

Intravascular hemolysis associated with *Candidatus Mycoplasma hematoparvum* in a non-splenectomized dog in the south region of Iran

Hassan Sharifiyazdi, Mohammad Abbaszadeh Hasiri*, Amin Hosein Amini

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

Article Info	Abstract
<p>Article history:</p> <p>Received: 13 April 2013 Accepted: 20 August 2013 Available online: 15 September 2014</p> <p>Key words:</p> <p>Dog Hemoplasma Hemotropic mycoplasma <i>Mycoplasma hematoparvum</i></p>	<p>A 2-year-old male Pekingese dog was referred to Shiraz University's Veterinary Teaching Hospital for anorexia and depression. The case had no history of surgery. Physical examination revealed no abnormalities except mild depression and fever. Small, coccoid, epicellular bacteria were detected on erythrocytes by microscopic examination of the Giemsa-stained blood smears. Abnormalities noted in the complete blood count included regenerative anemia characterized by a marked reticulocytosis. Examination of the plasma showed visual evidence of slight intravascular hemolysis. In addition, Howell-Jolly bodies, nucleated RBCs, increased immature neutrophils and thrombocytosis were found in this case. The urine was strongly positive for bilirubin, and the urine sediment had abundant bilirubin crystals. For polymerase chain reaction (PCR) purpose, total DNA was extracted from blood sample collected from dog. PCR was positive and phylogenetic analysis of concatenated data showed our isolate clustered within <i>Candidatus Mycoplasma hematoparvum</i> group. Treatment was performed by oral ciprofloxacin and prednisolone. The clinical signs improved after three days. Two month follow-up showed no recurrence. In conclusion, hemoplasmosis should be considered as a differential diagnosis in dogs with hemolytic process and pyrexia. The PCR evaluation for hemoplasma DNA should be included in the investigation of such cases to enable the rapid detection of this infection, which may be more common than previously estimated. Besides, ciprofloxacin might have an effect on treatment of hemoplasma in dogs, however, conducting further case studies are necessary to recommend successful treatment.</p> <p>© 2014 Urmia University. All rights reserved.</p>

همولیز داخل عروقی ناشی از کاندیداتوس مایکوپلازما هماتوپارووم در یک قلابه سگ بدون برداشت طحال در جنوب ایران

چکیده

سگ ۲ ساله، نر، نژاد پکینیز با علائم بی اشتها و بی حالی به بیمارستان دامپزشکی دانشگاه شیراز ارجاع داده شد. معاینه بالینی تب و افسردگی را نشان داد. آزمایش خون انجام و گسترش خونی جهت رنگ آمیزی گیمسا تهیه شد. در ارزیابی میکروسکوپی گسترش خونی رنگ آمیزی شده انگل‌های کوکوئید کوچک در سطح اریتروسیت‌ها شناسایی گردید. آزمایش خون کامل کم خونی جبرانی و رتیکولوسیتوزیس را نشان می‌داد. همولیز داخل عروقی در پلاسما دیده شد. بعلاوه اجسام هاول جولی، گلبول‌های قرمز هسته دار، افزایش نوتروفیل‌ها و ترومبوسیتوزیس مشاهده گردید. آزمایش ادرار کریستال‌های یبلی روبین فراوانی را نشان داد. در بررسی واکنش زنجیره‌ای پلیمرز (PCR) و تعیین سکانس، عفونت ناشی از کاندیداتوس مایکوپلازما هماتوپارووم تعیین گردید. درمان با سیپروفلوکساسین و پردنیزولون انجام شد. علائم بالینی بعد از ۳ روز بهبود یافت و پس از ۲ ماه پیگیری بازگشتی از بیماری مشاهده نگردید. هموپلاسموزیس باید به عنوان یکی از تشخیص‌های تفریقی تب و کم خونی همولیتیک در سگ‌ها در نظر گرفته شود. آزمایش PCR روش تشخیصی مناسبی برای رد یا تایید این بیماری است. بر اساس اطلاع نویسندگان، این مورد اولین گزارش عفونت کاندیداتوس مایکوپلازما هماتوپارووم در یک قلابه سگ بدون برداشت طحال با همولیز داخل عروقی در جنوب ایران بوده است.

واژه‌های کلیدی: ایران، سگ، مایکوپلازما هماتوپارووم، هموتروپیک مایکوپلازما، همولیز داخل عروقی

*Correspondence:

Mohammad Abbaszadeh Hasiri. DVM, DVSc
Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
E-mail: abbaszadeh@shirazu.ac.ir

Introduction

There are two species of hemotropic mycoplasma (also called hemoplasma) that infect dogs which are known so far: *Mycoplasma hemocanis* and *Candidatus Mycoplasma hematoparvum* (*CMhp*).^{1,2} The main form of transmission is probably through blood sucking arthropods such as the tick *Rhipicephalus sanguineus* whose main geographical distribution is associated with the Mediterranean and sub-Mediterranean climates.³ These extracellular parasites attach to the surface of canine erythrocytes, causing hemolytic anemia mostly through extravascular destruction of erythrocytes by the mononuclear phagocyte system.² Infection with these hemoplasmas generally only induces clinically significant anemia in splenectomized or immunocompromised dogs, although latent infections may cause subclinical anemia.^{2,4} *Candidatus M. hematoparvum* was first described in association with anemia in a splenectomized dog undergoing chemotherapy for leukaemia.⁵ Most non-splenectomized dogs infected with hemoplasma do not develop clinical evidence of disease and do not have sufficient number of organisms present in the blood to be recognized during routine blood film examinations. Besides, diagnosis of these pathogens by serological responses can be unspecific. Therefore, molecular techniques that are simpler, faster, less hazardous and usually more sensitive have been developed for hemoplasmas species detection.^{2,6}

The present case report describes the first report of hemolytic *Mycoplasma* infection in the south of Iran and hemolytic disorders in a non-splenectomized dog infected with *CMhp*.

Case Description

A 2-year-old male Pekingese dog was referred to Veterinary Teaching Hospital of Shiraz University for anorexia and depression. The case had no history of surgery. Body temperature was 40 °C, heart rate (130 beat per min), respiratory rate (20 breath per min) and body condition was normal. Physical examination revealed no abnormalities except mild depression.

Hematological examination was carried out using automatic cell counter (Exigo, Stockholm, Sweden) and blood smears were prepared for Giemsa staining.

Results

Small, coccoid, epicytellar bacteria were detected on erythrocytes by microscopic examination of the Giemsa-stained blood smears (Fig. 1).

Abnormalities noted in complete blood count included regenerative anemia characterized by a marked reticulocytosis ($278 \times 10^9 \text{ L}^{-1}$) and packed cell volume of 50.0%. Examination of the plasma showed visual evidence of slight

intravascular hemolysis (hemoglobinemia). In addition, Howell-Jolly bodies, nucleated RBCs (4 per 100 WBC), increased immature neutrophils (Band form; $0.36 \times 10^9 \text{ L}^{-1}$, reference interval: 0 to $0.30 \times 10^9 \text{ L}^{-1}$) and thrombocytosis ($705 \times 10^9 \text{ L}^{-1}$, reference interval: 211 to $621 \times 10^{11} \text{ L}^{-1}$) were found in this case.



Fig. 1. Coccoid bacteria were detected on the surface of erythrocytes of the affected dog, (Giemsa, 1000 \times).

The urine was strongly positive for bilirubin, and the urine sediment had abundant bilirubin crystals.

For polymerase chain reaction (PCR) purpose, total DNA was extracted from blood sample collected from dog using the DNeasy® Blood and Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of the 16S rDNA was performed using the lyophilized PCR micro tubes (Model Accupower PCR PreMix; BioNeer Co., Seoul, Korea) and oligonucleotides targeting 16S rDNA regions specific for HM (16S_HEMO forw: GGCCCATATTCCT (AG) CGGGAAG; 16S_HEMO rev: AC (AG) GGATTACTAGTGATTCCA).⁷ The amplified products were sequenced directly using the capillary DNA analyzer (Model ABI 3730; Applied Biosystems, Foster City, USA). The 16S rDNA sequence obtained was compared to GenBank entries using the BLAST tool provided by National Center for Biotechnology Information (NCBI).⁸ Different related *Mycoplasma* species were used for phylogenetic analysis. Multiple sequence alignments and construction of a phylogenetic tree were made with the neighbor-joining method using the software, MEGA (Version 4.0; Biodesign Institute, Tempe, USA).⁹ PCR was positive by producing a specific fragment of ~1000 bp from DNA of the blood as shown in Figure 2.

Phylogenetic analysis of concatenated data showed our isolate clustered within the *CMhp* group (Fig. 3). Furthermore, comparative sequence analysis using the obtained 16S rDNA sequence (Accession number KC 762746) demonstrated the highest homology (more than 99.0%) to *CMhp*, previously described by Novacco *et al.*¹⁰ (Accession numbers GQ129112, GQ129113; Italy) and Wengi *et al.*³ (Accession number EF416569; Switzerland). The difference between Iranian sequence and these isolates

was only a transition mutation of cytosine (C) to thymine (T) at position 902 based on the accession number KC762746. Also, comparison of an available sequence for *CMhp* (Accession number AY532390) originating from France with Iranian sequence showed the greatest difference (0.7%; 7 nucleotides).

Treatment was performed by oral ciprofloxacin (Farabi Pharmaceutical Co. Isfahan, Iran; 20 mg kg⁻¹, q24h for 7 days) and prednisolone (Aburaihan Co., Tehran, Iran; 0.5 mg kg⁻¹, q12h for 3 days). The clinical signs improved after three days. Two month follow-up showed no recurrence.

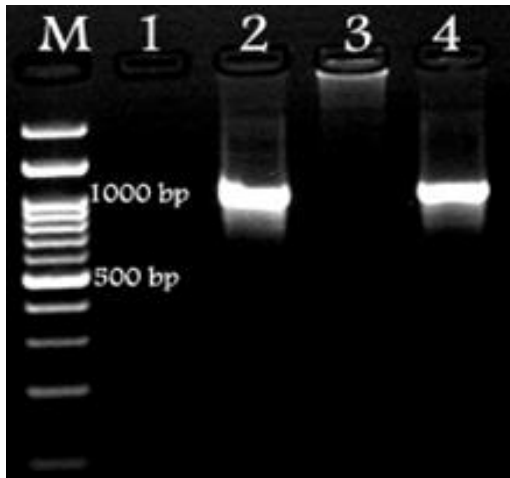


Fig. 2. PCR amplification provided from canine sample (Lane 4) compared with the molecular weight marker (Lane M: 100 bp) and positive control (Lane 2). Distilled water (Lane 1) and DNA from a healthy dog (Lane 3) are negative controls.

Discussion

This is the first case of canine infection with *CMhp* in the southern region of Iran. This organism, named *CMhp* is smaller than *M. hemocanis* and does not form chains on the erythrocyte surface of dogs.⁵ *Candidatus M. hemato-parvum* is genetically more closely related to *Candidatus M. hemominutum* than to *M. hemofelis*, and was isolated from the blood of a splenectomized dog with hematopoietic neoplasia.⁵ The infection has been confirmed by methods of molecular biology and specific PCR is the gold standard for detection of these infections.^{12,13}

Santos showed twenty (11.3%) out of 176 dogs living in rural areas were positive for hemoplasmas, whereas 6 of 104 (5.8%) dogs from urban areas harbored the organism.¹³ Our case lived indoors in an urban area. Blood samples from 460 dogs living in the south of France showed 9.6% were infected with *CMhp* whereas only 3.3% were infected with *M. hemocanis* and 2.6% were infected with both organisms.¹⁴ However, in Switzerland only 1.2% of dogs had positive real-time PCR results for the canine hemoplasmas. The prevalence in Europe is higher in the Mediterranean countries. It is postulated that the presence

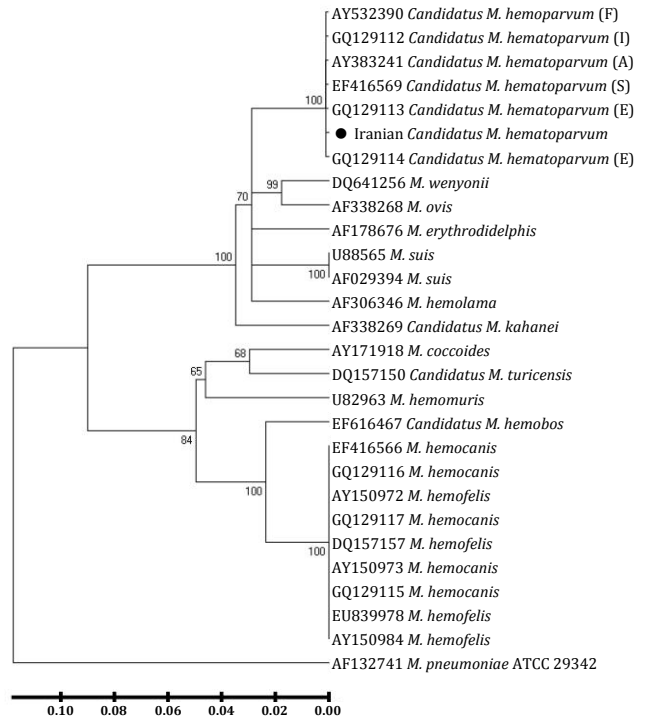


Fig. 3. Phylogenetic analysis using partial sequences of 16S rDNA showing the position of the sequenced canine hemoplasma isolates from Iran among the hemotropic mycoplasma group. Numbers in the relevant branches refer to the values of bootstrap probability of 1,000 replications. M.= *Mycoplasma*. S= Switzerland; F= France I= Italy, E= Spain and A= USA. The phylogenetic tree was constructed using the neighbor-joining method. A nucleotide sequence of the 16S rRNA gene of *M. pneumoniae* ATCC 29342 with accession number AF132741 was included as an out-group.

or absence of an appropriate vector for transmission of the organisms may explain these differences.²

Clinical cases of canine hemoplasmosis have occasionally been reported, but co-factors such as splenectomy or immunosuppression seem to play a role in pathogenesis of the disease. Rare cases of acute disease have occurred in dogs with intact spleen in which no evidence for immunosuppression was found.² However, additional immunological assays (not routinely available) were necessary to better characterize the underlying immunosuppressive conditions. In this case, hemolysis and fever existed in non-splenectomized dog. So it could be postulated that in intravascular hemolysis, hemoplasmas must be considered in differential diagnosis as a suspected causative agent.

On the other hand, most non-splenectomized dogs infected with hemoplasmas do not have sufficient numbers of organisms present in the blood to be recognized during routine blood film examinations and due to unspecific serological examination, PCR could be recommended. In the present case, we demonstrated this hemoplasma species in a hemolytic dog with regenerative response.

As mentioned above, a greater regenerative response occurs in hemolytic anemia than in other anemia because the iron and protein of the destroyed RBCs are readily available for erythropoiesis. The polychromasia, anisocytosis, Howell-Jolly bodies, and nucleated RBCs are consistent with regenerative anemia.¹⁵ In spite of normal PCV (50.0%), ongoing slight intravascular hemolysis was confirmed with light pink to red discoloration of plasma and remarkable regenerative response. Hemoglobinuria was not detected in this case because hemoglobinemia occurred below the threshold for urinary excretion of hemoglobin (> 150 mg dL⁻¹).

Recent research indicated that treatment of *M. hemofelis* infected cats with the fluoroquinolone may offer more effective long-term clearance of organisms than doxycycline.² Orally administered tetracyclines are reported to be effective in treating *M. hemocanis* infections but data are not available on the efficacy of treatment of *CMhp* infection in dogs.² We translated successful cat treatment for this case. The clinical signs improved within three days of initiating treatment and follow-up showed no regression.

This case was unique in several ways: first in describing *CMhp* infection using sequencing method in Iran. Since ticks and mites as important sources of *Hemobartonella* infection are frequent in this area, it seems this infection may be common in these dogs and all ticks should be removed from the environment; second, in associating hemoplasma infection with pyrexia and intravascular hemolysis in a non-splenectomized dog, and third, successful treatment.

In conclusion, hemoplasmosis should be considered as a differential diagnosis in dogs with hemolytic process and pyrexia. The PCR evaluation for hemoplasma DNA should be included in the investigation of such cases to enable the rapid detection of this infection, which may be more common than previously estimated. Besides, ciprofloxacin might have an effect on treatment of hemoplasma in dogs, however, conducting further case studies are necessary to recommend successful treatment.

References

1. Barker EN, Tasker S, Day M, et al. Development and use of real-time PCR to detect and quantify *Mycoplasma hemocanis* and "*Candidatus Mycoplasma hemato-parvum*" in dogs. *Vet Microbiol* 2009; 140: 167-170.
2. Greene CE. *Infectious diseases of the dog and cat*. 3rd ed. Philadelphia, USA: Saunders 2012; 310-319.
3. Wengi N, Willi B, Boretti FS, et al. Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. *Vet Microbiol* 2008; 126: 132-141.
4. Brinson JJ, Messick JB. Use of a polymerase chain reaction assay for detection of *Hemobartonella canis* in a dog. *J Am Vet Med Assoc* 2001; 218: 1943-1945.
5. Sykes JE, Bailiff NL, Ball LM, et al. Identification of a novel hemotropic mycoplasma in a splenectomized dog with hemic neoplasia. *J Am Vet Med Assoc* 2004; 224: 1946-1951.
6. Criado-Fornelio A, Martinez-Marcos A, Buling-Sarana A, et al. Presence of *Mycoplasma hemofelis*, *Mycoplasma hemominutum* and piroplasmids in cats from southern Europe: A molecular study. *Vet Microbiol* 2003; 93: 307-317.
7. Hoelzle K, Winkler M, Kramer MM, et al. Detection of *Candidatus Mycoplasma hemobos* in cattle with anemia. *VET J*; 2011; 187(3): 408-410.
8. BLAST: Basic Local Alignment Search Tool. Available at: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. 21 March, 2013.
9. Tamura K, Dudley J, Nei M, et al. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24: 1596-1599.
10. Novacco M, Meli ML, Gentilini F, et al. Prevalence and geographical distribution of canine hemotropic mycoplasma infections in Mediterranean countries and analysis of risk factors for infection. *Vet Microbiol* 2010; 142(3): 276-284.
11. Jensen WA, Lappin MR, Kamkar S, et al. Use of a polymerase chain reaction assay to detect and differentiate two strains of *Hemobartonella felis* in naturally infected cats. *Am J Vet Res* 2001; 62(4): 604-608.
12. Willi B, Boretti FS, Tasker S, et al. From hemobartonella to hemoplasma: Molecular methods provide new insights. *Vet Microbiol* 2007; 125: 197-209.
13. Santos AP. Hemoplasma infection in domestic cats from Porto Alegre, RS, Brazil [portuguese]. *Acta Scientiae Veterinariae* 37(1): 95-96.
14. Kenny MJ, Shaw SE, Beugnet F, et al. Demonstration of two distinct hemotropic mycoplasmas in French dogs. *J Clin Microbiol* 2004; 42: 5397-5399.
15. Cowgill ES, Neel JA, Grindem CB. Clinical application of reticulocyte counts in dogs and cats. *Vet Clin North Am Small Anim Pract* 2003; 33(6): 1223-1244.