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Original Article
Özgün Araştırma

First molecular detection of Canine Hemoplasmas in Sivas province in central part of Turkey

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Abstract: Canine hemoplasmas are vector-borne bacterial pathogens having worldwide distribution. There are two hemoplasmas species that cause disease in dogs. These are *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum. The aim of this study was to evaluate the prevalence of canine hemoplasmas among apparently healthy 194 owned-dogs in Sivas Province using species-specific polymerase chain reaction (PCR). According to our results, the overall prevalence of canine hemoplasmas was 14.94% (29/194). The molecular prevalence of *M. haemocanis* and *Ca.* M. haematoparvum was detected as 8.24% (16/194) and 10.82% (21/194) respectively while the prevalence of co-infections was 4.12% (8/194). In this study, *Ca.* M. haematoparvum which can infect humans was found more prevalent than *M. haemocanis*. To the best of our knowledge, it is the first molecular study on the determination of canine hemoplasmas in Sivas province in Turkey.

Keywords: Canine hemoplasmas, Dog, PCR, Sivas, Turkey.

Sivas ilinde Canine Hemoplasma'nin ilk moleküler tespiti

Özet: Canine hemoplasmas türleri vektör kaynaklı önemli bakteriyel patojenlerdendir. Etkenler dünyanın farklı bölgelerinde evcil köpeklerde tespit edilmiştir. Köpeklerde hastalığa neden olan iki önemli hemoplasma türü vardır. Bunlar sırasıyla *Mycoplasma haemocanis* ve *Candidatus* Mycoplasma haematoparvum'dur. Bu çalışmanın amacı Sivas ilinde 194 evcil köpekte canine hemoplasmas etkenlerinin yaygınlığının Polimeraz Zincir Reaksiyonu ile araştırılmasıdır. Bu çalışmada köpeklerin %14,94 (29/194)'ünde en az bir canine hemoplasmas türü ile enfekte olduğu tespit edilmiştir. Köpeklerin %8,24 (16/194)'ünde *M. haemocanis,* %10,82 (21/194)'ünde ise *Ca.* M. haematoparvum tespit edilmiştir. Köpeklerin %4,12 (8/194)'ünde ise miks enfeksiyon görülmüştür. Çalışmada köpeklerde *Ca*. M. haematoparvum'un *M. haemocanis*'e göre daha yaygın olduğu tespit edilmiştir. Bu çalışma ile bildiğimiz kadarıyla Sivas ilinde ilk kez köpeklerde moleküler yöntemlerle canine hemoplasmas türlerinin yaygınlığı araştırılmıştır.

Anahtar kelimeler: Canine hemoplasmas, Köpek, PCR, Sivas, Türkiye.

Introduction

Hemotrophic mycoplasmas (hemoplasmas) are tiny and pleomorphic bacterial agents that are cell-wall-deficient, obligate, and gram-negative. These pathogens invade red blood cells (RBCs) of different vertebrates, including dogs (Messick 2004; Chalker 2005; Sykes et al. 2005). Two hemoplasmas species are determined as canine-specific. These species are *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum (Messick 2004; Sykes et al. 2005). Although these species might affect different organs and tissues, infections are mostly asymptomatic in healthy dogs (Messick 2004; Chalker 2005). In some cases, progressive anemia can be seen in acute infections in immune-compromised or splenectomized dogs (Messick 2004; Sykes et al. 2005). Canine hemoplasmas rarely cause death (Messick 2004; Chalker 2005).

Transmission of canine hemoplasmas species is still not well defined, however *Rhipicephalus sanguineus*, brown dog tick, is thought to be a possible vector species of these species (Messick 2004; Baker and Tasker 2016; Aktas and Ozubek 2017). *M. haemocanis* and *Ca.* M. haematoparvum may also be transmitted mechanically via blood transfusions, fresh blood-contaminated fomites, and blood-sucking arthropods (Messick 2004; Willi et al. 2010; Baker and Tasker 2016).

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Microscopic, serological, and molecular methods have been used for the diagnosis of canine hemoplasmas infection so far (Messick 2004; Baker and Tasker 2016; Aktas and Ozubek 2018; Altay et al. 2020a). The microscopic techniques are successfully used for the diagnosis of clinical canine hemoplasmas infection (Baker and Tasker 2016) however, these techniques are not usable for the determination of chronic infections and may also cause false-positive results due to stain precipitation, basophilic stippling, or Howell-Jolly bodies (Kemming et al. 2004; Baker and Tasker 2016). Serological techniques and microscopic techniques are not fit to determine the pathogen species that cause canine hemoplasmas infection (Baker and Tasker 2016). Molecular identification techniques have been more preferred for the diagnosis of canine hemoplasmas compared to the microscopic and serological techniques. These methods; i) can determine the hemoplasmas species that caused infections in dogs ii) may find a small amount of nucleic acid that belongs to pathogens iii) can detect carrier animals (Messick 2004; Willi et al. 2010; Baker and Tasker 2016; Aktas and Ozubek 2017; Altay et al. 2020a).

Canine hemoplasmas species have been identified in different countries (Wengi et al. 2008; Compton et al. 2012; Baker and Tasker 2016; Maggi and Krämer 2019; Altay et al. 2020a), and in different parts of Turkey (Guo et al. 2017; Aktas and Ozubek 2017; Aktas and Ozubek 2018). There is a paucity of information on canine hemoplasmas in Sivas province. Due to this, the present study aimed to determine the molecular prevalence of canine hemoplasmas in Sivas province.

Material and Methods

Study Area and Material

Sivas province is placed in the central part of Turkey. Sivas has an approximately 28,400 km^2 geographical area. The average annual temperature is 8.9°C, the average precipitation, and relative humidity are 432 mm and 65% in Sivas, respectively.

The study material was composed of 194 (95 female, 99 male) apparently healthy owned-dog in four different parts of Sivas (Sivas City Center, Kangal, Yıldızeli, Hafik). The age, gender, and location data of the dogs were recorded. The blood samples were taken into blood collection tubes containing Etilendiamin tetraacetic acid (EDTA). The blood samples were stored at -20°C until DNA extraction.

Total Genomic DNA isolation and Polymerase Chain Reaction (PCR)

Total genomic DNA was obtained from 200 µL blood samples using PureLink Genomic DNA kit (Cat. No.: K1820-02, Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The genomic DNA samples were stored at -20°C until use.

PCR assay using species-specific primers was performed for determination of the presence and distribution of canine *Mycoplasma* species (Torkan ve ark. 2014). Further information on primers was detailed in Table 1. PCR was performed in a final volume of 50 µL including DNase-RNase-free sterile water (Cat No.: 129114, Qiagen®, Germany), 10× PCR buffer (Thermo Scientific™, Lithuanian), 2.5 mM $MgCl_2$ (25 mM) (Thermo Scientific™, Lithuanian), 200 µM of each dNTP (Cat. No.: PCCSKU1019, Procomcure Biotech GmbH), 1.25 U of Taq DNA polymerase (Cat. No.: EP0402, Thermo Scientific™, Lithuanian), 2 µL (10 pmol/µL) of each of the primers, and 5 µL template DNA. The PCR cycling conditions were done as described by Altay et al. (2020a). DNase-RNase-free sterile water (Cat. No.: 129114, Qiagen®, Germany) was used as negative control, the genomic DNA of *M. haemocanis* isolate (accession number: MK015018, Altay et al. 2020a) and *Ca.* M. haematoparvum (accession number: MK026012, Altay et al. 2020a) were used as positive controls for each PCR assay. Ten microliters of PCR products were electrophoresed on 1.5% agarose gel stained (100V, 45 min) with ethidium bromide and then were visualized by UV transilluminator (Figure 1). DNA extraction and PCR assay were conducted in different rooms for prevention of cross-contamination.

Table 1. Primers using in the present study.

Species	Primers 5'→3'	Target Gene	Length of Product	Reference
M. haemocanis	Forward GAAACTAAGGCCATAAATGACGC Reverse ACCTGTCACCTCGATAACCTCTAC	16S rRNA	309 bp	Torkan et al. 2014
Ca. M. haematoparvum	Forward ACGAAAGTCTGATGGAGCAATAC Reverse TATCTACGCATTCCACCGCTAC	16S rRNA	328 bp	Torkan et al. 2014

Statistical evaluation: Statistical analyses among various parameters were performed using the chi-square test. Differences were considered statistically significant if $p < 0.05$.

Results

All dog blood samples were screened for *M. haemocanis* and *Ca.* M. haematoparvum by PCR using the primers detailed in Table 1. The overall prevalence of hemoplasmas species was found to be 14.94% (29/194). The prevalence of *M. haemocanis* was detected as 8.24% (16/194), while the prevalence of *Ca.* M. haematoparvum was detected as 10.82% (21/194). Co-infections were found to be 4.12 % (8/194) (Table 2).

The prevalence among female dogs was detected as 14.73% (14/95), while among the male dogs it was 15.15% (15/99). Canine hemoplasmas were found to be 8.95% (6/67) between 0 and 2 years of age, 19.40% (13/67) between 3 and 4 years of age, and 16.66% (10/60) in dogs ≥ 5 years old (Table 2). The distribution of canine hemoplasmas in the sampling area was determined to be 18.84% (13/69) in Sivas city center, 13.33% (6/45) in Kangal, 11.11% (5/45) in Hafik, and 14.28% (5/35) in Yildizeli.

There were not statistically significant differences ($p<0.05$) between the prevalence of hemoplasmas species, sampling area, gender, and age groups.

Table 2. Comparison of canine hemoplasmas among gender, sampling area, and age groups.

Canine hemoplasmas species	Gender		Sampling Area				Age groups		
	Male	Female	Sivas City Canter	Hafik	Yıldızeli	Kangal	0-2	3-4	≥ 5
M. haemocanis	3	5	6	-	-	2	4	2	2
Ca. M. haematoparvum	8	5	3	3	4	3	-	7	6
Co-infections	4	4	4	2	1	1	2	4	2
Total	15 (15.15%)	14 (14.73%)	13 (18.84%)	5 (11.11%)	5 (14.28%)	6 (13.33%)	6 (8.95%)	13 (19.40%)	10 (16.66%)
p-value	**p>0.05**		**p>0.05**				**p>0.05**		

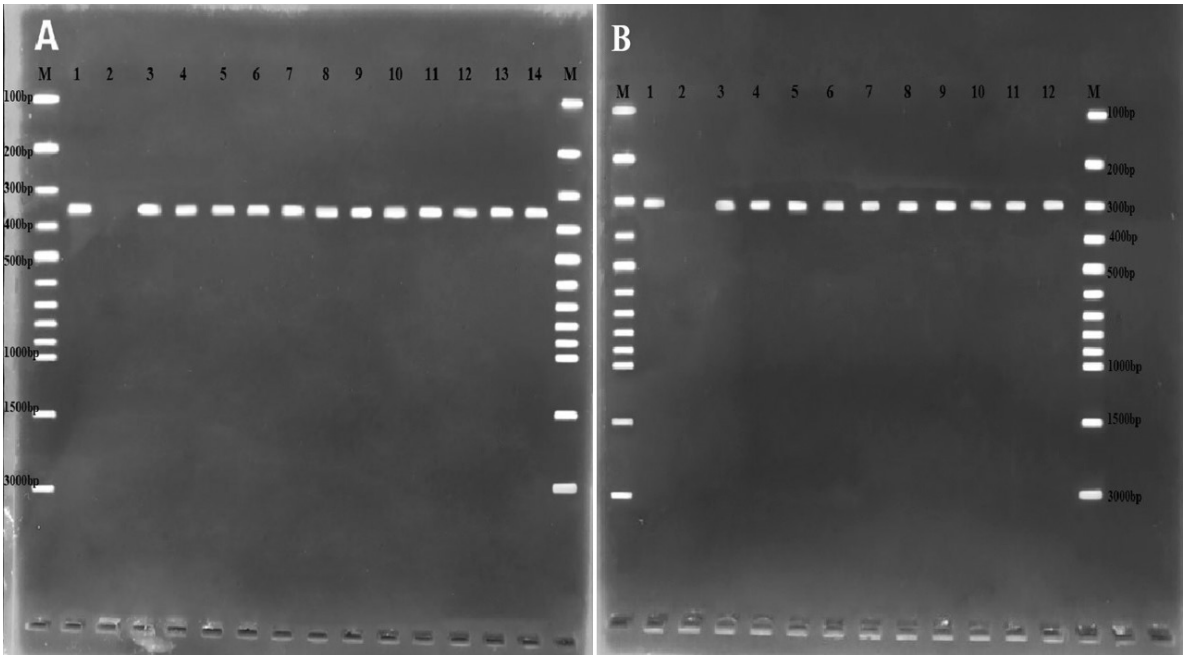

Figure 1. Agarose-gel electrophoresis of *Ca.* M. haematoparvum (A) and M. haemocanis (B) specific polymerase chain reaction. M. Marker, Lane A1. *Ca.* M. haematoparvum positive control DNA from dog, Lane A2. negative control distilled water, Lane A3-A14. *Ca.* M. haematoparvum positive dog blood samples, Lane B1. *M. haemocanis* positive control DNA dog, Lane B2. negative control distilled water, Lane B3-B12. M. haemocanis positive dog blood samples.

Discussion and Conclusions

Canine hemoplasmas are one of important tick-borne pathogens. Two hemoplasmas species, *M. haemocanis* and Ca. M. haematoparvum, are known to be the pathogens for domestic and wild canine species (Messick 2004; Sykes et al. 2004; André et al. 2011; Torkan et al. 2014; Baker and Tasker 2016). *M. haemocanis* was reported for the first time in 1928, while Ca. M. haematoparvum was identified in 2004 (Sykes et al. 2004). *M. haemocanis* and Ca. M. haematoparvum have a worldwide distribution (Wengi et al. 2008; Novacco et al. 2010; Compton et al. 2012; Baker and Tasker 2016; Maggi and Krämer 2019; Altay et al. 2020a), however there is little data available on the prevalence of these pathogens in Turkey (Guo et al. 2017; Aktas and Ozubek 2017; Aktas and Ozubek 2018). Sivas is the second-largest province and located in the central part of Turkey. There are found blood parasites such as *Theileria, Babesia, Anaplasma,* and *Dirofilaria immitis* in animal species like dogs, sheep, and cattle in different studies (Altay et al. 2017; Atas et al. 2018; Altay et al. 2020b). However, there is a lack of information molecular prevalence of canine hemoplasmas. Therefore, this study aimed to determine the prevalence of canine hemoplasmas in Sivas province using PCR.

There are different techniques to diagnose canine hemoplasmas species, but molecular identification techniques have been more preferred since they are faster and more specific than other identification techniques (Messick 2004; Willi et al. 2010; Torkan et al. 2014; Baker and Tasker 2016). *M. haemocanis* and *Ca.* M. haematoparvum have been detected in different geographical areas such as Turkey, Italy, Spain, Portugal, the USA, Sudan, and Kyrgyzstan using species-specific PCR (Inokuma et al. 2006; Novacco et al. 2010; Compton et al. 2012; Aquino et al. 2016; Aktas and Ozubek 2017; Guo et al. 2017; Ravagnan et al. 2017; Aktas and Ozubek 2018; Altay et al. 2020a). A few studies were carried out to determine the presence and distribution of canine hemoplasma infection in Turkey (Aktas and Ozubek 2017; Guo et al. 2017; Aktas and Ozubek 2018). These studies revealed that the prevalence of canine hemoplasmas was ranging from 15.3 to 38.3% in dog population in different parts of Turkey (Aktas and Ozubek 2017; Guo et al. 2017; Aktas and Ozubek 2018). In the present study, the overall prevalence was determined as 14.94% (29/194). Our result was similar to that of Aktas and Ozubek (2018) (15.3%), however the prevalence we found was lower than that in Konya %23.95% (Guo et al. 2017) and in Diyarbakır 38.3% (Aktas and Ozubek 2017). Like other vector-borne diseases, the prevalence of canine hemoplasmas might change depending on climate conditions in sampling areas, presence and distribution of vector species, and dog origin (stray or owned) (Baker and Tasker 2016; Guo et al. 2017; Aktas and Ozubek 2018; Maggi and Krämer 2019). The present study was performed using owned-dogs while the studies that have higher prevalence of canine hemoplasmas in Konya (Guo et al. 2017) and in Diyarbakır (Aktas and Ozubek 2017) were conducted using stray dog samples. Stray dogs are frequently exposed to ectoparasites such as ticks and fleas. Therefore, vector-borne pathogens may be more prevalent in stray dogs compared to owned-dogs. For this reason, we speculate that the lower prevalence (14.94%) compared to the ones in Diyarbakır (38.3%) and Konya (23.95%) could be related to that this study was performed using owned-dog blood samples.

In the present study, there were no statistically significant differences between the prevalence of *M. haemocanis* and *Ca.* M. haematoparvum. The prevalence of *M. haemocanis* was detected as 8.24% (16/194), while the prevalence of *Ca.* M. haematoparvum was found to be 10.82% (21/194). In the previous studies conducted in Turkey, *M. haemocanis* was found more prevalent than *Ca.* M. haematoparvum (Aktas and Ozubek 2017; Aktas and Ozubek 2018), however, in this study, *Ca.* M. haematoparvum was found more prevalent than *M. haemocanis*. These findings were similar to the studies in France (Kenny et al. 2004), Sudan (Inokuma et al. 2006), and the USA (Compton et al. 2012). Furthermore, *Ca.* M. haematoparvum can cause human infections (Maggi et al. 2013). Therefore, veterinarians and people who have intimate contact with dogs should be careful about this pathogen to protect their health.

According to our PCR results, the prevalence of canine hemoplasmas was found to be 15.15% (15/99) in the male dogs and 14.73% (14/95) in the female dogs. There was no significant association of canine hemoplasma infection with dog gender. This result was similar to the studies performed in different countries like France (Kenny et al. 2004), Italy (Ravagnan et al. 2017), and Turkey (Aktas and Ozubek 2017; Aktas and Ozubek 2018). In the present study, we found that the prevalence of canine hemoplasmas in the male dogs was slightly higher than in the female dogs. This finding may be related to that male dogs tend to have more aggressive contact with other dogs than female dogs, due to

these pathogens also can be transmitted with fresh blood (Sasaki et al. 2008; Barker et al. 2010).

Canine hemoplasmas were found in all age groups (Table 2). There was not found statistically significant differences between the prevalence of different age groups in the present study. This result was compatible with the studies conducted in different countries like Switzerland (Wengi et al. 2008), Italy (Ravagnan et al. 2017), Turkey (Aktas and Ozubek 2017; Aktas and Ozubek 2018), and the USA (Compton et al., 2012). Furthermore, our results revealed that the prevalence of canine hemoplasmas was more prevalent among the older age groups (3-4 years and ≥ 5 years) than younger age group (0-2 years) (Table 2). This finding could be attributed to that older animals are more exposed to ectoparasites (ticks or fleas) compared to the younger ones. Due to this, we thought that vector-borne pathogens like canine hemoplasmas are more prevalent in older animals. Normally, canine hemoplasmas should be more prevalent in ≥5 years' dogs than 3-4 years, but these pathogens were found more prevalent in 3-4 years' dogs (Table 2). This result could be related to the successful tick control by owners of these dogs that are older than 5 years old.

Mycoplasma haemocanis and *Ca.* M. haematoparvum were found in all sampling areas; Sivas city canter, Kangal, Hafik, and Yıldızeli, and the prevalences were 18.84%, 13.33%, 11.11%, and 14.28%, respectively. The difference between the sampling areas was not statistically significant. This result could be related to that the climate conditions of all sampling areas are similar. The climate conditions in geographical areas can directly affect distribution and abundance of vector species, and hence the prevalence of pathogens, especially vector-borne pathogens (Wengi et al. 2008; Novacco et al. 2010; Maggi and Krämer 2019). Probably because of that, we did not find statistically significant differences between the sampling areas.

In conclusion, vector-borne diseases (VBDs) are the main health problem in the world and are threat to both animal and human health. *M. haemocanis* and *Ca.* M. haematoparvum are one of important vector-borne diseases for dog populations. Moreover, these species also infect humans (Maggi et al. 2013). For this reason, determination of these pathogens in dog populations is vital to protect both animal and human health. To better understand the prevalence and the presence of *M. haemocanis* and *Ca.* M. haematoparvum in Sivas, there need to be more comprehensive surveys, including domestic and wild canine species.

Ethic statement: Permission was obtained from the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval number: 16.03.2021-513).

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Conflict of Interest: The authors declare that they have no conflicts.

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