. phagocytophilum* causes clinical disease in susceptible cattle and considerable economic loss to the farms in the region. Local animal health officers and veterinarians should be made aware of the presence of the pathogen, and include it in their diagnoses and treatment strategies. This pathogen also infects humans, causing granulocytic anaplasmosis. This study suggests that human anaplasmosis should be included in the differential diagnosis in this region in cases of influenza-like illness following a tick bite. This is also the first report of the presence of *A. bovis* in cattle in Turkey.

### Conflict of interest

The authors declare no conflicts of interest.

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