DOI 10.24425/pjvs.2024.149338

Original article

First detection of *Ehrlichia chaffeensis*, *Ehrlichia canis*, and *Anaplasma ovis* in *Rhipicephalus bursa* ticks collected from sheep, Turkey

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Abstract

Anaplasmosis and ehrlichiosis are important tick-borne rickettsial diseases of medical and veterinary importance that cause economic losses in livestock. In this study, the prevalence of *Anaplasma ovis, Ehrlichia canis* and *Ehrlichia chaffeensis* was investigated in ticks collected from sheep in various farms in Van province, which is located in the Eastern Anatolian Region of Turkey. The ticks used in this study were collected by random sampling in 26 family farm business in 13 districts of Van province. A total of 688 ticks were collected from 88 sheep and 88 tick pools were created. All ticks identified morphologically as *Rhipicephalus bursa*. Phylogenetic analysis of Chaperonin and 16S rRNA gene sequences confirmed *A. ovis, E. canis* and *E. chaffeensis* in this study. Of the 88 tick pools tested, 28.41% (25/88) were positive for at least one pathogen. Anaplasma DNA was detected in five of the 88 pools (5.68%), *E. canis* DNA was detected in 19 of the 88 pools (21.59%), and *E. chaffeensis* DNA was detected in one of the 88 pools (1.14%) of *R. bursa* ticks. To our knowledge, this is the first report describing the presence of *A. ovis*, *E. canis*, and *E. chaffeensis* in *R. bursa* ticks collected from sheep in Turkey. Further studies are needed to investigate other co-infections in sheep in Turkey.

Keywords: sheep, tick-borne parasitic diseases, Turkey

Introduction

Anaplasmosis and ehrlichiosis are important tick-borne rickettsial diseases of medical and veterinary importance that cause economic losses in livestock (Aktas et al. 2009, Zhang et al. 2014, Wang et al. 2021).

Anaplasmosis is a gram-negative, obligate intracellular bacterial disease that infects a variety of hosts (Aubry and Geale 2011, Tumwebaze et al. 2020). To date, seven *Anaplasma* species have been identified: *A. bovis, A. phagocytophilum, A. centrale, A. platys, A. marginale, A. ovis* and *A. capra* (Aktas et al. 2009, Liu et al. 2011, Tumwebaze et al. 2020, Wang et al. 2021). *A. ovis*, the most common cause of sheep anaplasmosis, has a worldwide distribution (Aubry and Geale 2011, Liu et al. 2011, Ma et al. 2011, Tumwebaze et al. 2020). The disease is mostly benign, but in endemic areas it can cause fever, depression, weakness, reduced milk production, weight loss, anemia, jaundice and abortion (Jalali et al. 2013, Belkahia et al. 2014, Yousefi et al. 2017, Enkhtaivan et al. 2019, Tumwebaze et al. 2020). In addition, *A. ovis* infection can predispose to other infectious or parasitic diseases that worsen the condition of animals and can lead to their death (Belkahia et al. 2014). Anaplasmosis is transmitted to mammalian hosts biologically by ticks and mechanically by biting flies and blood-contaminated fomites (Jalali et al. 2013, Yousefi et al. 2017).

Ehrlichia infection are tick-borne diseases that affect a variety of mammals worldwide, especially in tropical and subtropical regions (Zhang et al. 2017, Cicculli et al. 2019). The genus *Ehrlichia* consists of *E. chaffeensis, E. canis, E. ewingii, E. muris* and *E. ruminantium*, all of which can cause infections in both humans and domestic animals (Aktas et al. 2009, Cicculli et al. 2019). Canine monocytic ehrlichiosis is a serious tick-borne disease caused by *E. canis*. First described in Algeria in 1935, the disease has now been reported worldwide (Masala et al. 2012, Zhang et al. 2017). *E. chaffeensis* is the causative agent of human monocytic ehrlichiosis. It presents as a non-specific, febrile, influenza-like illness in humans. Characteristic clinical pathology findings are thrombocytopenia and leukopenia (Dugan et al. 2000, Wang et al. 2021). After infecting the host, *Ehrlichia* agents proliferate in monocytes and macrophages or peripheral blood neutrophils and spread to phagocyte cells of different organs such as the liver, spleen, lungs and lymph nodes, causing illness ranging from mild symptoms to a fatal condition in ruminants (Zhang et al. 2017).

In this study, the prevalence of *A. ovis, E. canis* and *E. chaffeensis* was investigated in ticks collected from sheep at various farms in Van province, which is located in the Eastern Anatolian Region of Turkey.

Materials and Methods

Study area and sample collection

This study was conducted in Van province in 2019-2020 (Fig. 1). Van province, which borders Iran, ranks first among the 14 provinces in the Eastern Anatolia Region of Turkey in terms of both population and small ruminant population (TUİK 2023). The ticks used in this study were collected by random sampling at 26 family farm businesses in 13 districts of Van province. A total of 688 ticks were collected from 88 sheep and 88 tick pools were created.

Tick morphology and DNA extraction

Ticks were placed in plastic tubes containing 1 mL to 3 mL 70% ethanol and kept at 4°C to 8°C until analysis. The ticks were examined by stereo light microscopy for determination of species. The detection of ticks was carried out using the method reported by Walker et al. (2000) and Estrada-Peña et al. (2004). Ticks inside the tubes were crushed using a sterile glass rod. DNA was extracted from tick samples using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA samples obtained were stored at -20°C for further analysis.

PCR amplification

Conventional PCR was used for *A. ovis* screening and nested PCR was used for *E. canis* and *E. chaffeensis* screening in the obtained DNA. The PCR method was used to detect *A. ovis* based on the Chaperonin gene, and to detect *E. canis* and *E. chaffeensis* based on the 16S rRNA gene. The PCR primers and cycling conditions used in this study are shown in Table 1. Subsequently, 1.5% agarose gel was prepared and stained with redSafeTM Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnum, Korea), PCR products were run at agarose gel, and images were obtained using gel imaging device (Syngene bioimaging system).

Sequence analysis and phylogeny

Two-way sequence analysis of positive samples was performed by a private company (BM Labosis, Ankara, Turkey). The DNA sequences obtained were checked and aligned in the BioEdit program and made available for analysis (Hall 1999). The edited sequences were then submitted to NCBI for BLASTn search, sequence alignment and analysis (Altschul et al. 1990). In order to create phylogenetic trees, data sets were aligned in the BioEdit program and a model test was

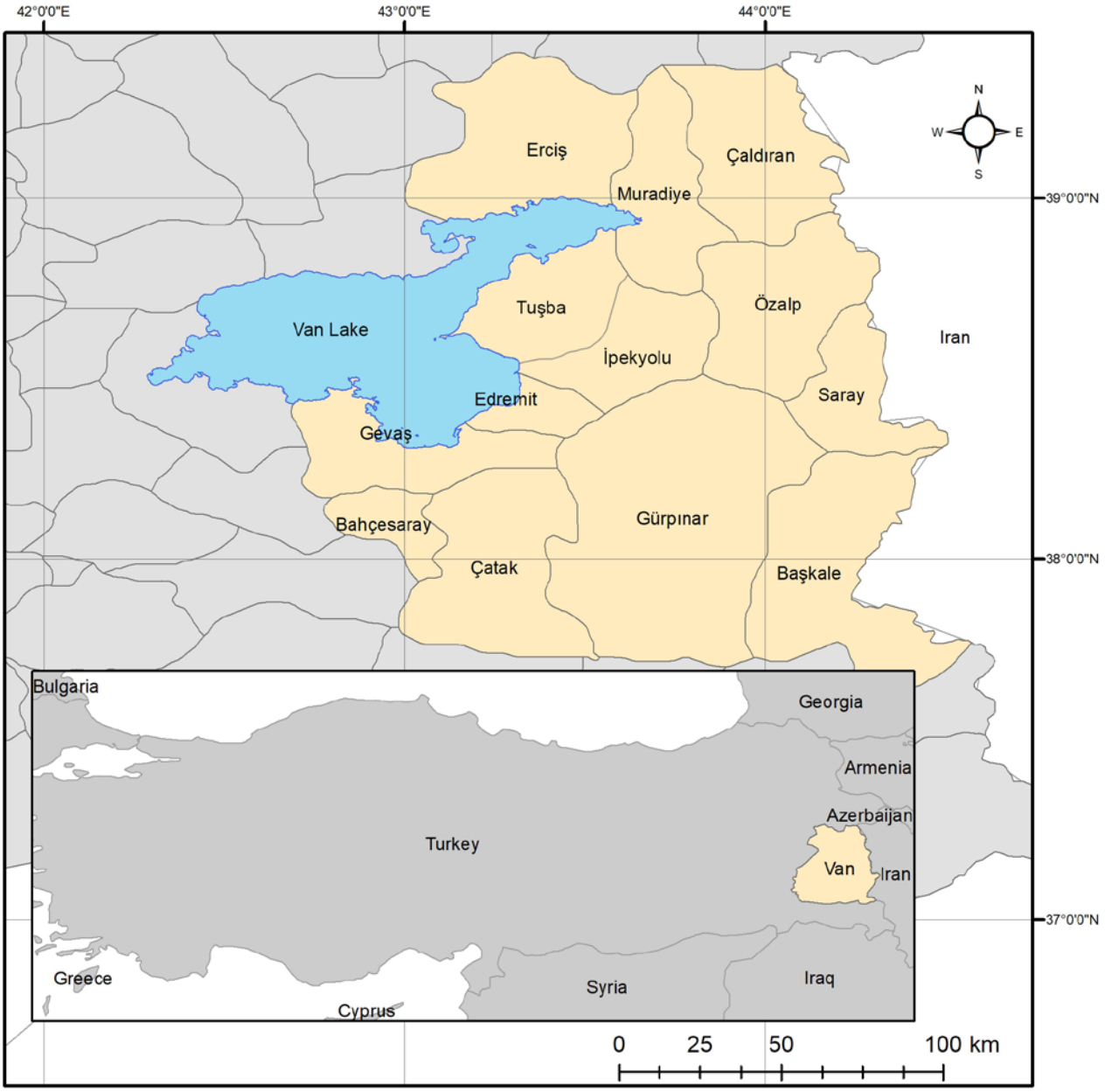

Fig. 1. Van Province map showing the sampling location.

Table 1. Primers used in this study to detect *Anaplasma ovis, Ehrlicia canis* and *Ehrlichia chaffeensis*.

Pathogen	Target gene	Primers (5′→ 3′)		Product (bp)	Annealing temperature (◦C)	Ref
A. ovis	Chaperonin	JH0011	TAAAAGCCAAGGAGGCTGTG	181	60	(Haigh et al. 2008)
		JH0012	TTGCTCTCCTCGACCGTTAT			
E. chaffeensis	16S rRNA	ECC	AGAACGAACGCTGGCGGCAAGC	458	55	(Murphy et al. 1998, Lee et al. 2005)
		ECB	CGTATTACCGCGGCTGCTGGCA			
		HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	396	55	
		HE3	TATAGGTACCGTCATTATCTTCCCTAT			
E. canis	16S rRNA	ECC	AGAACGAACGCTGGCGGCAAGC	458	55	(Murphy et al. 1998, Makino et al. 2015)
		ECB	CGTATTACCGCGGCTGCTGGCA			
		ECAN	CAATTATTTATAGCCTCTGGCTATAGGA	398	55	
		HE3	TATAGGTACCGTCATTATCTTCCCTAT			

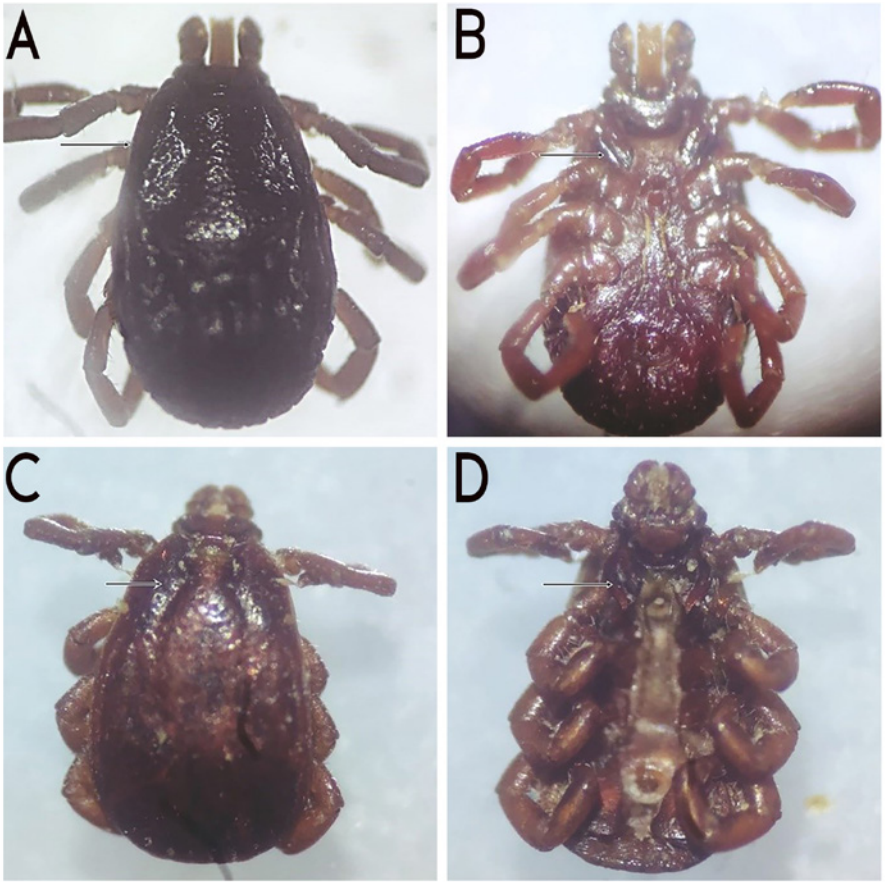

Fig. 2. *Rhipicephalus bursa* ticks collected in sheep. A) Female dorsal. Dense dotted scutum. B) Female ventral. Coxa I C) Male dorsal. Long and deep lateral groove D) Male vental. Coxa I.

performed using the Maximum Likelihood statistical method in the IQTREE program, and according to the Bayesian Information Criterion, the most appropriate model was determined to be TIM2+F+G4 for *Anaplasma* and TN+F+R2 for *Ehrlicia* and phylogenetic trees were created according to this model with 1000 bootstraps (Minh et al. 2013, Trifinopoulos et al. 2016).

Statistical analysis

The data obtained in the study were analyzed using the SPSS V16.0 (IBM, Chicago, IL, USA) program. The relationship between grouped variables was calculated using the Chi-square test. The difference was considered statistically significant when $p<0.05$.

Ethical approval

This study was approved by Van Yuzuncu Yıl University Animal Experiments Local Ethics Committee (Approval number2023/03-09).

Results

In total, 688 adult ticks (312 male and 376 female) were collected and identified morphologically as *Rhipicephalus bursa* (Fig. 2). Of the 88 tick pools tested, 28.41% (25/88) were positive for at least one pathogen. *Anaplasma* DNA was detected in five of the 88 pools (5.68%), *E. canis* DNA was detected in 19 of the 88 pools (21.59%), and *E. chaffeensis* DNA was detected in one of the 88 pools (1.14%). Two co-infections by two pathogens (*E. canis* + *E. chaffeensis* and *A. ovis* + *E. canis*) were also detected. In relations to the locations, the highest prevalence was detected in Çatak and İpekyolu districts (40%), while the lowest prevalence was detected in Bahçesaray, Edremit and Gevaş districts (0%). ($p>0.05$) (Table 2).

As a result of this study, 25 sequences obtained from PCR positive samples were deposited in GenBank. BLAST analysis showed that *A. ovis, E. chaffeensis* and *E. canis* obtained in this study have 99.28-100%, 100% and 99.38-100% identity, respectively, when compared with the data sets in GenBank (Table 3). Phylogenetic analysis of Chaperonin and 16S rRNA gene sequences confirmed *A. ovis, E. canis* and *E. chaffeensis* in this study (Fig. 3, 4).

Table 2. Prevalence of *Anaplasma ovis, Ehrlicia canis* and *Ehrlichia chaffeensis*.

District	Tick Pool	*Anaplasma ovis*		*Ehrlichia canis*		*Ehrlichia chaffeensis*	
	(n)	(n)	%	(n)	%	(n)	%
Bahçesaray	3	0	0.00	0	0.00	0	0.00
Başkale	13	1	7.69	4	30.77	0	0.00
Çaldıran	7	0	0.00	1	14.29	0	0.00
Çatak	5	0	0.00	2	40.00	0	0.00
Edremit	2	0	0.00	0	0.00	0	0.00
Erciş	11	0	0.00	2	18.18	0	0.00
Gevaş	3	0	0.00	0	0.00	0	0.00
Gürpınar	12	0	0.00	4	33.33	0	0.00
İpekyolu	5	1	20.00	1	20.00	0	0.00
Muradiye	6	0	0.00	2	33.33	0	0.00
Özalp	11	1	9.09	2	18.18	1	9.09
Saray	7	1	14.29	1	14.29	0	0.00
Tuşba	3	1	33.33	0	0.00	0	0.00
Overall	**88**	**5**	**5.68**	**19**	**21.59**	**1**	**1.14**

Table 3. DNA sequences deposited in GenBank as a result of this study.

Obtained Sequences						Reference sequences from GenBank
Pathogen	Host	Target Gene	Accession number	Length (bp)	Identitzy (%)	Accession number
A. ovis	Sheep	Chaperonin	OR077274	184	100	LC553520, LC553522
A. ovis	Sheep	Chaperonin	OR077275	131	98.47	JF260866, JF260869
A. ovis	Sheep	Chaperonin	OR077276	143	99.28	LC553520, LC553522
A. ovis	Sheep	Chaperonin	OR077277	138	99.28	LC553520, LC553522
A. ovis	Sheep	Chaperonin	OR077278	132	100	LC553520, LC553522
E. chaffeensis	Sheep	16S rRNA	OR066438	340	100	KY644145, KY644143
E .canis	Sheep	16S rRNA	OR095091	323	99.38	JQ260853, JQ260849
E .canis	Sheep	16S rRNA	OR066439	361	100	OP268428, OP268421
E .canis	Sheep	16S rRNA	OR066440	376	100	OP268428, OP268418
E .canis	Sheep	16S rRNA	OR066441	340	99.71	KY434112, KX165358
E .canis	Sheep	16S rRNA	OR066442	369	100	MN396361, MH620200
E .canis	Sheep	16S rRNA	OR066443	340	99.71	MG793442, MH234591
E .canis	Sheep	16S rRNA	OR066444	376	100	OP268428, OP268417
E .canis	Sheep	16S rRNA	OR066445	385	100	OP268428, OP268417
E .canis	Sheep	16S rRNA	OR066446	393	99.74	OP268428, OP268418
E .canis	Sheep	16S rRNA	OR066447	386	99.74	OP268428, OP268418
E .canis	Sheep	16S rRNA	OR066448	386	99.74	OP268428, OP268418
E .canis	Sheep	16S rRNA	OR066449	373	100	MH620200, MK507008
E .canis	Sheep	16S rRNA	OR066450	385	100	MH620200, MK507008
E .canis	Sheep	16S rRNA	OR066451	392	100	OP268428, OP268417
E .canis	Sheep	16S rRNA	OR066452	375	100	OP268428, OP268417
E .canis	Sheep	16S rRNA	OR066453	340	100	MG793442, MH234591
E .canis	Sheep	16S rRNA	OR066454	357	100	MG793442, MH234591
E .canis	Sheep	16S rRNA	OR066455	340	100	MG793442, MH234591
E .canis	Sheep	16S rRNA	OR066456	381	100	MH620200, MK507008

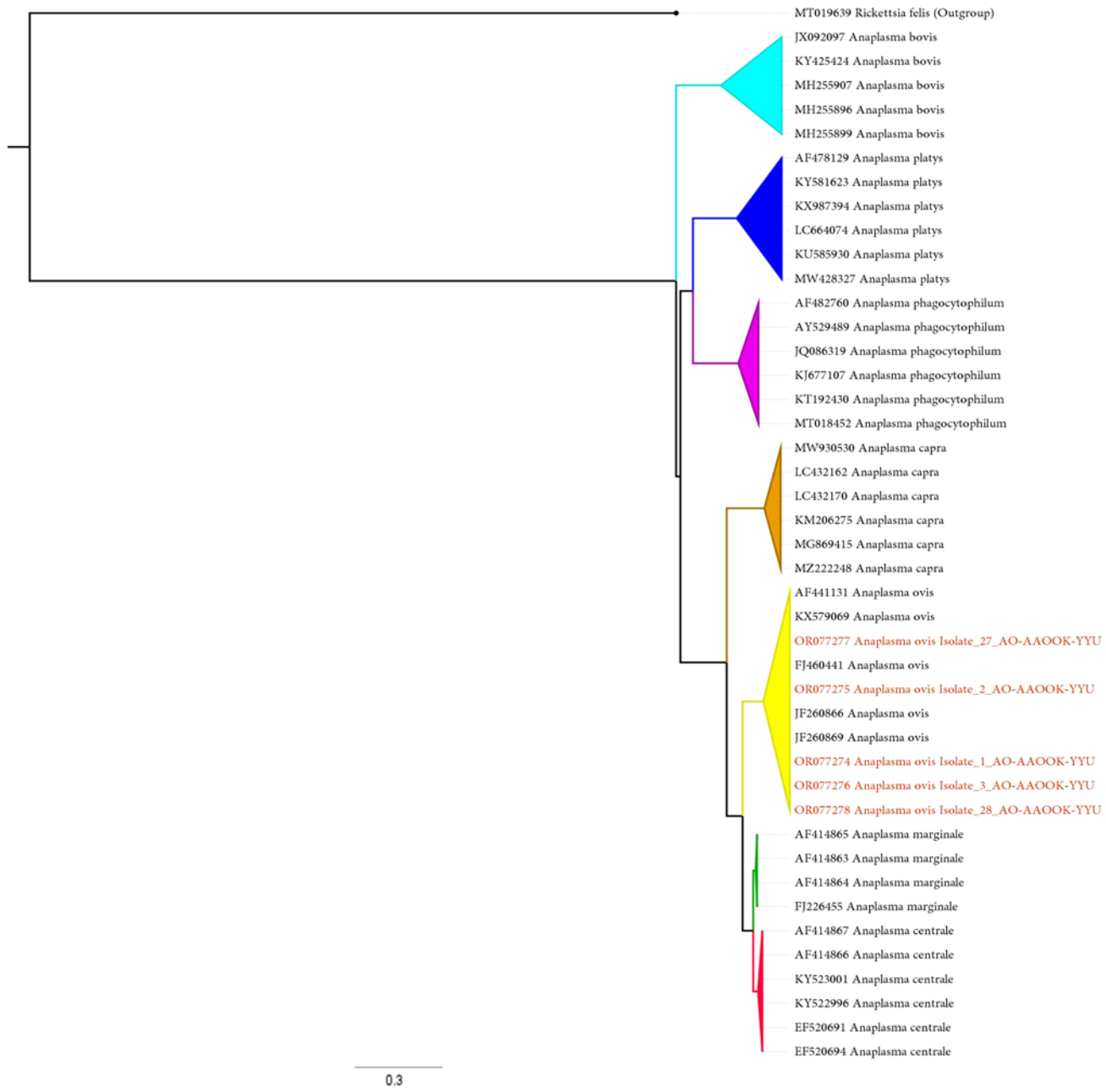

Fig. 3. Phylogenetic relationships of *Anaplasma ovis* isolates using Maximum Likelihood method analysis based on the Chaperonin gene region. Numbers in nodes represent Bootstrap values (1000 bootstrap). *Rickettsia felis* was used as an outgroup.

Discussion

Tick-borne pathogens of sheep represent a serious threat to veterinary and public health worldwide (Li et al. 2020).

Anaplasmosis caused by *A. ovis* in sheep is widespread in Asia, Europe, the Mediterranean, and North and South America (Enkhtaivan et al. 2019). *A. ovis* prevalence has been found in different regions of the world including Italy 82.9% (Alessandra and Santo 2012), Tunisia 70.1% (Belkahia et al. 2014), Iran 87.4% (Jalali et al. 2013), Portugal 82.5% (Renneker et al. 2013), Iraq 66.7% (Renneker et al. 2013), Sudan 41.7% (Renneker et al. 2013), China 69.2% (Ma et al. 2011), Mongolia 69% (Enkhtaivan et al. 2019), France 7.9% (Dahmani et al. 2017) and Egypt 9.1% (Tumwebaze et al. 2020). In a previous study conducted with blood samples in Turkey, the prevalence of *A.ovis* was reported to be 31.4% (Renneker et al. 2013).

Although microscopic and serological methods are used in the diagnosis of *Anaplasma*, molecular methods are increasingly used as more sensitive and specific diagnostic tools to detect and differentiate *Anaplasma* species in carrier animals (Jalali et al. 2013). In this

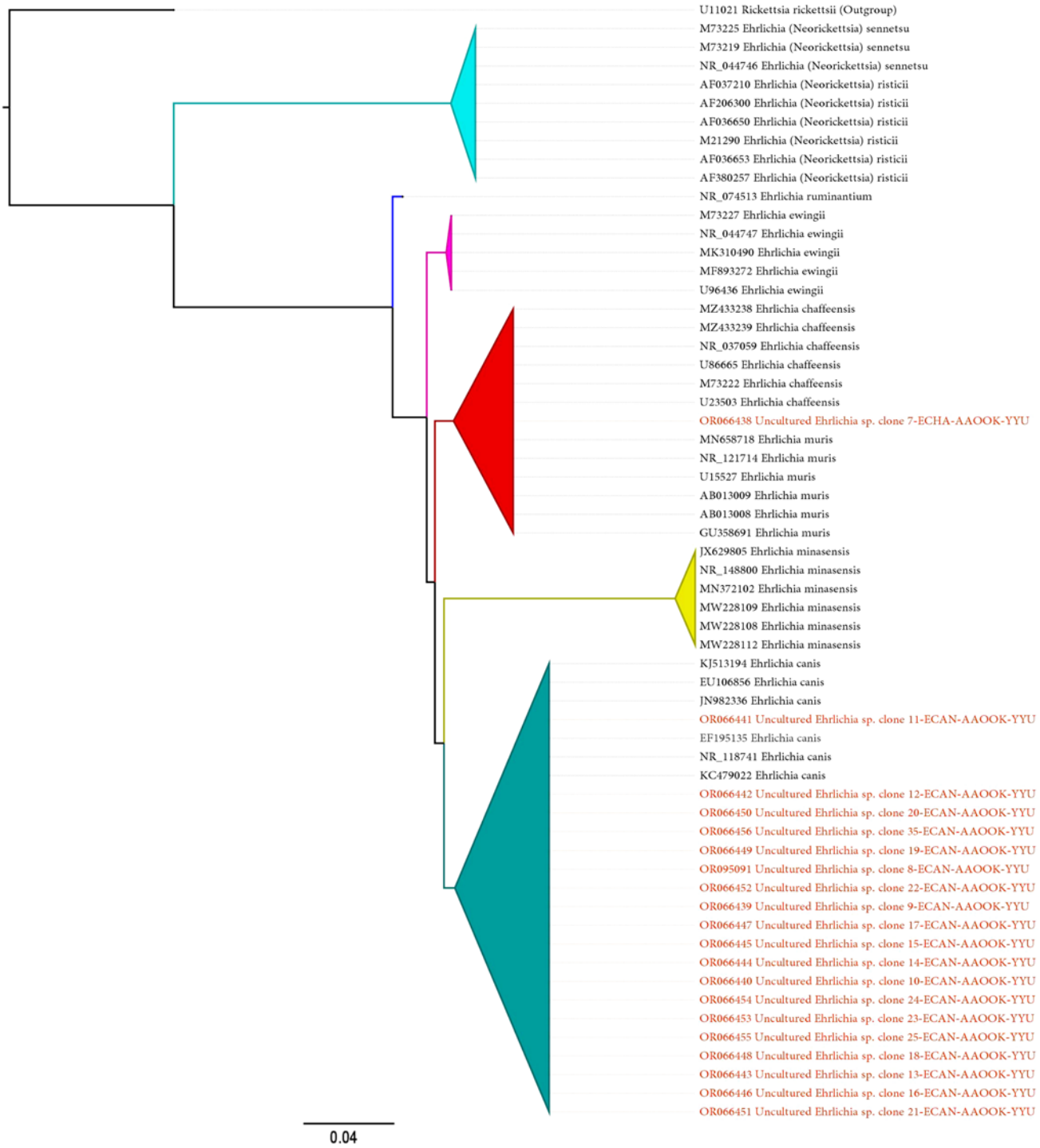

Fig. 4. Phylogenetic relationships of *Ehrlicia canis* and *Ehrlicia chaffeensis* isolates using Maximum Likelihood method analysis based on 16S rRNA gene region. Numbers in nodes represent Bootstrap values (1000 bootstrap). *Rickettsia rickettsii* was used as an outgroup.

study, the conventional PCR method was used to investigate the presence of *A. ovis*. Ovine anaplasmosis is mainly caused by *A. ovis* and *A. marginale* (Li et al. 2020). In this study, *A. ovis* DNA was found in 5 tick pools (%5.68). For confirmation of the PCR results, phylogenetic studies of *A. ovis* and for the determination of the genetic characterisation of *A. Ovis* Chaperonin gene was used in sequencing. BLAST analysis of *A. ovis* showed 100% and 98.4% similarity to reported strains of *A. ovis* in Africa (LC553520, LC553522) and Mongolia (JF260866, JF260869) respectively. The result of present study indicate that the prevalence was lower than the other studies conducted in Italy (Alessandra and Santo 2012), Tunisia (Belkahia et al.

2014), Iran (Jalali et al. 2013), and Portugal (Renneker et al. 2013) but similar to the prevalence in France (Dahmani et al. 2017) and Eygpt (Tumwebaze et al. 2020).

Although *A. ovis* has been detected in several tick species, the vectorial capacity of these ticks is unknown as most of these studies analyzed ticks collected from animal bodies (Enkhtaivan et al. 2019). Confirmed tick vectors of *A. ovis* include *R. bursa* and *Dermacentor andersoni* (Kocan and Blouin 2008, Noaman 2012, Enkhtaivan et al. 2019). *Dermacentor* spp. and *R. bursa* are vectors of *A. ovis* respectively in the New World as well as the Old World (Belkahia et al. 2014). Some tick species such as *D. nuttalli* in Mongolia (Enkhtaivan et al. 2019), *Rhipicephalus sanguineus, Hyalomma marginatum marginatum,* and *Hyalomma anatolicum anatolicum* in Iran (Noaman 2012), *R. turanicus, R. sanguineus* and *R. annulatus* in Tunusia (Belkahia et al. 2014), *R. turanicus* in Italia (Torina et al. 2008), and *R. sanguineus* in Turkey (Aktas et al. 2009) have been proposed previously as vectors of *A. ovis*. In a study conducted in France, it was determined that all ticks collected from sheep were *R. bursa* and it was reported that 7.9% of these ticks had *A. ovis* DNA (Dahmani et al. 2017). In a previous study conducted to determine tick species in Turkey, 20.2% of 2518 ticks collected were reported to be *R. bursa* (Yılmaz and Deger 2011). The fact that all tick samples collected in this study were identified as *R. bursa* supports such previous research (Yılmaz and Deger 2011, Dahmani et al. 2017, Enkhtaivan et al. 2019)

E. chaffeensis is the etiological agent of human monocytic ehrlichiosis, which is characterized by non-specific clinical manifestations such as fever, headache, myalgia, chills, malaise, anorexia and vomiting in infections (Cao et al. 2000). *E. chaffeensis* was reported in humans in Italy 8% (Santino et al. 1998), in yaks in China 0.6% (Wang et al. 2021), in goats in Georgia 73.7% (Dugan et al. 2000), in ticks in the USA 2.6% (Cohen et al. 2010) and in ticks in Korea 4.2% (Lee et al. 2005). In this study, *E. chaffeensis* DNA was detected in one tick pool (1.14%). This result is similar to the results of Wang et al. (2021) and Cohen et al. (2010). BLAST analysis of *E. chaffeensis* showed 100% similarity to reported strains of *E. chaffeensis* in Argentina (KY644143, KY644145). Among the tick species that may serve as vectors for *E. chaffeensis*; *Amblyomma americanum* (Anderson et al. 1993, Cohen et al. 2010), *Amblyomma testudinarium* (Cao et al. 2000), *Dermacentor variabilis* (Roland et al. 1998), *Ixodes pacificus* (Kramer et al. 1999) and *Haemaphysalis longicornis* (Lee et al. 2005) have been reported. In this study, *E. chaffeensis* was detected in *R. bursa* ticks. In the literature review, no report on the presence of *E. chaffeensis* in *R. bursa* ticks was found.

Canine monocytic ehrlichiosis is a serious tickborne disease caused by *E. canis* (Masala et al. 2012). *E. canis* was reported in dogs in Tailand 21.5% (Piratae et al. 2015), in Brazil 26.8% (Macieira et al. 2005), in Mexico 38.46% (Ojeda-Chi et al. 2019), in Italy 6.4% (Solano-Gallego et al. 2006) and in Egypt 9.7% (Selim et al. 2020). In the present study, *E. canis* DNA was detected in 19 tick pools (21.59%). This result was similar to the results of Macieira et al. (2005) and Piratae et al. (2015). BLAST analysis of *E. canis* showed 100% similarity to reported strains of *E. canis* in Mexico (OP268421), the USA (MH620200), and Cuba (MK507008). The principal vector of *E. canis* is known to be the Brown dog tick *R. sanguineus* (Masala et al. 2012). However, it has been associated with various tick species such as *R. bursa* (Masala et al. 2012, Dahmani et al. 2017), and *Dermacentor variabilis* (Johnson et al. 1998), which may serve as a vector for *E. canis*. In this study, *E. canis* was detected in *R. bursa* ticks, similarly to the study conducted by Masala et al. (2012) and Dahmani et al. (2017). Differences between studies may be due to geographical conditions, climate, different animal species, tick control programs, methodology, antibiotic applications and vector density.

Conclusion

Tick-borne diseases are becoming increasingly important throughout the world. The vector role of *R. bursa* ticks to *A. ovis*, *E. canis*, and *E. chaffeensis* has not been sufficiently investigated in Turkey. The results of the sequencing analysis in this study revealed the presence of *A. ovis*, *E. canis*, and *E. chaffeensis* in sheep. To our knowledge, this is the first report describing the presence of *A. ovis*, *E. canis*, and *E. chaffeensis* in *R. bursa* tick collected from sheep in Turkey. The current results therefore present valuable information about ovine tick-borne infections in Turkey. Further studies are needed to investigate other co-infections in sheep in Turkey.

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