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Molecular detection and differentiation of canine hemoplasma infections using RFLP-PCR in dogs in southern Iran

Mohammad A. Hasiri*, Hassan Sharifiyazdi, and Tayebeh Moradi

Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

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ABSTRACT

Two hemoplasma species are known in dogs: Mycoplasma haemocanis (Mhc) and Candidatus *Mycoplasma haematoparvum* (CMhp). The aim of the present study was to develop a novel restriction fragment length polymorphism (RFLP)-PCR method based on the 16S rDNA gene, using endonuclease *Hind III*, for detection and differentiation of canine hemoplasmas. Also, analysis of risk factors, clinical features and hematologic changes of positive cases was performed in dogs living in the Shiraz area of Iran. Blood samples were collected from anemic (packed cell volume (PCV) ≤35; n = 26) and control dogs (PCV >35; n = 27) and were examined for the presence of canine hemoplasmas, using RFLP-PCR and 16S rDNA Sanger sequencing. The presence of Mhc (4 out of 53 cases; 7.5%) and CMhp (3 out of 53 cases; 5.7%) was confirmed by RFLP analysis of the amplified 16S rDNA and sequencing. No association was found between hemoplasma infection and anemia, health status, age, breed, gender, type of housing or the presence of other dogs in this study. Only the platelet number in Mhc infected dogs was statistically higher compared to CMhp positive and hemoplasma negative dogs. The present report documents the occurrence of Mhc and CMhp in southern Iran, and these hemotropic Mycoplasma infections must be expected even in the absence of clinical symptoms or hematologic abnormalities in dogs. For the first time, it has been indicated that RFLP-PCR assay is able to successfully distinguish hemotropic *Mycoplasma* in dogs.

Key words: hemoplasma, *Mycoplasma haemocanis* (Mhc), Candidatus *Mycoplasma haematoparvum* (CMhp), dog, Iran

Introduction

Hemotropic Mycoplasma (also called hemoplasma) species are facultative intracellular, cell wall-less, epierythrocytic parasites, comprising the group of uncultivable *Mycoplasma* species (MESSICK, 2004).

*Corresponding author:

M. Abbaszadeh Hasiri, Assistant Professor of Small Animal Internal Medicine, Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, P.O. Box 1731, 71345, Shiraz, Iran, Phone: +98 71 3228 8660, +98 91 7707 0881, Fax: +98 71 3228 6950, E-mail: abbaszadeh@shirazu.ac.ir; vetmail@gmail.com

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Two species of hemotropic mycoplasma that infect dogs are known so far: *Mycoplasma hemocanis* (Mhc) and Candidatus *Mycoplasma hematoparvum* (CMhp) (BARKER et al., 2009; GREENE, 2012). Transmission through blood sucking arthropods, such as the tick *Rhipicephalus sanguineus* or by ingestion (or transfusion) of infected blood have been proven (DANTAS-TORRES, 2008; WENGI et al., 2008). These extracellular parasites attach to the surface of canine erythrocytes, causing hemolytic anemia, mostly through extravascular destruction of erythrocytes by the mononuclear phagocyte system (GREENE, 2012). Infection with these hemoplasmas generally only induces clinically significant anemia in splenectomized or immunocompromised dogs, although latent infections may cause subclinical anemia (BRINSON and MESSICK, 2001; GREENE, 2012). Most non-splenectomized dogs infected with hemoplasma do not develop clinical evidence of disease and do not have sufficient numbers of organisms present in the blood to be recognized during routine blood film examinations. Therefore, molecular techniques that are simpler, faster and usually more sensitive have been developed for hemoplasma species detection (CRIADO-FORNELIO et al., 2003; GREENE, 2012).

The objective of this study was the molecular characterization of hemotropic *Mycoplasma* species and differentiation of Mhc and CMhp in a population of 26 anemic and 27 non-anemic (PCV $\geq$ 35) dogs, in the south of Iran by RFLP-PCR. In addition, complete blood count (CBC) parameters and epidemiological factors were evaluated to determine if there was an association between them and *Mycoplasma* infection status.

Materials and methods

Sample collection: In total, 26 anemic dogs (PCV $\leq$35) and 27 control dogs (PCV >35) were selected from cases referred to the School of Veterinary Medicine, Shiraz University, Fars province, Iran during a one-year period (October 2013 to September 2014). Complete histories, including: age, breed, gender, type of housing (indoor, outdoor), presence of another dog in the household and whether they were symptomatic or not, were recorded. A physical examination, which included TPR (temperature, pulse and respiratory rate), was performed. A blood sample (4 mL) was obtained via cephalic vein puncture, and placed into tubes containing EDTA.

PCR assay. Amplification of the 16S rDNA sequences for both Mhc and CMhp was performed using the following primers: 5'- GGCCCATATTCCT(AG)CGGGAAG -3' and 5'- AC(AG)GGATTACTAGTGATTCCA-3' (HOELZLE et al., 2011). These primers amplified a ~1000 bp PCR product encompassing the polymorphic site for the Hind III restriction enzyme. The PCR reaction (25 µL) was performed in 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl2, 100 µM of each dNTP, 20 pmol of each primer (Cinnagen Inc., Tehran, Iran), and 2 U Taq DNA polymerase (Cinnagen Inc., Tehran, Iran) using 2 µL of DNA extracted from the blood as a template. The target gene was amplified using a DNA thermo cycler (MJ mini, BioRad, USA) as follows: Initial denaturation at 94 °C for 5 min,

followed by 45 amplification cycles (94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s) and a final extension cycle (72 °C for 5 min). Finally, the PCR products from positive cases were sequenced using the Sanger method (ABI 3730 Capillary DNA analyzer, Applied Biosystems, Foster City, CA, USA) to find possible existing patterns of polymorphism in the 16S rDNA gene. Sequences from the hemoplasma species identified in this study were used in the phylogenetic analyses, along with other related sequences of hemotropic *Mycoplasma*, with the Clustal W and neighbor-joining methods using MEGA4 software (TAMURA et al., 2007).

Finally, on the basis of the obtained pattern of polymorphism in this gene in Iran and data already existing in GenBank, an appropriate PCR-RFLP method was designed. Two canine hemoplasma sequence analysis showed a polymorphism for restriction enzyme (Hind III; 5'-A/AGCTT- 3'). RFLP analysis predicted a unique fragment without digestion for Mhc, so this species could be clearly distinguished from CMhp, which produced two fragments of 873 bp and 123 bp. Accordingly, PCR products were digested with endonucleases Hind III (Jena Bioscience, Germany) to differentiate two canine hemoplasma agents. Enzyme digestion was performed in a 20 µL mixture containing 12 µL of the 16S rDNA PCR product, 0.5 µL (2 U) of enzyme, 2 µL buffer and 5.5 µL of distillated water at 37 °C for at least 5 hrs. After digestion with Hind III, the presence of PCR products was determined by electrophoresis of 10 µL of each reaction product in 1.2% (w/v) agarose gel with trisborate EDTA electrophoresis buffer, and visualized under UV light.

Statistical analysis. The data were analyzed using SPSS software (version 15) by means of chi-square, Fischer exact test and Mann-Whitney test. Differences between means were considered statistically significant if the P value was less than 0.05.

Results

The PCR yielded an amplicon of approximate length of 1001 bp and 975 bp for CMhp and Mhc, respectively. All positive PCR products (n = 7) were purified and sequenced using both forward and reverse primers. Partial 16S rDNA sequence analysis revealed that 4 (7.5%) and 3 (5.7%) samples were positive for Mhc and CMhp, respectively. All samples positive by the PCR assay were subjected to RFLP analysis of the amplification product. There was a complete correlation between the results obtained with the sequencing method and RFLP typing design. Fig. 1 shows the different RFLP patterns for two species of Mhc and CMhp after digestion with Hind III restriction enzyme. No atypical or ambiguous RFLP patterns occurred, and all positive samples could be correctly assigned to a species according to the RFLP pattern. The 16S rDNA sequences obtained in this study were submitted to the GenBank, with accession numbers KU886262 to KU886264.

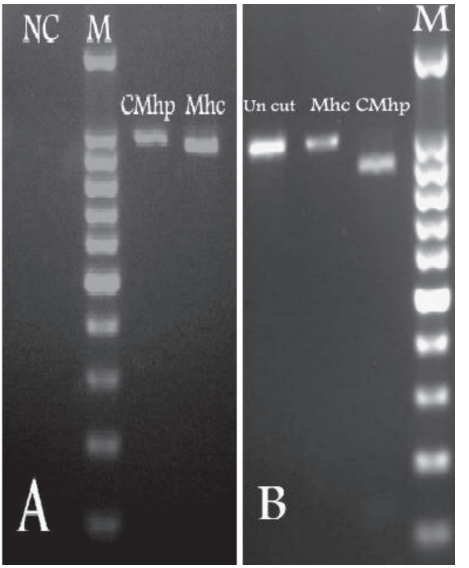

Fig. 1. (A) Gel electrophoresis of PCR products amplified from positive cases for Candidatus *Mycoplasma haematoparvum* (CMhp) and *Mycoplasma haemocanis* (Mhc) compared to molecular marker (M) and negative control (NC). (B) RFLP patterns after digestion of PCR product using Hind III for Mhc and CMhp compared to uncut PCR product and molecular marker (M).

Sequencing results showed that all the isolates for Mhc (n = 4) shared similar sequences in amplified regions of the 16S rDNA gene (KU886264), whereas a point mutation of T to C in position 902 (based on our accession numbers) was detected in Iranian CMhp, and two genotypes A(KU886262) and B (KU886263) of these organisms were characterized in Iran. Furthermore, multiple alignments of other isolates of CMhp reported in GenBank showed additional polymorphisms base d on 16S rDNA analysis (Table 1).

Phylogenetic analysis of the sequence alignment using a neighbor-joining algorithm is shown in Fig. 2.

Out of 53 blood samples (27 non-anemic dogs and 26 anemic dogs), 7 (13.2%) were positive for one *Mycoplasma* species. The results showed that only 3 (11.53%) anemic dogs were detected as positive cases. Unexpectedly, 4 (14.81%) of the non-anemic dogs were also positive by PCR assay. The lowest hematocrit levels (10.5% and 19.3%) were detected in dogs with Mhc infection. The detailed changes in the hematologic parameters

of examined dogs based on PCR results are presented in Tables 2 and 3. Factors such as age, breed, gender and the presence of another dog in the same household, showed no significant differences between infected or non-infected dogs (P>0.05). Although six (85.7%) of the infected dogs were kept outdoors (in a yard or garden), the type of housing (indoor, outdoor) was not statistically significant either (P = 0.40).

Table 1. Intraspecies variation in the Iranian CMhp 16S rDNA sequences (genotype A: KU886262 and genotype B: KU886263) and other existing CMhp sequences in the GenBank database

Nucleotid Positions	Main Base	Nuleotide polymorphisms in 16S rDNA for Candydatus *Mycoplasma hematoparvum* CMhp								
		KC762746	AY383241	GQ129113	GQ129112	GQ129114	EF416569	AY532390	KU886262	KU886263
314	G	G	G	G	G	A	G	G	G	G
379	T	T	T	T	T	T	T	G	T	T
493	T	T	T	T	T	T	T	G	T	T
503	T	T	T	T	T	T	T	G	T	T
745	G	G	G	G	G	G	G	T	G	G
746	T	T	T	T	T	T	T	G	T	T
751	T	T	T	T	T	T	T	G	T	T
902	T	C	T	T	T	T	T	T	C	T

Table 2. Comparison of hematological variables (Mean ± SE) in dogs with haemotropic *Mycoplasma* (either Mhc or CMhp) infection and PCR negative dogs

Factor		Groups		P value
		PCR-positive dogs (n = 7)	PCR-negative dogs (n = 47)	
Mean ± SE	PCV (%)	32.88 ± 4.96	35.14 ± 1.74	P>0.05
	RBC (10^{12}/L)	4.30 ± 0.69	4.60 ± 0.20	P>0.05
	Hb (g/dL)	11.65 ± 1.73	12.25 ± 0.62	P>0.05
	MCV (fL)	78.01 ± 2.18	75.39 ± 1.33	P>0.05
	MCH (pg)	27.98 ± 1.08	26.25 ± 0.50	P>0.05
	MCHC (%)	35.81 ± 0.59	34.77 ± 0.25	P>0.05
	RDW (%)	17.05 ± 0.83	16.71 ± 0.57	P>0.05
	Platelet (10^9/L)	465 ± 102.58	428.06 ± 28.75	P>0.05
	MPV (fL)	7.57 ± 0.47	8.36 ± 0.20	P>0.05
	WBC (10^9/L)	14.74 ± 2.73	14.46 ± 1.74	P>0.05

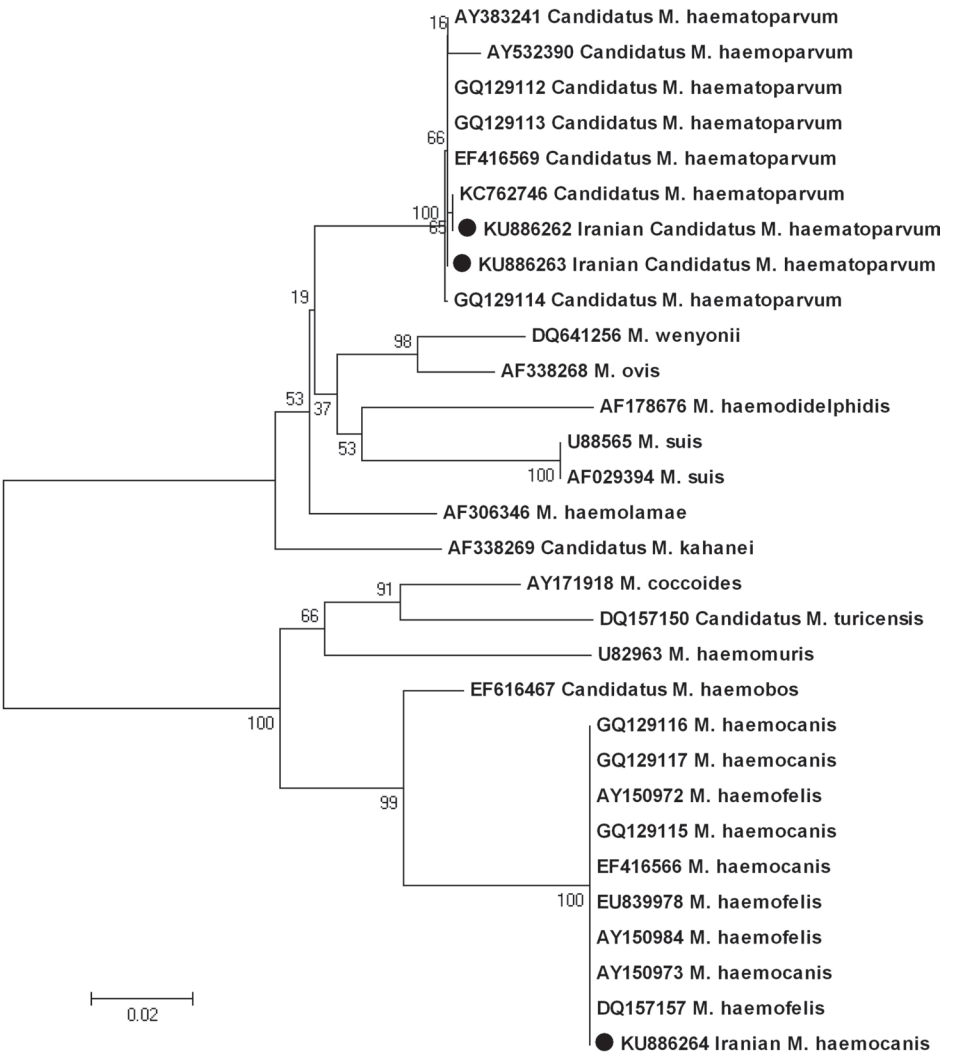

Fig. 2. Phylogenetic tree based on 16S rDNA sequences, constructed according to the Neighbor-Joining method, showing the position of Iranian Mhc and CMhp compared to other hemotropic *Mycoplasma* species. Bootstrap values, calculated from 1,000 repetitions, are placed at each branch point.

The number of platelets were significantly increased in Mhc infected dogs compared to the CMhp positive dogs (P = 0.01) (Table 3).

Table 3. Comparison of hematological variables (Mean ± SE) in dogs with Mhc, CMhp and negative cases

Factor		Group			P value
		Dogs positive for CMhp (n = 3)	Dogs positive for Mhc (n = 4)	Control group (n = 47)	
Mean ± SE	PCV (%)	38.53 ± 3.44	28.65 ± 8.14	35.14 ± 1.74	P>0.05
	RBC (10^{12}/L)	4.99 ± 0.48	3.79 ± 1.17	4.60 ± 0.20	P>0.05
	Hb (g/dL)	13.5 ± 1.22	10.27 ± 2.87	12.25 ± 0.62	P>0.05
	MCV (fL)	77.50 ± 3.38	78.40 ± 3.29	75.39 ± 1.33	P>0.05
	MCH (pg)	27.10 ± 0.87	28.65 ± 3.83	26.25 ± 0.50	P>0.05
	MCHC (%)	35 ± 0.37	36.42 ± 0.94	34.77 ± 0.25	P>0.05
	RDW (%)	16.40 ± 0.95	17.55 ± 1.33	16.71 ± 0.57	P>0.05
	Platelet (10^9/L)*	221.33 ± 26.56	647.75 ± 102.48	428.07 ± 28.75	P≤0.05
	MPV (fL)	7.86 ± 0.86	7.35 ± 0.61	8.36 ± 0.20	P>0.05
	WBC (10^9/L)	14.16 ± 2.82	15.17 ± 4.69	14.46 ± 1.74	P>0.05

* indicate P = 0.01

Discussion

Hemoplasmas are obligate epierythrocytic organisms that attach to the erythrocytes of dogs. In some cases, hemoplasma infection is associated with hemolytic anemia of variable severity, ranging from nonclinical hemolysis to severe anemia (WILLI et al., 2007).

In this study, we applied a novel RFLP-PCR for detection and differentiation of two distinct haemotropic mycoplasma in dogs from Shiraz, Iran.

The results showed that the discriminatory power of RFLP analysis of 16S rDNA was perfect and this method allows the accurate typing of canine hemotropic mycoplasma species. To our knowledge, this is the first discrimination of the hemotropic mycoplasma in a dog population by a RFLP approach.

Despite the identification of canine hemoplasma agents in dogs in Iran (TORKAN et al., 2014), 16S rDNA gene sequencing and phylogenetic analysis of both causative agents had not been identified in this area. Based on this, a decision was made to check the phylogenetic position of both canine hemotropic mycoplasmas based on the 16S rDNA gene. Sequencing results and the RFLP PCR pattern of the samples obtained in the present study, with those previously reported for different hemotropic mycoplasma, revealed that both canine hemotropic mycoplasma exist in the southern part of Iran.

This study represents an expansion of the phylogenetic data available for mycoplasmas, including hemoplasmas, based on 16S rDNA sequence data. A previous phylogenetic study revealed that hamoplasmas had been classified into Haemominutum and Haemofelis clusters based on 16S rDNA analyses (KENNY et al. 2004). Similarly, our phylogenetic tree based on this molecular marker showed that Iranian canine hemotropic *Mycoplasma* species were divided into two major groups. Further studies will be required, including phylogeny with non-16S rRNA gene sequences, to describe more fully the relationship between the canine and feline isolates.

In addition, the comparison between the sequence alignments of the Iranian 16S rDNA for the CMhp undertaken in this study, showed the occurrence of genetic polymorphisms and heterogeneity in this ribosomal locus, in which two different genotypes of CMhp (A and B) were characterized in Iran. Despite the occurrence of this genetic variation in Iranian CMhp, 16S rDNA sequences in Mhc, were completely conserved (100% identity) in the present study.

In the current study, 13.2% of samples were positive for hemoplasma. It was hypothesized that this high prevalence may be associated with the presence of *Rhipicephalus sanguineus*, a proposed vector for canine hemoplasmas. CMhp and Mhc infections were diagnosed at 5.7% and 7.5%, respectively. Similarly, TORKAN et al. (2014) found the prevalence of CMhp and Mhc infections among dogs in Isfahan (central Iran) was 10% and 13%, respectively (TORKAN et al., 2014). A survey conducted in Brazil to compare infection rates for Mhc between urban and rural areas showed that twenty (11.3%) out of 176 dogs living in rural areas were positive, whereas 6 of 104 (5.8%) dogs from urban areas harbored the organism (SANTOS, 2008). In France, blood samples from dogs (n = 460) were analyzed by PCR to evaluate hemoplasma infection status. Seventy-one dogs (15.4%) were positive. Of these, 44 (9.6%) were infected with an organism closely related to CMhp; 15 (3.3%) were infected with Mhc, and 12 dogs (2.6%) were infected with both organisms (KENNY et al., 2004). Although both canine hemoplasma species were detected in the population examined in the present study, they were not detected in the same dog concurrently. The present results are in agreement with previous studies in Sudan and France, where Mhc was more prevalent than CMhp (INOKUMA et al., 2006; KENNY et al., 2004). In another study conducted in Europe, canine hemoplasmas were detected in 82 (9.6%) out of 850 blood samples (NOVACCO et al., 2010). The hemoplasma prevalence was significantly higher in Portugal (40%) than in Italy (9.5%) and Spain (2.5%) (NOVACCO et al., 2010).

In a recent investigation performed in the United States, hemoplasma prevalence was 1.3% (7 out of 506), with Mhc and CMhp prevalence of 0.6% and 0.8%, respectively (COMPTON et al., 2012). Difficulties exist in comparing results from different studies because small sample sizes might not truly represent whole populations, and different

types of animals are sampled in different studies (e.g., variable percentages of healthy and sick animals, demographics). Vector distribution could also influence results (DANTAS-TORRES, 2008).

In the present research, there was no association between canine hemotropic mycoplasma infection with the presence of another dog in the household, fever, respiratory signs such as sneezing or coughing, and gastrointestinal signs such as vomiting or diarrhea. Some research indicates that kennel-kept dogs were significantly more frequently infected with hemoplasmas than dogs living in private homes (NOVACCO et al., 2010). Although in the present study six (85.7%) of the infected dogs were kept outdoors (in a yard or garden), type of housing (indoor, outdoor) was not statistically significant either. However a larger sample size is required for accurate statistical analysis to evaluate this finding.

Five infected dogs were male and 5 positive cases were older than 2 years old, but the gender or age factors had no significant effect on infection occurrence. In contrast to our results, NOVACCO et al. (2010) showed that PCR-positive dogs for canine hemotropic mycoplasma infections were significantly younger than PCR negative dogs (NOVACCO et al., 2010). In the present study, although breed type or general signs (depression and/or lethargy) showed no statistical differences between infected or non-infected dogs, infection prevalence was higher in mixed breed dogs (than pure breeds) or those that had depression and/or lethargy. NOVACCO et al. (2010) found that crossbred dogs are more likely to be PCR-positive than purebred dogs. Similar to our study, some research showed that the prevalence of canine hemoplasma infections did not differ between clinically healthy and sick dogs (COMPTON et al., 2012; NOVACCO et al., 2010) and there was no association between anemia and hemoplasma infection (NOVACCO et al., 2010; ROURA et al., 2010). Some of these disagreements between studies may also be related to different sample sizes and the various stages of infection. As the stages of infection in the PCR-positive dogs were unknown, we could not determine whether the dogs represented chronic carriers that may have recovered from acute illness and thus lacked clinical signs, or acutely infected animals.

Although the anemia and infection had no statistical relation in our results, the fewest hematocrits (10.5% and 19.3%) were detected in dogs with Mhc infection. This could be attributed to the more severe hemolytic anemia induced by Mhc compared to CMhp during the acute phase of infection. Interestingly, statistical analysis showed a significant increase in platelet count in Mhc infected dogs (647.75×10^9/L) compared to CMhp positive dogs (221.33×10^9/L) ($P = 0.01$). Platelet elevation may be related to secondary thrombocytosis in response to hemolytic anemia (BUSS et al., 1994).

Conclusion

We conclude from the present study that both forms of canine hemoplasma infection must be expected in the south of Iran. The hemoplasma infected dogs may not exhibit clinical signs clearly attributable to a specific canine haemoplasma agent. Accordingly, the RFLP-PCR presented in this study provides a rapid and easy-to-use method in a wide variety of laboratories, to detect and discriminate between two species of haemoplasma in dogs.

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SAŽETAK

U pasa su poznate dvije vrste hemoplazama: *Mycoplasma haemocanis* (Mhc) i Candidatus *Mycoplasma haematoparvum* (CMhp). Cilj je ovog istraživanja bio razviti novu metodu za dokaz i razlikovanje pasjih hemoplazama temeljenu na određivanju polimorfizma dužine restrikcijskog fragmenta (PDRF) gena 16S rDNA uporabom endonukleaze *Hind III*. Analizirani su i rizični čimbenici, kliničke osobitosti i hematološke promjene u inficiranih pasa na području Shiraza u Iranu. Uzorci krvi bili su prikupljeni od anemičnih (hematokrit ≤35; n = 26) i kontrolnih pasa (hematokrit >35; n = 27) te pretraženi na prisutnost pasjih hemoplazama metodom RFLP-PCR i Sangerovom metodom sekvenciranja 16S rDNA. Prisutnost Mhc (4 od 53 slučaja; 7,5%) i CMhp (3 od 53 slučaja; 5,7%) bila je potvrđena analizom polimorfizma restrikcijskog fragmenta 16S rDNA i sekvenciranjem. Nije ustanovljena veza između infekcije hemoplazmama i anemije te zdravstvenog stanja, dobi, pasmine, spola, načina držanja i prisutnosti drugih pasa. Jedino je broj trombocita u pasa inficiranih vrstom *Mycoplasma haemocanis* bio statistički značajno veći od onih inficiranih Candidatus *Mycoplasma haematoparvum* i pasa negativnih na hemoplazme. Ovo izvješće potkrepljuje prisutnost Mhc i CMhp u južnom Iranu. Infekcije hemotropnim mikoplazmama mogu se očekivati i u pasa bez kliničkih znakova ili hematoloških poremećaja. Prvi put je pokazano da se metodom RFLP-PCR mogu uspješno razlikovati hemotropne mikoplazme u pasa.

Ključne riječi: hemoplazma, *Mycoplasma haemocanis* (Mhc), Candidatus *Mycoplasma haematoparvum* (CMhp), pas, Iran