Abstract
This study was conducted to determine the presence and distribution of Anaplasma ovis and Anaplasma phagocytophilum in small ruminants in Istanbul, Tekirdag, Edirne and Kırklareli provinces in Thrace region of northwestern Turkey during May-September in 2014. A total of 423 blood samples (216 sheep and 207 goats) were collected randomly from small ruminants regardless of the clinical symptoms. Species-specific polymerase chain reaction (PCR) assays, targeting the major surface protein 4 (msp4), were employed for identification of A. ovis and A. phagocytophilum and selected products were confirmed via sequencing. A total of 230 small ruminants (54.37%) were found to be infected with A. ovis and/or A. phagocytophilum. The rates of infected animals for A. ovis and A. phagocytophilum were 50.83% (215/423) and 8.51% (36/423) respectively. Coinfection rate in small ruminants was determined as 4.96% (21/423). Sequence diversity rates of 0-0.94% for A. ovis and 0.41-2.49% for A. phagocytophilum have been observed. This is the first detection of A. ovis and A. phagocytophilum in sheep and goats in Thrace region of northwestern Turkey via polymerase chain reaction and sequence characterization. Further researches are needed to determine the vectors, vector-host interactions and genotypic variants that may affect the presence and distribution of Anaplasma species in the region.

Keywords: Anaplasma ovis, Anaplasma phagocytophilum, Sheep, Goat, msp4, Thrace, Turkey

INTRODUCTION
The family Anaplasmataceae belongs to order Rickettsiales of class α-Proteobacteria. The genus Anaplasma comprises six species; Anaplasma centrale, A. marginale, A. bovis (formerly Ehrlichia bovis), A. ovis, A. phagocytophilum (formerly Ehrlichia equi, E. phagocytophila and Human Granulocytic Ehrlichiosis [HGE] agent) and A. platys 1. Anaplasma species are Gram negative bacteria parasitizing in the blood cells of mammals. The life cycle of Anaplasma include the
reproduction stages taking place in both vector ixodid ticks and vertebrate animals [2]. Ticks belonging to the genera *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* are the main biological vectors of *Anaplasma* species [3].

*Anaplasma ovis* and *A. phagocytophilum* are medically-important species, pathogenic for small ruminants [3]. *Anaplasma ovis* causes ovine anaplasmosis in small ruminants, which is associated with significant morbidity and mortality, especially in goats [4]. Moreover, *A. ovis* can cause severe clinical disease in bighorn sheep as well as predisposing animals to other pathogens [5-7]. *A. ovis* infections have been reported to be endemic worldwide including Europe [8], China [9] and United States of America [10]. *A. ovis* has been reported from Turkey’s neighboring countries Greece [11], Cyprus [12] and Iran [13].

*Anaplasma phagocytophilum* is the causative agent of tick borne fever in ruminants and granulocytic anaplasmosis in humans, equines and canines [1,14]. *A. phagocytophilum* can cause subclinical or severe infection in sheep and it is seldom fatal unless complicated by other infections. In addition to crippling, direct and production losses, *A. phagocytophilum* can cause abortion and impaired matogenesis in sheep [15-17]. *A. phagocytophilum* has been reported in China [18], United States of America, Europe, Asian part of Russia and north Africa [19]. *A. phagocytophilum* has also been reported from Turkey’s neighboring countries Bulgaria [19], Greece [20] and Iran [21]. In Turkey, early records of *A. ovis* have been reported in small ruminants by using direct microscopy [22,23]. *A. phagocytophilum* in sheep [24,25], in goats [25], in ixodid ticks [26-28], in cattle [24,29,30] and in dogs [31] as well as *A. ovis* in sheep [25], in goats [25] and in ixodid ticks [26,32] have been reported by nucleic acid detection in various regions of Turkey.

Morphological and serological techniques are not reliable to differentiate *Anaplasma* and *Ehrlichia* species due to morphological similarities and antigenic cross reactions between species [33]. Detection of the bacterial nucleic acids via polymerase chain reaction (PCR) provide tools with high sensitivity and specificity and thus, are widely used in definitive diagnosis of *Anaplasma* species. These techniques also have the advantages of detecting the positive hosts in the early acute phase of the infection as well as the carrier stages [34,35].

This study was undertaken to investigate the presence and the distribution of *Anaplasma ovis* and *A. phagocytophilum* in sheep and goats in Thrace region by species-specific PCRs, where no previous information on *Anaplasma* is available.

**MATERIAL and METHODS**

**Research Area and Sample Collection**

The study was conducted between May and September 2014 in four representative provinces (Istanbul, Tekirdag, Edirne and Kirkareli) in Thrace region of northwestern Turkey.

Totally 423 blood samples (216 sheep and 207 goats) were collected randomly from 2-4 aged small ruminants regardless of showing any clinical symptoms. Ten ml blood sample was collected in tubes containing ethylene diamine tetra acetic acid (EDTA) in (K2E BD Vacutainer®) from each individual and transferred to laboratory in cold chain. Blood samples were stored in -20°C until DNA extraction.

**PCR and Sequencing**

Total genomic DNA extraction was performed by using a commercial kit (High Pure® PCR Template Preparation Kit Roche Diagnostics GMBH) according to the manufacturer’s instructions.

For the identification of *A. ovis*, species-specific primer sets AovisMSP4Fw (5′-TGAAGGGAGCGGGGTATG-3’) forward and AovisMSP4Rev (5′-GAGTAAATTGACACCGGGACTCT-3’) reverse were used for amplification of the *A. ovis* major surface protein (msp4) gene 347-bp coding region [36]. For the identification of *A. phagocytophilum*, species-specific primer sets MAP4AP5 (5′-ATGAATTACAGGAATGTGTTAGG-3’) forward and MSP4AP3 (5′-TAAATGAAAGCAAATCTTGCT-3’) reverse were used for amplification of the *A. phagocytophilum* msp4 gene 849-bp coding region [37].

Protocols described by Torina et al.[36] and de la Fuente et al. [37] were optimized for PCR amplifications. The final PCR conditions were established as: reaction buffer 1x, 0.4 µM of each primer, 1.5 mM of MgCl₂, 0.2 mM of dNTP, 1.25 U of Taq DNA Polymerase (ThermoScientific, Waltham, M.A.). PCR reactions were performed in an automated PCR thermal cycler (Axygen, Corning, N.Y.). For *A. ovis*, the thermal profiles for PCR were optimized as: 2 min at 94°C for denaturation followed by 35 cycles with denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The final extension step was 5 min at 72°C. For *A. phagocytophilum*, 15 min at 95°C for denaturation followed by 40 cycles with denaturation at 94°C for 30 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 min. The final extension step was 7 min at 72°C. The programs were terminated by storing the reaction mixtures at 4°C. PCR products were visualized via observation under UV light in a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide.

For the confirmation of positive PCR results, randomly-selected PCR products for *A. ovis* and *A. phagocytophilum*, were cleaned up using High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and were sequenced via sense and antisense primers. employing an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). Obtained sequences were handled using CLC
Main Workbench v7.5.2 (CLCBio, Aarhus, Denmark) and by MEGA software v5.2 [38].

**Statistical Analysis**

Chi square test was used for statistical analysis by SPSS v13 and P<0.05 was accepted statistically significant.

**RESULTS**

Among 423 small ruminants examined, 230 (54.37%) were found infected with *A. ovis* and/or *A. phagocytophilum*. The percentages of positive animals for *A. ovis* and *A. phagocytophilum* were 50.83% (215/423) and 8.51% (36/423) respectively. Coinfection rate of *A. ovis* and *A. phagocytophilum* in small ruminants was 4.96% (21/423).

The distribution of *A. ovis* and *A. phagocytophilum* in sheep and goats according to the sampling provinces in Thrace region of northwestern Turkey and the significance level of differences among sampling provinces were presented in Table 1.

According to Table 1, the percentages of *A. ovis* in sheep and goats were 58.8% (127/216) and 42.51% (88/207) respectively, whereas the percentages of *A. phagocytophilum* in sheep and goats were 11.11% (24/216) and 6.45% (12/207) respectively. There was a statistically-significant difference among the provinces in Thrace region of Turkey for prevalence of *A. ovis* in sheep (P<0.001), *A. ovis* in goats (P=0.028), *A. phagocytophilum* in goats (P=0.015) and *A. phagocytophilum* in goats (P=0.008) (Table 1).

Representative sequences of the msp4 gene were obtained for *A. ovis* and *A. phagocytophilum* and submitted to GenBank (accession no. KT251211 for *A. ovis* and Table 1.

### Table 1. The distribution of *A. ovis* and *A. phagocytophilum* in sheep and goats by sampling provinces in Thrace region of northwestern Turkey and the significance level of differences among sampling provinces

| Parasite (Host) | Total | Sampling Provinces | P Value |
|-----------------|-------|--------------------|---------|
|                 | n     | Istanbul           | Tekirdag | Edirne | Kirklareli |
| *A. ovis* (Sheep) | 127/216 | 58.8 | 14/52 | 26.92 | 28/50 | 56.00 | 47/62 | 75.81 | 38/52 | 73.08<sup>a</sup> <sup>b</sup> | <0.001 |
| *A. ovis* (Goat) | 88/207 | 42.51 | 14/52 | 26.92 | 21/50 | 42.00 | 24/53 | 45.28 | 29/52 | 55.77<sup>a</sup> | 0.028 |
| *A. phagocytophilum* (Sheep) | 24/216 | 11.11 | 3/52 | 5.77 | 5/50 | 10.00 | 4/62 | 6.45 | 12/52 | 23.08<sup>a</sup> | 0.015 |
| *A. phagocytophilum* (Goat) | 12/207 | 5.8 | 1/52 | 1.92<sup>a</sup> | 1/50 | 2.00 | 2/53 | 3.77 | 8/52 | 15.38<sup>a</sup> | 0.008 |

<sup>a, b, c</sup> Differences among percentages represented with different letters in the same line is significant (P<0.05)

**Fig 1.** Agarose gel electrophoresis of *A. ovis* and *A. phagocytophilum* PCR products extracted from sheep and goat blood samples. Lines: M: 100-bp DNA marker; 1: *A. ovis* negative control (PCR-grade water); 2: *A. ovis* DNA extracted from sheep; 3: *A. ovis* DNA extracted from goat; 4: *A. ovis* positive control; 5: *A. phagocytophilum* negative control (PCR-grade water); 6: *A. phagocytophilum* DNA extracted from sheep; 7: *A. phagocytophilum* DNA extracted from goat; 8: *A. phagocytophilum* positive control

**Şekil 1.** Koyun ve keçi kan örneklerinden elde edilen *A. ovis* ve *A. phagocytophilum* ait PCR ürünlerini agaroz jel elektroforezi. Ok ucu 347-bp ve ok 849-bp PCR ürünlerini göstermektedir. Sıralar: M: 100-bp DNA işaretleyicisi; 1: *A. ovis* negatif kontrol (PCR-kalite su); 2: *A. ovis* DNA örnekleri; 3: *A. ovis* DNA örnekleri; 4: *A. ovis* pozitif kontrol; 5: *A. phagocytophilum* negatif kontrol (PCR-kalite su); 6: *A. phagocytophilum* DNA örnekleri; 7: *A. phagocytophilum* DNA örnekleri; 8: *A. phagocytophilum* pozitif kontrol
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Comparison of A. ovis sequences with several selected homolog regions from various sources revealed very limited diversity, with 99.06-100% nucleotide similarities. However, A. phagocytophilum sequences demonstrated 0.41-2.49% divergence, and observed to constitute a distinct cluster, separated from sequences from Poland, Slovenia and Italy (Fig. 2).

**DISCUSSION**

Anaplasmosis is a tick borne disease caused by various species of *Anaplasma* with a significant impact on animal breeding due to the economic burden resulting from morbidity and mortality associated with the disease. Thus, the epidemiology, diagnosis and regional prevalence of Anaplasmosis remain as an important issue for mitigating the impact of the disease in the current practice of veterinary parasitology and microbiology [3].

The circulation of various *Anaplasma* species have been investigated in Turkey. Recent studies reported the presence of *A. phagocytophilum* nucleic acids in sheep [24,25], in goats [25] in ixodid ticks [26-28], in cattle [24,29,30] and in dogs [31], as well as *A. ovis* in sheep [25], in goats [25] and in ixodid ticks [28,32] in discrete regions. In East Black Sea region of Turkey, *A. phagocytophilum* seroprevalence by IFAT has been observed 14.86% (107/720) and specific DNA ratio by nested PCR has been found 12.35% (22/178) in sheep [24]. In East Anatolia region of Turkey, 71.32% (301/422) small ruminants have been reported to be infected by *A. ovis* and/or *A. phagocytophilum*. The percentages of positive animals for *A. ovis* and *A. phagocytophilum* have been reported 67.06% (283/422) and 19.66% (83/422) respectively. Coinfections of *A. ovis* and *A. phagocytophilum* have been reported in 15.40% (65/422) of analysed small animals. The percentages of *A. ovis* in sheep and goats were 67.35% (196/291) and 66.41% (87/131) respectively, whereas the number of *A. phagocytophilum* in sheep and goats were 18.90% (55/291) and 21.37% (28/131) respectively [25].

In this study, *A. ovis* and/or *A. phagocytophilum* infections were revealed in a total of 230 (54.37%) small ruminants investigated. The detection rates of *A. ovis*
and A. phagocytophilum in small ruminants were 50.83% (215/423) and 8.51% (36/423) respectively. Moreover, A. ovis and A. phagocytophilum coinfection frequency was noted as 4.96% (21/423). The prevalences of A. ovis in sheep and goats were 58.8% (127/216) and 42.51% (88/207) respectively, whereas the prevalences of A. phagocytophilum in sheep and goats were 11.11% (24/216) and 5.8% (12/207) respectively (Table 1).

The results according to the study location demonstrated A. ovis to be the most abundant in Edirne (75.81%) and Kırklarelî (55.77%) in sheep and goats respectively. A. phagocytophilum detection frequencies were highest in Kırklarelî with 23.08% and 15.38% observed for sheep and goats respectively (Table 1). The prevalence of A. ovis in sheep in Edirne was significantly higher compared to Istanbul and Tekirdag provinces, whereas it was significantly higher in Kırklarelî than Istanbul. No statistically-significant difference was noted of A. ovis detection rates in sheep among Kırklarelî, Edirne and Tekirdag provinces (P < 0.05) (Table 1). In Istanbul, prevalence of A. ovis in sheep was statistically lower than the other provinces while the prevalence of A. ovis in goats was only statistically lower than Kırklarelî (P < 0.05) (Table 1).

A comparison of A. phagocytophilum detection rates revealed a significantly higher the prevalence of A. phagocytophilum in goats in Kırklarelî province than the remaining provinces, while the prevalence of A. phagocytophilum in sheep was statistically higher than Istanbul and Edirne. A. phagocytophilum prevalence in sheep in Tekirdag was statistically similar to other provinces in the study (P < 0.05) (Table 1). In Europe, Ixodes ricinus (European sheep tick) acts as the main vector of A. phagocytophilum [39]. It has been reported that the Ixodes ricinus in Istanbul metropolitan area and in Kırklarelî were infected with A. phagocytophilum at a rate of 2.7% and 17.5% respectively [27]. In our study, the prevalence of A. phagocytophilum in Istanbul in sheep and goats were 5.77% and 1.92% respectively while the prevalence of A. phagocytophilum in Kırklarelî in sheep and goats were 23.08% and 15.38%, respectively.

The identities of the PCR products for A. ovis and A. phagocytophilum were verified by sequencing of the amplicons obtained from selected samples in the study. Despite the high level of similarity observed for A. ovis, A. phagocytophilum sequences demonstrated divergence up to 2.49% (Fig. 2). Several genotypes and variant clusters, some of which are associated with the host species have been characterized for A. phagocytophilum [40,41]. Moreover, phylogenetic analyses of the msp4 region were reported to differentiate strains of A. phagocytophilum obtained from ruminants from those obtained from humans, dogs, and horses [37]. However, sequence data from several regions have been utilized for a more precise interpretation of phylogenetic relations among A. phagocytophilum isolates. Given that sequence data was available only from selected samples and employed for confirmatory purposes, a thorough analysis of A. phagocytophilum sequence variations was not possible. Limited divergence was reported from various targets such as 16S rRNA and ankA sequences from Turkey [25,31].

So far, Anaplasma infections in small ruminants have not been documented in Thrace region of Turkey. Herein, we reported the presence and the distribution of Anaplasma ovis and A. phagocytophilum in sheep and goats in Thrace region of northwestern Turkey for the first time by using species-specific PCRs (Table 1). Potential vectors of Anaplasmosis are known to be endemic in Thrace region of Turkey [42-44]. Therefore further researches are needed to determine the vectors, vector-host interactions and genotypic variants that may affect the presence and distribution of Anaplasma species in Thrace region of northwestern Turkey.

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REFERENCES

1. Dumler JS, Barbet AF, Bekker CPJ, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Ronald PR: Reorganisation of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and ‘HGE agent’ as subjective synonyms of Ehrlichia phagocytophila. Int J Syst Evol Microbiol, 51, 2145-2165, 2001.
2. Rikihisa Y: The tribe Ehrlichiaceae and ehrlichial diseases. Clin Microbiol Rev, 4, 286-308, 1991.
3. Rymaszewska A, Grenda S: Bacteria of the genus Anaplasa - characteristics of Anaplasa and their vectors: A review. Veterinarni Medicina, 53 (11): 573-584, 2008.
4. Friedhoff KT: Tick-borne diseases of sheep and goats caused by Babesia, Theileria or Anaplasma spp. Parasitologia, 39, 99-109, 1997.
5. Tibbits T, Goff W, Forrey W, Stiller D: Susceptibility of two rocky mountain bighorn sheep to experimental infection with Anaplasma ovis. J Wildl Dis, 28 (1): 125-129, 1992. DOI: 10.7589/0090-3558-28.1.125
6. Kocan KM, de la Fuente J, Blouin EF, Garcia-Garcia JC: Anaplasma marginale (Rickettsiales: Anaplasmataceae): Recent advances in defining host-pathogen adaptations of a tick-borne Rickettsia. Parasitology, 129, 285-300, 2004. DOI: 10.1017/S0031182003004700
7. Uilenberg G: International collaborative research: Significance of tick-borne hemoraphic diseases to world animal health. Vet Parasitol, 57, 19-41, 1995. DOI: 10.1016/0304-4017(94)03107-8
8. Ioannou I, Sandalakis V, Kassinis N, Chochlakis D, Papadopoulos B, Loukaides F, Tselentis Y, Psaroulaki A: Tick-borne bacteria in mouflons and their ectoparasites in Cyprus. J Wildl Dis, 47, 300-306, 2011. DOI: 10.7589/0090-3558-47.2.300
9. Liu Z, Ma M, Wang Z, Wang J, Peng Y, Li Y, Guan G, Luo J, Yin H: Molecular survey and genetic identification of Anaplasma species in goats from central and southern China. Appl Environ Microbiol, 78, 464-
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