Original Article

The molecular detection of hemotropic Mycoplasma species in dogs

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Abstract

Hemotropic mycoplasmas are uncultured bacteria that cause different degrees of hemolytic anemia in infected hosts. Molecular approaches are used to diagnose hemotropic mycoplasmas since they are difficult to identify using conventional methods. The aim of this study was to use molecular approaches to determine hemotropic mycoplasmas in dogs. Blood samples were collected in tubes (with EDTA) from 100 dogs. After DNA extraction, 100 DNA samples were investigated by PCR. *Hemotropic mycoplasma* was detected in 12 (12%) samples and positive samples were subjected to Sanger sequencing. Mycoplasma haemocanis was detected in 6 (50.0%) of the samples, *Candidatus* Mycoplasma hematoparvum in 5 (41.6%), and *Candidatus* Mycoplasma haemominutum in 1 (8.4%) of the samples after typing 12 PCR positive amplicons using Sanger sequencing. Anemia was found in 5 (83.3%) *Mycoplasma haemocanis* positive dogs while 3 (60.0%) were *Candidatus* Mycoplasma haematoparvum positive dogs. As a consequence of this research, hemotropic mycoplasmas were determined in dogs using molecular methods.

Keywords: Anemia, dog, hemotropic mycoplasma, polymerase chain reaction, sequencing

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Introduction

Hemotropic mycoplasmas are small, pleomorphic bacteria that do not have a cell wall and have been detected in the blood of different kinds of animals (Soto *et al*., 2017). Previously, hemotropic mycoplasmas were classed as rickettsial organisms. Phenotypic characteristics such as the difficulty of development needs and the lack of a cell wall further supported this relation. However, molecular sequencing and phylogenetic studies indicated that these species are more closely connected to the *Mycoplasmataceae* family and a new classification has been renamed to reflect their mycoplasmal origin (Tasker, 2010).

Mycoplasma haemocanis and *Candidatus* Mycoplasma haematoparvum have been described as hemotropic mycoplasma agents in dogs (Soto *et al*., 2017). In addition, some studies have reported that *Candidatus* Mycoplasma haemominutum has been detected in dogs from China, France and Japan (Kenny *et al*., 2004; Zhuang *et al*., 2009; Obara *et al*., 2011).

Transmission can occur through contaminated blood transfusions and dog fights. Blood-sucking arthropods like fleas and ticks have been considered as potential vectors (Zarea *et al*., 2022). Hemotropic mycoplasma disease, which can vary from asymptomatic infection to sudden death has clinical signs such as acute hemolytic anemia, anorexia, lethargy, dehydration and weight loss (Ravagnan *et al*., 2017).

Cytological analysis of Romanowsky, Diff-Quick, or filtered Giemsa-stained blood smears are used to diagnose hemotropic mycoplasma infection. Single, paired and chain organisms can be shown on the surface of erythrocytes. Cytological diagnosis has a low sensitivity and specificity. Due to the occurrence of cross reactions, studies on serological diagnostic tests have shown that they have not been efficient for the diagnosis of hemotropic mycoplasma infections yet (Tasker *et al*., 2018). Polymerase Chain Reaction (PCR) exhibits higher sensitivity and specificity than serological and cytological investigation for the detection of hemotropic mycoplasma infections (Tasker, 2010; Tasker *et al*., 2018).

The aim of this study was to determine hemotropic mycoplasmas in dogs with molecular methods.

Materials and Methods

Specimen collection: Five ml EDTA-anticoagulated blood samples were obtained from 100 (50 females, 50 males) dogs which were brought to veterinary clinics and hospital for investigation of hemotropic mycoplasma from Aydin province in Türkiye. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Aydin Adnan Menderes University (64583101/2017/074). Table 1 shows the age and gender distributions of male (n = 50) and female (n = 50) dogs whose blood was collected for investigation (Table 1).

Table 1 Data on dog samples by age and gender

Whole Blood Samples	Age Ranges										Total
	0-1 Age		2-5 Age		6-10 Age		11-15 Age		> 15 Years		
	F	M	F	M	F	M	F	M	F	M	
Dog (n=100)	16	18	27	21	4	7	3	2	0	2	100

F: Female, M: Male

Table 1 shows that 32 % of female dog samples are 0-1 year old and 54 % are 1-5 years old, whereas 36 % of male dog samples are 0-1 year old and 42 % are 1-5 years old.

Anemia was determined using hematological analysis and the results were evaluated according to Raskin and Wardrop (2010). According to Raskin and Wardrop, reference values for RBC (red blood cell), Hb (hemoglobin) and HCT (hematocrit) are stated as 5.5-8.5 (10^{12}/l), 12,0-18,0 (g/dl) and 37,0-55,0 (%) respectively. Dogs with RBC, Hb and HCT values below the reference range were evaluated and diagnosed as having anemia. QuickGene DNA whole blood kit S (Kurabo, Japan) was used to extract DNA from all blood samples and until PCR analysis, DNA samples were kept at -20°C.

PCR and sequencing: Extracted DNA samples were subjected to PCR analysis. The 16S rRNA gene primers (16S Myco322s 5'- GCC CAT ATT CCT ACG GGA AGC AGC AGT -3 ', 16S Myco938as 5'- CTC CAC CAC TTG TTC AGG TCC CCG TC -3') were used for the detection of canine hemotropic mycoplasma (Varanat *et al*., 2011). Positive control DNA was obtained from the clinically infected dog blood sample, which was validated using Sanger sequencing and BLAST (100% similarity to *M. haemocanis* [MG594502]). As a negative control, sterile deionized water was used. The PCR mixture for each reaction contained 5 µl of extracted DNA samples and 12.5 µl PCR master mix, 0.5 µl (30 µM) from each primer, 7 µl deionized water (Varanat *et al*., 2011). PCR was performed with initial denaturation at 94°C for 5 mins, followed by 55 cycles of denaturation at 94 ° C for 15 secs, annealing at 68°C for 10 secs, extension at 72°C for 15 secs and final extension 72 ° C for 30 secs (Varanat *et al*., 2011). The PCR products were detected by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The bands were visualized with UV trasilluminator (Infinity™ VX2, Collégien, France) (Varanat *et al*., 2011). ExoSAP-IT™ (AppliedBiosystems, CA, USA) was used for the PCR positive amplicons' enzymatic purification. Purified amplicons were subjected to sequence PCR and the sequence PCR amplicons were purified using by Sephadex (Merck, Darmstadt, Germany) with spin columns (Sanger *et al*., 1977). The ABI Prism 310 Genetic Analyzer (AppliedBiosystems, FosterCity, CA, USA) was used to analyze purified amplicons. The obtained sequences' identity was evaluated by checking against existing sequences using by BLAST.

Table 2 Distribution of anemia findings by age and gender based on hemogram results

Sample No	Age/Gender	RBC*	Hb*	HCT*
1	2 Mo/ F	4,13	10,2	22,2
2	2 Mo/ F	3,58	10,1	20,2
3	3 Mo/ F	3,14	9,7	19,5
4	3 Mo/ F	5,15	11,8	34,1
5	6 Mo/ F	4,3	12	24,3
6	6 Mo/ F	5,14	11,3	32,1
7	7 Mo/ F	4,68	10,6	30,06
8	7,5 Mo/ F	5,29	11,5	33,16
9	9 Mo/F	4,49	11,1	28,5
10	1 A/ F	4,86	10,9	31,3
11	1 A/ F	4,67	10,6	29,2
12	1 A/ F	4,19	10,5	28,8
13	1 A/ F	5,23	11,8	36,1
14	1,5 A/ F	4,05	10,2	28
15	1,5 A/ F	5,39	11,9	32,3
16	2 A/ F	4,57	11,6	26,8
17	2 A/ F	3,76	8,1	23,5
18	2 A/ F	3,76	9,3	25,1
19	2 A/ F	2,18	5,5	17,5
20	3 A/ F	4,27	10,6	31,1
21	3 A/ F	3,79	9	29,3
22	3 A/ F	4,81	11,4	30,5
23	3 A/ F	5,24	11,8	34,8
24	3 A/ F	5,15	11,9	33,7
25	5 A/ F	4,93	11,7	36
26	5 A/ F	4,73	11,5	29,3
27	5 A/ F	4,47	11,2	27,9
28	5 A/ F	5,46	18	35,9
29	7 A/ F	2,18	5,4	16,4
30	10 A/ F	4,81	10,8	34,8
31	13 A/ F	3,4	6,6	21
32	2 Mo/ M	3,77	8,2	21,7
33	2 Mo/ M	1,43	4,6	7,6
34	2 Mo/ M	4,03	11	21,8
35	2 Mo/ M	2,68	8	14,7
36	2 Mo/ M	3,42	9,9	21,1
37	2 Mo/ M	3,47	9,6	19,2
38	4 Mo/ M	5,38	11	31,51
39	6 Mo/ M	3,15	9	19,7
40	10 Mo/ M	3,15	7,4	21,93
41	1 A/ M	4,04	11,7	25,7
42	1 A/ M	5,05	13,8	28,8
43	1 A/ M	3,85	10,7	22,2
44	1 A/ M	5,1	11,5	33,5
45	2 A/ F	4,2	10,9	28,4
46	2 A/ M	4,93	11,4	30,5
47	2 A/ M	4,47	9,9	29,43
48	2 A/ M	5,38	11,8	36,7
49	2 A/ M	3,2	8,4	24,7
50	3 A/ M	5,32	16	31,9
51	3 A/ M	5,36	10,2	36,8
52	4 A/ M	2,55	6,3	19,6
53	5 A/ M	4,61	8,6	26,19
54	5 A/ M	4,1	10,5	26,3
55	5 A/ M	4,11	10,9	25,5
56	7 A/ M	4,98	11,8	31,5
57	7 A/ M	5,12	11,7	36
58	8 A/ M	3,92	8,3	24,9
59	9 A/ M	3,5	6,5	23,7
60	15 A/ M	5,09	11,9	34,9
61	17 A/ M	3,22	8,8	23,13
62	18 A/ M	3,66	8,7	25,02

*Reference values= RBC: 5,5-8,5 10^{12}/l; Hb: 12,0-18,0 g/dl; HCT: 37,0-55,0 (%); F: Female; M: Male; A: Age; Mo: Month

Results

Hematological Analysis: Hematological analysis was performed to diagnose anemia (Abacus Junior Vet, Diatron, Budapest, Hungary). The results were compared to hematologic reference intervals for healthy dogs and blood samples from dogs were considered as anemic with low erythrocyte and

hemoglobin values (Raskin and Wardrop, 2010). The age ranges and gender of dogs with anemia are indicated in Table 2 as a result of hemotological study performed on canine blood samples.

Low red blood cell rates were found in 62 (62 %) (32 % female, 30 % male) samples as a result of hematological analysis performed on 100 whole blood samples and these samples were considered as positive for anemia. Ninety percent of female dogs and 76.6 % of male dogs found to be anemic were between the ages of 0 and 5.

Canine Hemotropic Mycoplasma 16S rRNA PCR Results: As a result of the PCR analysis, 12/100 (12%) samples were detected positive at 600 bp on the agarose gel.and these samples were considered as hemotropic mycoplasma (Fig. 1). In 16S rRNA PCR, 88/100 (88%) of samples were found to be negative for hemotropic mycoplasma.

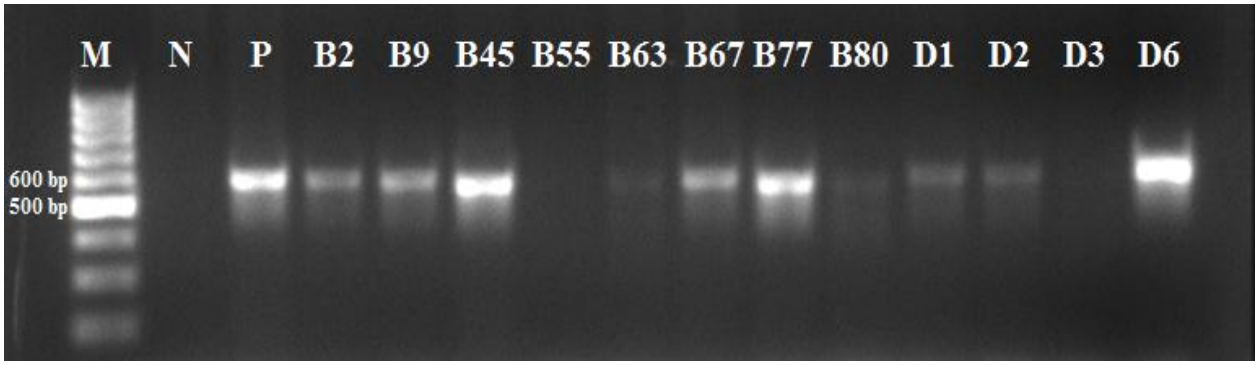

Figure 1 Canine hemotropic mycoplasma 16S rRNA PCR agarose gel image.
M: 1000 bp DNA ladder; N: Negative control (Sterile deionized water); P: Positive control (sequenced *M.haemocanis* DNA; Accession Number MG594502.); B2, B9, B45, B63, B67, B77, B80, D1, D2, D6; Hemotropic mycoplasma PCR positive samples (600 bp); B55, D3: Hemotropic mycoplasma PCR negative samples.

PCR analysis revealed that 10 (83.3%) of the 12 hemotropic mycoplasma positive dogs were aged from 2 to 5, one (8.3%) was aged 0 to 1 and one (8.3%) was aged from 6 to 10 (Table 3).

It was seen in canine hemotropic mycoplasma 16s rRNA PCR that 3 (25%) of positive dogs were female and 9 (75.0%) of positive dogs were male after PCR analysis. Anemia was found in 8/12 (66.7%) positive PCR samples but it was not found in the remaining 4/12 (33.3%) samples, according to the hemogram analysis.

Sequence Results: The Sanger sequencing method was used to identify 12 canine hemotropic mycoplasma 16S rRNA PCR positive samples. *Mycoplasma haemocanis* was found in 6/12 (50.0%) samples; *Candidatus* Mycoplasma hematoparvum was found in 5/12 (41.6%) samples; and *Candidatus* Mycoplasma haemominutum was found in 1 (8.4%) sample. The distribution of canine hemotropic mycoplasma species identified by Sanger sequence method are shown in Table 4 according to gender and age ranges. It was determined that 25.0% (n=3) of the 12 isolates that were detected were female and 75.0% (n=9) were male (Table 4).

Table 3 Distribution of canine hemotropic mycoplasma 16S rRNA PCR positive samples by gender and age ranges

PCR positive samples	Age Ranges										Total
	0-1 Age		2-5 Age		6-10 Age		11-15 Age		> 15 Years		
	F	M	F	M	F	M	F	M	F	M	
Dog (n=12)	1	-	2	8	-	1	-	-	-	-	12

F: Female; M: Male

Table 4 Overall results for canine hemotropic mycoplasma

Sample	Age/Gender	Anemia	PCR	Sanger Sequence	Accesion Number	Similarity Rate(%)
B2	3 A / M	-	+	*CMhp*	MG594500	97,56
B9	3 A/ M	+	+	*Mhc*	MG594502	97,03
B20	6 Mo/F	+	+	*CMhp*	MG594500	98,41
B38	7 A / M	+	+	*CMhp*	MG594500	98,74
B45	3 A / M	+	+	*Mhc*	MG594502	100,00
B50	5 A / M	+	+	*CMhp*	MG594500	97,17
B67	2 A / M	+	+	*Mhc*	MG594502	99,46
B77	5 A / M	+	+	*Mhc*	MG594502	100,00
D2	2 A / F	-	+	*Mhc*	MG594502	99,23
D6	2 A / M	+	+	*Mhc*	MG594502	99,74
D12	2 A / M	-	+	*CMhp*	MG594500	97,72
D18	4 A / F	-	+	*CMhm*	KU852583	97,72

Mhc: *Mycoplasma haemocanis*; *CMhp*: *Candidatus Mycoplasma haematoparvum*; *CMhm*: *Candidatus Mycoplasma haemomunitum*; (-): Negative; (+): Positive; F: Female; M: Male; A: Age; Mo: Month

Discussion

Increasing knowledge of bacterial genomic sequences and phylogenetic relationship has led to the reclassification of these organisms as hemotropic mycoplasmas in the genus *Mycoplasma* (Neimark *et al*., 2001). Because of the difficulties in growing these agents *in vitro*, research into hemotropic mycoplasmas is limited. In addition, for the sensitive detection of canine hemotropic mycoplasmas, species-specific conventional and real-time TaqMan PCR analysis have been developed. These analyses allow research further into the pathophysiology and epidemiology of hemotropic mycoplasma infections in dogs (Willi *et al*., 2010).

Hemotropic mycoplasma prevalence was reported in dogs as follows; 1.2% in Switzerland (Wengi *et al*., 2008), 15.4% in France (Kenny *et al*., 2004), 9.5% in Italy (Novacco *et al*., 2010), in Spain 14.9% (Roura *et al*., 2010), 10.6% in Greece (Tennant *et al*., 2011), 12.3% in Romania (Hamel *et al*., 2012) , 7.7% in Nigeria (Aquino *et al*., 2016), 6.9% in Brazil (Valle *et al*., 2014), 12.2% in India (Abd Rani *et al*., 2011), and in the USA 1.3% (Compton *et al*., 2012), 22.9% (Guo *et al*., 2017) and 15.3% (Aktas and Ozubek, 2018) in Türkiye. The prevalence of hemotropic mycoplasma was detected to be 12% in this study, which is similar to the prevalence values observed by Guo *et al*. (2017), and Aktas and Ozubek (2018).

Aktaş and Ozubek (2018) conducted research in Elazig, Erzurum, Ankara, Nevsehir, Adapazar, Izmit, Mersin, Giresun, and Izmir, whereas Gou *et al*. (2017), conducted study in Konya province. Aktas and Ozubek (2018) reported the prevalence of *M. haemocanis* is 2.5% in Elazig; 5.9% in Erzurum; 4.1% in Ankara; 2.0% in Nevsehir; Adapazarı 3.4%; 1.8% in Izmit; 6.7% in Mersin; 8.0% in Giresun and 6.7% in Izmir and prevalence of *Candidatus* Mycoplasma haematoparvum in accordance with the provincial order indicated above by 3.3%; 2.0%; 2.0%; 0%; 1%; 0%; 9.4%; 6.0% and 15.0%. In this study, hemotropic mycoplasma isolates were classified as 6 (6.0%) *Mycoplasma haemocanis*, 5 (5.0%) *Candidatus* Mycoplasma haematoparvum and 1 (1.0%) *Candidatus* Mycoplasma haemominutum and our results were similar to the studies previously obtained in Türkiye.

Other studies reported that there is no substantial link between anemia and hemotropic mycoplasma infections (Wengi *et al*., 2008; Roura *et al*., 2010; Tennant *et al*., 2011; Ravagnan *et al*., 2017). In this study, 8 (66.6%) of the 12 positive samples were determined to be anemic, whereas the remaining 4 (33.4%) samples were not anemic. Unlike previous studies, anemia was detected in animals infected with the hemotropic mycoplasma as a result of this research.

In this study, it was determined that the age distribution of hemotropic mycoplasma positive dogs with anemia (n=8) was between 6 months and 7 years, and 7 of them were male and 1 was female. In hemotropic mycoplasma positive dogs without anemia (n=4), the age range was between 2 and 4 years, and 2 were female and 2 were male (Table 4). The average age of hemotropic mycoplasma positive dogs is approximately 3 years old.

Di Cataldo *et al*. (2021), reported that they found hemotropic mycoplasma at a rate of 17.8% in male dogs and 5.6% in female dogs. Moreover, when the age ranges of the dogs in the study group were evaluated, 14.7% positivity was found in adult dogs that are 1 year old and older and 5.5% positivity was found in young dogs under 1 year old. Di Cataldo *et al*. (2021), attributed that the high risk of exposure to the pathogen as they age and the high contact with the external environment increased the rate of hemotropic mycoplasma infection in male and adult dogs.

Barbosa *et al*. (2021), stated in their study on hemotropic mycoplasma that gender and presence of wild animals were not statistically significant in the transmission of infection to dogs. However, they reported that ticks, tick bites and dogs fighting play an important role in the contagion of hemotropic mycoplasma infections. Ravagnan *et al*. (2017), stated that identified both Mhc and CMhp were in free-range dogs in northern and northeastern Italy and there was not significant relationship among hemoplasma infection and gender, age or habitat. Cortese *et al*. (2020), reported that male sex and tick infestations are high risk factors for Mhc, while adult and large-sized dog breeds are high risk factors for CMhp. In this study, it was shown that hemotropic mycoplasma positivity was higher in male and adult dogs (3 years and older). As a conclusion of this study, it was inferred that gender and age factors cannot be considered as risk factors in hemotropic mycoplasma infections.

Studies have shown that *Mycoplasma haemocanis* was more common than *Candidatus* Mycoplasma haematoparvum in Nigeria (Aquino *et al*., 2016), in Australia (Barker *et al*., 2010), in Spain (Roura *et al*., 2010), in Greece (Tennant *et al*., 2011), in Iran (Torkan *et al*., 2013). In studies conducted in France (Kenny *et al*., 2004), the Sudan (Inokuma *et al*., 2006), and the United States (Compton *et al*., 2012), *Candidatus* Mycoplasma haematoparvum was found to be the most common canine hemotropic mycoplasma. The prevalence of *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum was found to be similar in this study.

Guo *et al*. (2017), used the PCR test to detect vector-borne pathogens in different dog groups in Türkiye. As a result of the sequence analyses performed on 4 PCR positive samples, *Mycoplasma haemocanis* similarity was detected at the rates of 99.67%-99.83%. Aktas and Ozubek (2018) stated that the prevalence of hemotropic mycoplasma in dogs was 15.3%, after PCR analysis in Türkiye. While *Mycoplasma haemocanis* was found to be 99.6% similar to the isolate previously described in Türkiye, *Candidatus* Mycoplasma haematoparvum was found to be 99.7% similar to the isolate previously described in Türkiye. In this study, 6 obtained sequences show similarities with *Mycoplasma haemocanis* between rates 93.7%- 100% in the Genbank database, 5 sequences were detected 91.17%- 98.74% homology to *Candidatus* Mycoplasma haematoparvum and 1 sequence was similar to *Candidatus* Mycoplasma haemominutum with 97.72% homology.

In conclusion, the clinical manifestations of hemotropic mycoplasmas in dogs can range from asymptomatic to anemia, lethargy and sudden death.

Since the identification of these organisms cannot be achieved through conventional methods, molecular techniques have become increasingly crucial. Consequently, in this study, hemotropic mycoplasmas were detected in apparently healthy dog samples using molecular techniques. These findings highlight the importance of employing sensitive and specific diagnostic tools for the timely identification and intervention of hemotropic mycoplasma infections, even in asymptomatic animals. Furthermore, it is recommended that veterinary professionals be trained in the agents of hemotropic mycoplasma infection and that new epidemiological studies be conducted to better understand the prevalence and distribution of hemotropic mycoplasma agents in light of the results of this study.

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