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Investigation of Vector-Borne Diseases in Dogs

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Abstract: In this study, a total of 186 blood samples were collected from kennel dogs consisting of 104 male and 82 female in five provinces (Mersin, Adana, Hatay, Gaziantep and Batman) of Turkey, and evaluated using molecular methods for the presence of canine vector-borne diseases (CVBDs). Overall, 10.8% of the sampled dogs were found to be infected with one or more CVBD pathogens investigated. *Ehrlichia canis* (17/186; 9.1%) was the most common CVBD pathogen, followed by *Babesia canis vogeli* (5/186; 2.7%) and *Hepatozoon canis* (1/186; 0.5%), respectively. Co-infection of *E. canis* with *B. canis* was detected in 3 (1.6%) dogs. Infection with *Rickettsia* spp., *Coxiella burnetii*, *Borrelia burgdorferi s.l., Francisella tularensis*, *Bartonella* spp., *Leishmania* spp., *Diroflaria immitis*, *Diroflaria repens*, and *Acanthocheilonema reconditum* were not detected. No sex association with CVBDs was determined (p>0.05). The result of the study indicates the presence of three CVB pathogens, including the first report of *B. canis* and *H. canis* in the studied provinces.
Keywords: *Canine vector-borne pathogen, Dog, Molecular characterization.*

Köpeklerde Vektör Kaynaklı Hastalıkların Araştırılması

Özet: Bu çalışmada, Türkiye'nin beş farklı ilindeki (Mersin, Adana, Hatay, Gaziantep ve Batman) köpek barınaklarından alınan 186 (104'ü erkek ve 82'si dişi) kan örneği vektör kaynaklı nakledilen patojenler yönünden moleküler yöntemlerle araştırıldı. İncelenen örneklerin %10.8'inin en az bir veya birden fazla patojen ile enfekte olduğu tespit edildi. *Ehrlichia canis* (17/186; %9.1) en yaygın vektör aracılı nakledilen patojen olup, bunu sırasıyla *Babesia canis* vogeli (5/186; %2.7) ve *Hepatozoon canis* (1/186; %0.5) izledi. *E. canis* ve *B. canis* ortak enfeksiyonu 3 (%1.6) köpekte tespit edildi. *Rickettsia* spp., *Coxiella burnetii*, *Borrelia burgdorferi s.l*., *Francisella tularensis*, *Bartonella* spp., *Leishmania* spp., *Diroflaria immitis*, *Diroflaria repens* ve *Acanthocheilonema reconditum* enfeksiyonu saptanmadı. Vektör aracılı nakledilen patojenler yönünden pozitif bulunan köpeklerde yaş ve cinsiyet yönünden istatistiksel olarak önemli bir fark belirlenmedi (p> 0.05). Çalışılan illerde köpeklerde vektör aracılı nakledilen patojenlerden üçünün varlığı gösterilmiş ve çalışılan illerde ilk kez *B. canis* ve *H. canis* varlığı tespit edilmiştir.
Anahtar Kelimeler: *Köpek, Moleküler karakterizasyon, Vektör kaynaklı patojen.*

Introduction

Canine vector-borne diseases (CVBDs) constitute a large group of diseases that are of great importance on canine health status. CVBDs are caused by a variety of pathogens of bacteria, viruses, protozoa, and helminths, transmitted by arthropods (e.g. ticks, fleas, lice, mosquitoes, phlebotomine sandflies) (Otranto et al., 2009a). Besides their importance for canine health, CVBDs have an impact on public health due to their zoonotic character (Maggi and Krämer, 2019). CVBDs have a wide range of clinical manifestations, changing from asymptomatic cases to serious health implications, depending on the pathogenicity of the causative agent, the susceptibility of the host, the presence of single or co-infections, which makes diagnosis, control and treatment of CVBDs more challenging for veterinarian practitioners (Otranto et al., 2009b).

Distribution and incidence of many CVBDs have been attributed to a plethora of anthropogenic factors, including climate change, globalization, a significant increase in international trade, tourism, travel, and the rapid growth of human, expansion of canine and wildlife reservoir populations (Duscher et al., 2014; Maggi and Krämer, 2019). Among these factors, climate changes are the main factors involved in the density and life cycles of vectors as well as their habitats (Fouque and Reeder, 2019). Apart from the life cycles of vectors, environmental temperature also affects the survival rates of microorganisms carried by vectors and definitive hosts (Semenza and Menne, 2009). Due to the dynamic nature of the abovementioned factors, continuous surveillance for the determination of the prevalence, incidence, and spatial distribution

of CVBDs is an integral part of the prevention, and control programs (Self et al., 2019).

In previous studies involving dog populations in Turkey, the presence of many CVBDs has been reported by molecular methods (Aktaş et al., 2015; Düzlü et al., 2014; Guo et al., 2017; Güven et al., 2017; Karagenç et al., 2005; Orkun et al., 2018). However, the majority of these studies focused on either a particular pathogen(s) or in a restricted area. Therefore, this study aimed to determine the current situation of vector-borne pathogens causing babesiosis, hepatozoonosis, leishmaniasis, toxoplasmosis, anaplasmosis, filariasis, rickettsiosis, bartonellosis, ehrlichiosis, Q-fever, borreliosis, and tularemia using molecular methods in shelter dogs in five different cities in Turkey.

Materials and Methods

Ethical approval: The study was conducted in compliance with the Animal Ethical Committee of Hatay Mustafa Kemal University with the decision number of 2020/02-12.

Study area and sample collection: The study was conducted on shelter dogs in five provinces (Hatay, Adana, Mersin, Gaziantep, and Batman) of Turkey. The blood samples (2-3 ml) were collected into EDTA-coated vacutainer tubes from 186 dogs between May 2020 and August 2020. During sampling time, data regarding sex and age were also recorded, as presented in Table 1. All the dogs included in the study were clinically healthy and not infested with ectoparasites.

Table 1. Sample distribution according to sex, age and locations

Location	Sex	Age (year)				Total
		1<	1-3	3-6	>6	
Hatay	Female	1	8	6	4	19
	Male	4	8	9	1	22
	Total	5	16	15	5	41
Mersin	Female	3	5	4	3	15
	Male	5	6	6	4	21
	Total	8	11	10	7	36
Adana	Female	3	11	3	5	22
	Male	5	7	7	4	23
	Total	8	18	10	9	45
Batman	Female	3	10	5	3	21
	Male	2	11	10	6	29
	Total	5	21	15	9	50
Gaziantep	Female	0	1	2	2	5
	Male	0	5	3	1	9
	Total	0	6	5	3	14
Total	Female	10	35	20	17	82
	Male	16	37	35	16	104
	Total	26	72	55	36	186

DNA isolation and PCR analysis: Genomic DNA was extracted from blood samples using PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, California, USA) by the manufacturer's recommendations. Extracted DNA samples were stored at -20 ^{o}C until molecular analysis. Conventional and real-time PCR was performed to detect bacterial and protozoal pathogens, and DNase-RNase-free sterile water was used as a negative control, and positive control DNA extracted from the pathogens were included in each reaction. The PCR methods, target genes, and primer sequences used in the study are given in Table 2.

Table 2. Primers used in this study

Pathogen	Methods	Target gene	Primer sequences	Product size (bp)	Reference
Anaplasma spp., *Ehrlichia* spp.	Real time-PCR/ PCR	*gro*EL	ESpF- TACTCAGAGTGCTTCTCAATGT ESpR- GCATACCATCAGTTTTTTCAAC	362	Bell and Patel (2005)
Rickettsia spp.	Real-time-PCR Taqman prob	23S rRNA	PanR8F- AGCTTGCTTTTGGATCATTTG G PanR8R- TTCCTTGCCTTTTCATACATCTAGT PanR8-P- Fl-CCTGCTTCTATTTGTCTTGCAGTAACACGCCA-BHQ1	111	Kato et al. (2013)
Coxiella burnetii	Real-time-PCR/ Sybr-green	*omp*A	CoxF- CAGAGCCGGGAGTCAAGCT CoxR- CTGAGTAGGAGATTTGAATCGC	82	Jaton et al. (2013)
Francisella tularensis	Real-time-PCR Taqman prob	*tul*4	Tul4F-ATTACAATGGCAGGCTCCAGA Tul4R-TGCCCAAGTTTTATCGTTCTTCT Tul4P-TCTAAGTGCCATGATACAAGCTTCCCAATTACTAAG BHQ1)	91	Versage et al. (2003)
Bartonella spp.	Real-time-PCR/Sybr-green	*ssr*A	ssrA F-GCTATGGTAATAAATGGACAATGAAATAA ssrA R-GCTTCTGTTGCCAGGTG	301	Diaz et al. (2012)
Babesia spp. *Hepatozoon* spp, *Theileria* spp., *Hemolivia mauritanica*	PCR	18S rRNA	BJ1- GTCTTGTAATTGGAATGATGG BN2- TAGTTTATGGTTAGGACTACG	411–452	Casati et al. (2006)
Leishmania spp.	Real-time-PCR /Evagreen	ITS1	LSGITS1-F1-CATTTTCCGATGATTACAC LSGITS1-R1-CGTTATGTGAGCCGTTATC	220 to 275	De Almeida et al. (2017)
Pan-filarial	PCR	5.8 S-ITS2-228S	DIDR-F1-AGTGCGAATTGCAGACGCATTGAG DIDR-R1-AGCGGGTAATCACGACTGAGTTGA	484-578	Rishniw et al. (2006)
Diroflaria immitis	PCR	COI	DICOI-F1-AGTGTAGAGGGTCAGCCTGAGTTA DICOI-R1-ACAGGCACTGACAATACCAAT	203	
Acanthocheilonema reconditum	PCR	COI	ARCOI-F1AGTGTTGAGGGACAGCCAGAATTG ARCOI-R1-CCAAAACTGGAACAGACAAAACAAGC	208	
Diroflaria repens	PCR	COI	DRCOI-F1AGTGTTGATGGTCAACCTGAATTA DRCOI-R1GCCAAAACAGGAACAGATAAAACT	209	

Sequencing and phylogenetic analysis: The successfully amplified PCR products were purified and bidirectionally sequenced at a commercial sequencing service provider (Macrogen, Netherlands). Obtained nucleotide sequences were compared with registered GenBank sequences using BLAST analysis (www.ncbi.nlmn.nih.gov/BLAST). The sequences were edited and aligned using BioEdit software (Hall, 1999). The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers MN250296, MN364708-MN364722 for *E. canis*, MT908962-MT908966 for *B. canis*, and MT909554 for *H. canis*.

Phylogenetic analysis: Phylogenetic relationships between the sequences were inferred using the maximum likelihood method (ML) with the MEGAX.0 software (Kumar et al. 2018).

Statistical analysis: Statistical differences between vector-borne pathogens and variables including sex and age for significance were assessed through Pearson's Chi-square using SPSS v.14·0 software. A P-value less than 0·05 was regarded as statistically significant.

Results

The overall infection rate of CVBD pathogens was 10.8% (20/186). Frequency of positivity of *E. canis*, *B. canis* and *H. canis* was 9.1% (17/186), 2.7% (5/186), and 0.5% (1/186), respectively. No positivity for other CVBD pathogens was detected (Table 3). Simultaneous infection by two CVBD pathogens were only observed in 3 (15%) of the infected dogs (Table 4). No difference between positivity to CVBD pathogens, sex was determined ($p>0.05$). While the highest number of positivity was determined from the province of Mersin with 30.6%, the lowest was Hatay (4.9%). No CVBD pathogen was detected in the province of Batman. Phylogenetic trees were illustrated in Figure 1-3.

Table 3. The frequency of CVBD pathogens detected by PCR and DNA sequencing according to provinces

Province	No of dogs tested	Pathogens identified			Total
		E. canis	*B. canis*	*H. canis*	
Mersin	36	11	4	0	15
Adana	45	4	0	0	4
Hatay	41	0	1	1	2
Batman	50	0	0	0	0
Gaziantep	14	2	0	0	2
Total	**186**	**17**	**5**	**1**	**23**

Table 4. Distribution and frequency of CVBD pathogens in sampled dogs, detected by DNA amplification and DNA sequencing

Infection status	Identified pathogen	n	%
Single infection	*E. canis*	14	7.5
	B. canis	2	1.1
	H. canis	1	0.5
Mixed infection	*E. canis* + *B. canis*	3	1.6
Negative		166	89.2
Total		**186**	**100**

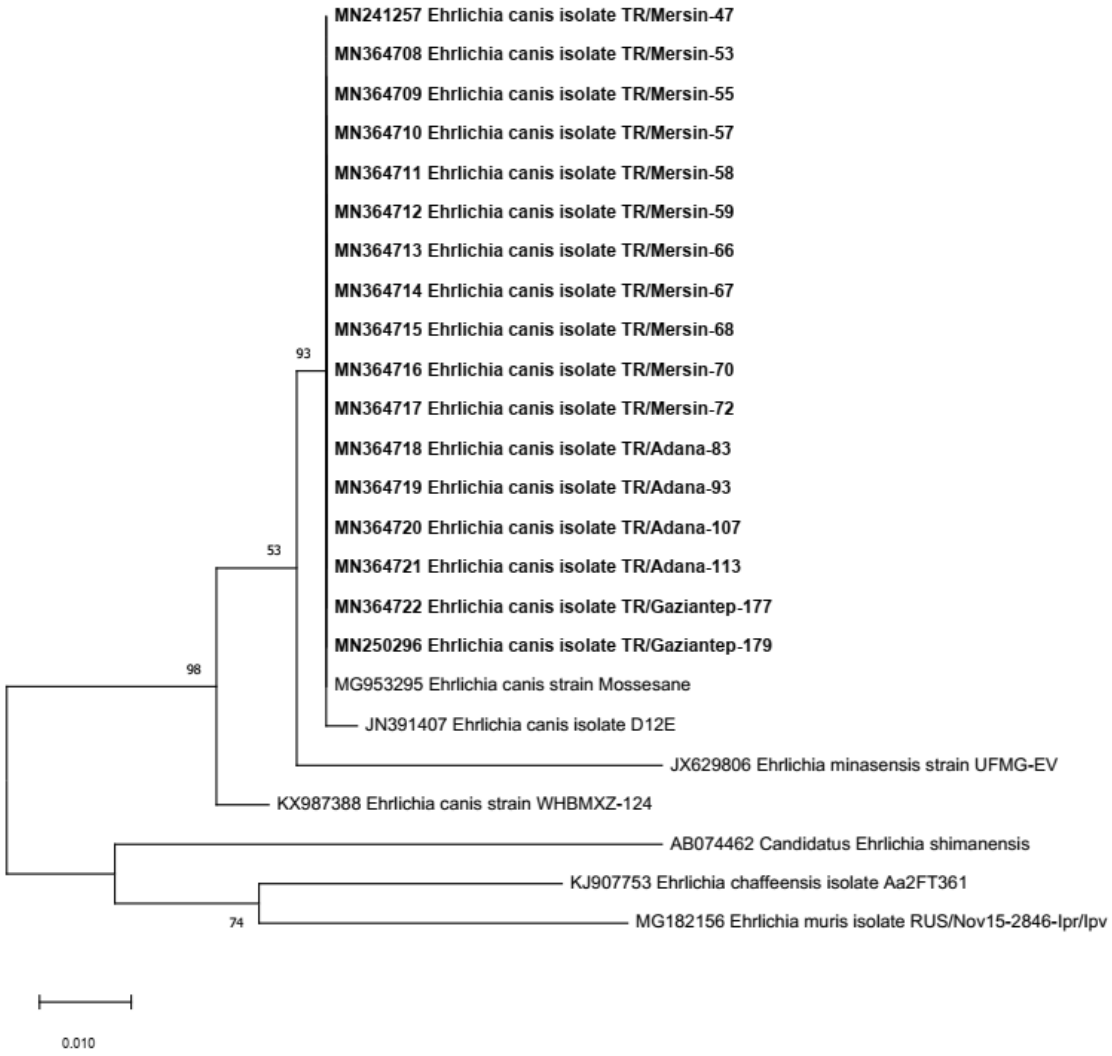

Figure 1. Phylogenetic tree based on aligned sequences 16S rRNA of *E. canis* isolates using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with 1000 bootstrap. The *E. canis* sequences generated in this study are indicated in bold. GenBank accession numbers of sequences and names of lineages are given before species names.

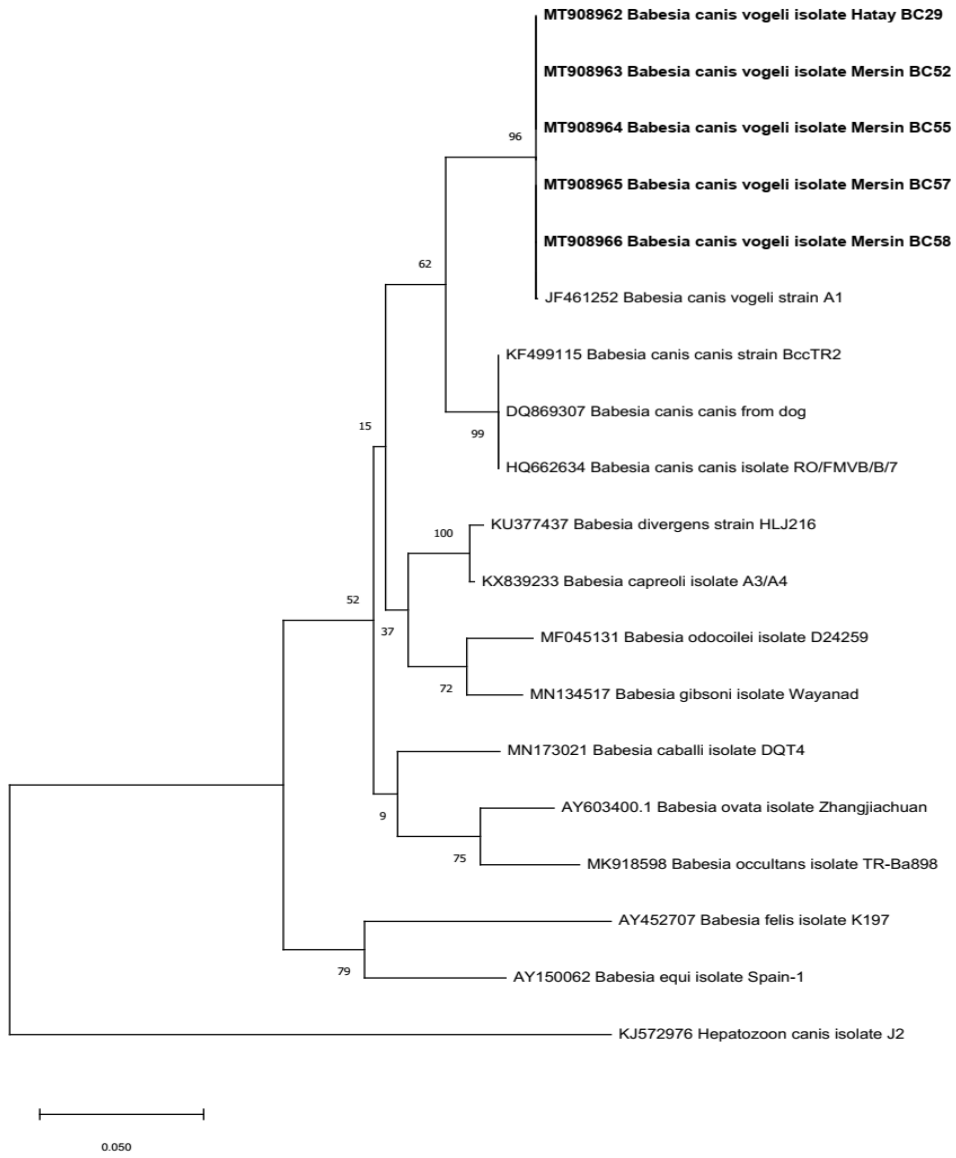

Figure 2. Phylogenetic tree based on aligned sequences 18S rRNA of *B. canis* isolates constructed by using Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with 1000 bootstrap. The *B. canis* sequences generated in this study are indicated in bold. GenBank accession numbers of sequences and names of lineages are given before species names.

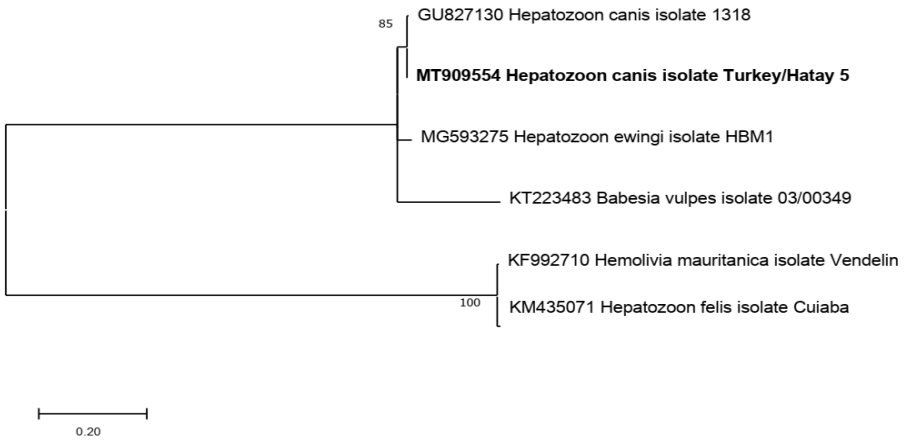

Figure 3. Phylogenetic tree based on aligned sequences 18S rRNA of *H. canis* isolate constructed by using Maximum Likelihood method and Tamura 3 Model model (Tamura, 1992) with 1000 bootstrap. The *H. canis* sequence generated in this study are indicate in bold. GenBank accession numbers of sequences and names of lineages are given before species names.

Discussion

CVBDs constitute a varied and complex group of diseases posing an important threat for both animal and human health, and the geographic distribution and incidence of CVBDs are on the rise worldwide (Baneth et al., 2012). *E. canis* (9.1%), the etiological agent of canine monocytic ehrlichiosis (CME), was the most common CVBD pathogen detected in the study. Apart from being an important veterinary pathogen, human infections with *E. canis* have been also reported (Perez et al., 1996; Perez et al., 2006; Bouza-Mora et al., 2017). The main vector of the agent is the brown dog tick *Rhipicephalus sanguineus* (*s.l.*), which is also dominant species in dog populations in Turkey (Aktaş et al., 2013). In previous studies, various rates of positivity have been reported depending on the regions of Turkey. In the Aegean region of Turkey, the prevalence of *E. canis* was reported as 41.5% (Karagenç et al., 2005). Düzlü et al. (2014) reported a prevalence rate of 14.5% in Kayseri; Guo et al. (2017) no positivity for *E. canis* was reported in Konya. Güven et al., (2017) reported a prevalence rate of 9.77% in Erzurum. Aktaş et al. (2015) investigated tick-borne bacterial and protozoal diseases in dog blood sample collected from 10 provinces located in different regions of Turkey using reverse line blotting (RLB) and sequencing, and found positivity for *E. canis* in only four provinces, percentage of which were ranging between cities as 8.1-% and 28%. The spatial distribution of *E. canis* observed in dogs in different provinces of Turkey could be attributed to different dog populations sampled and climatic conditions affecting the vector dynamics.

Canine babesiosis is an important CVBD infection with a worldwide spread. Dogs can be infected by different Babesia species including large Babesia species (*B. canis*, *B. rossi*, and *B. vogeli*) and small Babesia species (*B. gibsoni*, *B. conradae*, and *Babesia vulpes*). In previous studies, low prevalence rates have been reported in Turkey. Aysul (2006) investigated Babesia species in dogs and reported a prevalence rate of 3.8% for *B. canis vogeli* by RLB In İstanbul. In a comprehensive study, Aktaş et al. (2015) tested a total of 757 dog blood samples from different provinces and found only one (0.1%) dog to be infected with *B. canis* in Eastern Anatolia of Turkey. In another study carried out in the same province, Güven et al. (2017) reported a prevalence rate of 5.3% (7/133). Guo et al. (2017) reported a prevalence rate of 2.1% in Konya in Central Anatolia. In the present study, for the first time, the presence of *B. canis* was detected in two cities (Mersin and Adana) located in Southern Turkey. These findings are important to show the presence of a vector carrying the agent in the region.

Canine hepatozoonosis (CH) is currently known to be caused by two hepatozoon species (*H. canis* and *H. americanum*). *H. canis* infections are widespread in Europe, Asia, Africa, and South America with a prevalence rate varying 7.5% and 52% (Baneth, 2011). Although the brown dog tick *Rhipicephalus sanguineus* (*s.l.*) is known as the main vector of *H. canis*, *Haemaphysalis sulcata*, *Dermacentor marginatus* and *Ixodes ricinus* were also reported as other possible vectors (Aktaş *et al*. 2013; Aktaş 2014). *H. americanum* infections are restricted to North America since the vector of the Gulf Coast tick *Amblyomma maculatum* is found only in the southeastern states of America (Little at al. 2009). In this study, only one *H. canis* positive sample was detected, resulting in a positive rate of 0,5% (1/186), which is similar to that of Bölükbaş et al. (2016) findings (0.5%, 1/200) in Samsun. In contrast to the results of the current study, high or higher prevalence rates of *H. canis* infection in Turkey have been reported in previous studies. Karagenç et al. (2006) reported the prevalence of the infection in the Aegean coast of Turkey as 10.6% by microscopy and 25.8% by PCR. Also, the authors found that 36.8% of serum samples were positive for antibodies against *Hepatozoon* spp. by IFAT. In a study covering 10 Turkish provinces, Aktaş et al. (2015) examined a total of 694 dog blood samples by PCR and found 22.3% of the dogs to be positive for *H. canis*, ranging from 3.9 to 42.8% according to provinces sampled. In Erzurum, out of 133 dog blood samples, 43 (32.3%) were found to be positive for *Hepatozoon* spp., and seven of the positive samples were confirmed as *H. canis* based on DNA sequencing (Güven et al. 2017). *H. canis* positivity was reported as 4.2% in Konya (Guo et al., 2017), 5.3% in Kayseri (Düzlü et al. 2013), 4% in Ankara (Aktaş et al., 2015b). In contrast, Orkun et al. (2018) reported higher positivity (49.5%) for *H. canis* infection in 103 stray dogs living in a shelter in Ankara. The differences observed in *H. canis* prevalence rates abovementioned studies could be attributed to the fact that the distribution of the vector and population density (Otranto et al., 2011), sampling methodology, and characteristics of the targeted dog population (Gomes et al., 2010).

Co-infections are a common event in vector-borne infections in endemic areas, especially for those dogs living mostly outdoors (Otranto et al., 2009). Moreover, co-infections with CVBD pathogens are reported to be associated with severe clinical manifestations and hematological abnormalities (De Tommasi et al., 2013). Co-infections with other vector-borne pathogens have been reported in previous studies in low rates in Turkey (Aktaş et al., 2015; Düzlü et al., 2014; Guo et al., 2017; Güven et al., 2017). Similarly, a low rate of

co-infection with *E. canis* and *B. canis* (3/186, 1.6%) was detected in the current study. Infections with multiple vector-borne pathogens may be attributed to the dogs' simultaneous exposure to different vectors or multiple pathogen carrying vector species (e.g. ticks) (Fang et al., 2015; Kordick et al., 1999).

The results of the current study indicated that *E. canis, B. canis vogeli* and *H. canis* species were present in dogs in different provinces of Turkey, with *E. canis* being the most common species among CVBD pathogens. To our knowledge, for the first time, *B. canis* and *H. canis* were detected in some of the studied provinces. Regarding the changing vector dynamics all over the world, continuous and detailed studies are needed to detect emerging and re-emerging vector-borne pathogens and to develop the necessary control strategies for these diseases.

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