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Short communication

The first clinical and laboratory evidence of co-infection by *Anaplasma phagocytophilum* and *Ehrlichia canis* in a Brazilian dog

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ABSTRACT

Information on *Anaplasma phagocytophilum* in Brazil is very restricted. The aim of this study was to report clinical, parasitological, hematological and molecular evidence of a natural *A. phagocytophilum* infection of an urban Brazilian dog. The dog was an eight-month-old male French bulldog. Veterinary clinical examinations were performed three times: in April, June and December 2013. Biochemical and hematological analyses were performed during all examinations, and blood samples were collected for parasitological surveys in June and December. Morulae were present within neutrophils in blood smears from June. Both samples were PCR positive for *A. phagocytophilum* and *Ehrlichia* spp. Phylogenetic analysis revealed that the phylogenetic topology placed samples from this study in close proximity to other *A. phagocytophilum* isolates. *Ehrlichia* isolates from this dog were 100% identical to *E. canis* isolates, thus *E. canis* and *A. phagocytophilum* co-infection was diagnosed in this dog. Lethargy and skin lesions were the clinical signs observed in this dog. Abnormal hematological parameters, among those, severe thrombocytopenia, were observed in all three occasions. This finding highlights the growing importance of *A. phagocytophilum* in South America.

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Introduction

Dogs in Brazil are affected by various tick-borne pathogens with *Ehrlichia canis* being the most commonly detected. In Minas Gerais region 24–65% of dogs were seropositive against *E. canis* (Costa-Júnior et al., 2009) while in other states the seropositivity varied between 16 and 75% (Vieira et al., 2011; Spolidorio et al., 2013; Paulan et al., 2013).

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an emerging zoonotic tick-borne pathogen that is a Gram-negative obligate intracellular bacterium of granulocytes (Dumler et al., 2001). This pathogen is frequently reported in humans, animals in North America, Europe and Asia (Teglas and Foley, 2006; Zhang et al., 2013). In Brazil, this bacterium has been detected in dogs and ticks using a real-time PCR technique (Santos et al., 2013), in carnivorous birds using conventional PCR (Machado et al., 2012). According to the information available to us, human granulocytic anaplasmosis has not been reported in Brazil.

The occurrence of anaplasmosis in dogs has been geographically associated with HGA (Human Granulocytic Anaplasmosis) (Madewell and Gribble, 1982). The features of granulocytic anaplasmosis in dogs include malaise, lethargy, fever, anorexia, weakness, indisposition, nervous tension, lymphadenomegaly, hepatomegaly and splenomegaly. Hematologic abnormalities, such as mild to severe thrombocytopenia and other leukogram changes have been described in dogs with clinical disease (Dumler et al., 2001). Some clinical manifestations and laboratory findings such as fever, anorexia, lymphadenomegaly, anemia and thrombocytopenia are also observed in *E. canis* infections (Hoskins, 1991).

Due to the difficult clinical diagnosis and the low sensitivity of blood smears, in areas where *A. phagocytophilum* infections are rare, the diagnosis of granulocytic anaplasmosis requires the use of multiple techniques (Carrade et al., 2009). Considering the importance of *A. phagocytophilum* in veterinary and public health, the aim of this study was to report the first clinical case of infection by *A. phagocytophilum* supported by parasitological, hematological, and

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molecular analysis of naturally infected Brazilian dog co-infected with *E. canis.*

Materials and methods

The study was submitted to, and approved by, the Ethics Committee for Animal Research (CETEA) of the Universidade Federal de Minas Gerais (UFMG) under protocol number 175/2010.

Case presentation

Anamnesis

An eight-month-old male French bulldog was referred to the veterinary hospital of the Veterinary School at the Universidade Federal de Minas Gerais in the city of Belo Horizonte in the state of Minas Gerais in Brazil. The dog was born in a kennel in the city of Rio de Janeiro (latitude: 22°54′10″ S; longitude: 43°12′27″ W) and after weaning lived in a kennel in an urban area to Belo Horizonte (latitude: 19°55′15″ S; longitude: 43°56′16″ W). The area is highly urbanized without parks around. When the animal appeared to be sick, the owner kept the dog in an apartment in the same city without other dogs. Veterinary clinical examinations were performed three times: in April, June and December 2013.

Clinical signs and clinical examination results

In April weight loss, depression, pruritus, tick (*Rhipicephalus sanguineus*) and flea infestation were observed. The dog weighted 10.5 kg and skin alterations were observed (hair loss in the thorax and right hind limb, peeling, hypotrichosis and thickening) and skin scraping did not indicate mite infection. The veterinary clinician used the clinical and laboratorial findings to make a diagnosis of *E. canis* infection, and demodicosis due to the skin lesions. The dog was medicated using doxycycline (50 mg, BID for 24 days) and prednisone (10 mg, BID for 3 days). Topical 0.25% fipronil spray was also administered.

In June dog was admitted to the hospital with pruritus and persistent flea infestation. The dogs' weight was 10.3 kg and its skin condition had worsened in skin alterations (i.e., ventral skin suffusions and crusts with chafing). Skin scraping revealed *Demodex canis* infection. The dog was medicated using ivermectin (6 mg, once per week for four weeks) and topical 0.25% fipronil spray (biweekly for two months and monthly thereafter).

In December, the examination revealed weight gain, improvement of the in skins lesion, but the owner reported persistent flea infestation and no sign of ticks for 4–5 months. The dog weighted 10.5 kg and skin alterations had resolved. Skin scraping did not reveal mite infection. Physical examinations revealed normal parameters (i.e., behavior, temperature, nutritional status, cardiorespiratory frequency, pulse, lymph nodes, color of mucous membranes) in all three veterinary consultations.

Laboratory diagnostics

Biochemical and hematological analyses were performed according to Jain (1993). In April, the dog exhibited anemia, thrombocytopenia, and leukopenia with neutropenia. Serum biochemical tests revealed an increase in alanine aminotransferase (ALT). In June, bone marrow aspiration was performed for parasitological examination. Hematological analysis revealed the persistence of anemia and thrombocytopenia, and a considerable increase in total leukocyte numbers and basophilia were observed. In December, hematological analysis revealed the persistent thrombocytopenia and leukopenia with neutrophilia.

Parasitological tests

Samples were taken for parasitological examinations in June and December and blood smears for microscopy were made from peripheral blood (ear tip), subjected to quick Romanowsky staining (Panótico Rápido; Laborclin, Pinhais, PR, Brazil) and examined under the optical microscope at 1000× magnification. For each sample, all the slides were observed.

Bone marrow aspirate was performed during the second examination; one aliquot was used for PCR assays, and the other aliquot was used for bone marrow smears. Considering that canine visceral leishmaniasis (*Leishmania infantum*) is endemic in Belo Horizonte (Leal et al., 2014), laboratory test was performed to detect this protozoan.

Molecular diagnostic

DNA was extracted from blood samples during the second and third clinical examinations and from bone marrow during the second clinical examination. DNA was extracted from the whole blood samples (250 μL) using an illustra blood genomic Prep Mini Spin Genomic DNA purification kit (GE Healthcare®, Piscataway, NJ, USA) according to the manufacturer's instructions.

The nested PCR reaction was performed. Sets of primers were used to detect the following hemoparasite species: *msp2/p44* and *msp4* gene of *A. phagocytophilum*, 16S rRNA gene of *Ehrlichia* spp. and 18S rRNA from the Piroplasmida order (Table 1). Twice-distilled water was used as the negative control. As a positive control, DNA was extracted from embryonic tick cells (strain IDE8) infected with *A. phagocytophilum* and blood samples from dogs individually experimentally infected with *E. canis* and *Babesia canis*, respectively.

In all PCR assays, the reaction mixture in the first round contained 7.5 μL of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 0.6 μL of a solution containing the mixed primers (10 mM) and 5.4 μL of nuclease free water. A 1.5 μL aliquot of the DNA template was added to the reaction mixture to obtain a final volume of 15 μL. The reaction mixtures in the second-round assays were similar, except the templates were the products from the first-round PCR reactions (1.5 μL). Amplifications were done using a touchdown PCR programme as previously described by the authors who designed the primers (Table 1). The PCR amplicons were separated by electrophoresis on 1% agarose gels (40 min, 100 V), stained with GelRed™ (Biotium, Hayward, CA, USA), and visualized under ultraviolet light.

The products from the second-round PCR reactions were purified using a QIAquick PCR Purification Kit (Qiagen Biotecnologia Brasil, São Paulo, Brazil) according to the recommendations of the manufacturer. The purified amplicons were sequencing using an Applied Biosystems model ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) and the Applied Biosystems BigDye® Direct Cycle Sequencing Kit (v. 3.1), with the POP-7™ polymer as the separating matrix and the primers employed in the second-round PCR reaction. Sequences were aligned, edited, and analyzed using MEGA 6.0 software at the URL http://asparagin.cenargen.embrapa.br/phph/ (Tamura et al., 2013). The identity of each sequence was confirmed by a comparison to sequences available at GenBank using BLAST software (Altschul et al., 1990).

Results

Hemoparasite tests

The dog in the present study was negative for *Leishmania infantum* infection, as indicated by IFAT, ELISA, bone marrow smears and molecular analysis of blood and bone marrow.

Table 1
Specific primers used for the detection of hemoparasites.

Specificity	Primers (5′–3′)	Name	Target	Size (bp)	References
Anaplasma phagocytophilum					
First round	ATGAATTACAGAGAATTGCTTGTAGG TTAATTGAAAGCAAATCTTGCTCCTATG	MSP4AP5 MSP4AP3	*msp4*	849	de la Fuente et al. (2005)
Second round	CTATTGGYGGNGCYAGAGT GTTCATCGAAAATTCCGTGGTA	msp4f msp4r	*msp4*	381	Bown et al. (2007)
Anaplasma phagocytophilum	CCAGCGTTTAGCAAGATAAGAG GCCCAGTAACATCATAAGC	msp2-3F msp2-3R	*msp2/p44*	334	Zeidner et al. (2000)
Granulocyte/platelet *Anaplasma/Ehrlichia*					
First round	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC	GE3a GE10r	16S rRNA	932	Massung et al. (1998)
Second round	AACGGATTATTCTTTATAGCTTGCT GGCAGTATTAAAAGCAGCTCCAGG	GE9f GE2	16S rRNA	546	Massung et al. (1998)
Monocyte *Ehrlichia* spp.					
First round	ACGGACAATTGCTTATAGCCTT ACAACTTTTATGGATTAGCTAAAT	NS16SCH1F NS16SCH1R	16S rRNA	1195	Kawahara et al. (2009)
Second round	GGGCACGTAGGTGGACTAG CCTGTTAGGAGGGATACGAC	NS16SCH2F NS16SCH2R	16S rRNA	443	Kawahara et al. (2009)
Piroplasmida					
First round	CGGGATCCAACCTGGTTGATCCTGC CCGAATTCCTTGTTACGACTTCTC	RIB-19 RIB-20	18S rRNA	1700	Zahler et al. (2000)
Second round	ACCTCACCAGGTCCAGACAG GTACAAAGGGCAGGGACGTA	BAB-rumF BAB-rumR	18S rRNA	430	Silveira et al. (2011)

Morulae were observed only within neutrophils in blood smears from the second clinical examination (Fig. 1), and no hemoparasites were observed in bone marrow smears.

Molecular assays

Molecular analyses of *msp2*|*p44* and *msp4* gene from *A. phagocytophilum* were positive in blood samples from both examinations and negative in the bone marrow sample. The blood samples from both examinations were positive for the *Ehrlichia* spp. 16S rRNA gene, but the bone marrow sample was negative for this gene. All of the samples were negative for the Piroplasmida 18S rRNA gene.

The nucleotide sequences amplified from parasites present in the whole blood were deposited in GenBank under the following accession numbers: *A. phagocytophilum*: KJ664225 (*msp4* gene); *E. canis* (16S rRNA gene) KJ664224. A Blastn sequence analysis revealed that the *msp4* gene from *A. phagocytophilum* was 99% identical to the sequences obtained from a human in Slovenia, a horse in Germany and other Brazilian dog isolates from this study group (GenBank accession numbers: JF893886.1; JF893888.1; and KF445232.1–KF445234.1, respectively). The *Ehrlichia* isolate from the present study was 100% identical to other *E. canis* isolates from dogs (GenBank accession numbers: KC479024.1; KC479023.1; KC479022.1; and EF195134.1).

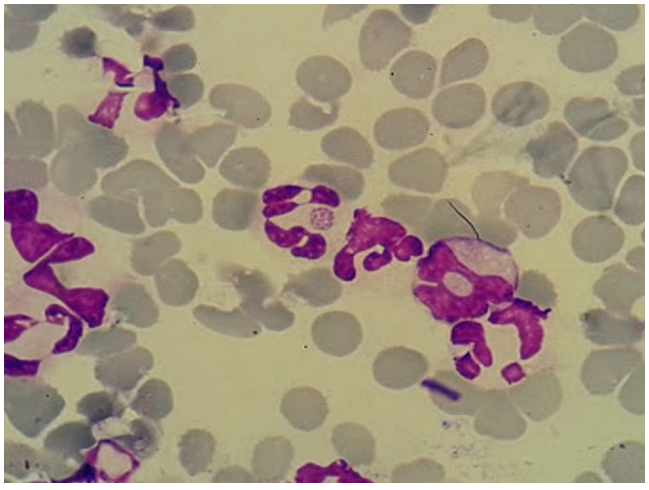


Fig. 1. Blood smears of a dog infected with *Anaplasma phagocytophilum*, showing morulae within parasitophorous vacuole in granulocytic cells. Romanowsky's stain, mag. 1000×.

Discussion

The clinical symptoms caused by *A. phagocytophilum* and *E. canis* infections are very similar. Thus, multiple diagnostic modalities are needed to confirm the diagnosis of granulocytic anaplasmosis, primarily in non-endemic areas such as Brazil. In the present study, at the first veterinary consultation, the dog showed unspeciffic clinical signs. PCR confirmed *E. canis* and *A. phagocytophilum* co-infection that likely exacerbated the clinical symptoms and affected the results of the laboratory analyses.

All blood examinations revealed abnormal parameters. Changes in hemogram, primarily thrombocytopenia, are frequently reported during granulocytic anaplasmosis and monocytic ehrlichiosis (Moreira et al., 2003). White cell analysis revealed that leukopenia occurred in April and December and monocytopenia was observed during the third examination. Cytopenia has been detected in infected humans and dogs, but the underlying mechanisms that are not fully understood. Opportunistic infections have also been documented during granulocytic anaplasmosis (Carrade et al., 2009). Mild to moderate hepatic injury can cause serum ALT to increase during *A. phagocytophilum* infection (Dumler et al., 2007).

Anaplasma phagocytophilum in tick is transmitted transstadialy. In Brazil, Santos et al. (2013) described *A. phagocytophilum* infection in *Amblyomma cajennense* and *R. sanguineus* from domesticated dogs, which is the hometown of the dog in this study. This tick is also the main transmission vector of *E. canis*. In this study, *R. sanguineus* infestation was present during the first clinical exam of the dog. In Belo Horizonte, *R. sanguineus* is the most common tick species in urban areas and produces three generations per year, but it has been found throughout the year (Silveira et al., 2009).

The dog exhibited skin alterations that were initially thought to be caused by *E. canis* and *D. canis*, but as Berzina et al. (2014) reported, immunological response to persistent *A. phagocytophilum* infection also can contribute to the skin lesions.

Immunosuppression could be result to prednisone administration. Prednisone was administered 61 days after the first examination due to the observed skin lesions. In dogs experimentally infected with *A. phagocytophilum*, the use of prednisolone could be associated with the development of positive PCR results, the reappearance of morulae on blood smears, thrombocytopenia and in some dogs, slight lymphocytosis (Egenvall et al., 2000). Although *D. canis* is considered a normal inhabitant of the skin, clinical signs can appear in immunosuppressed individuals (Ravera et al., 2013). During the second examination, the dog was positive for *D. canis* in skin scrapings.

The treatment of choice for both granulocytic anaplasmosis and monocytic ehrlichiosis in dogs is doxycycline, and reinfection has not been reported in dogs (Carrade et al., 2009). In this study, morulae were present in granulocytes 38 days after the end of doxycycline treatment and *A. phagocytophilum* and *E. canis* DNA persisted until eight months after treatment. Persistent *A. phagocytophilum* infection was demonstrated up to five and a half months after inoculation in experimentally infected dogs (Egenvall et al., 2000). Most veterinary clinicians in Brazil use clinical and/or laboratory findings to make a presumptive diagnosis of *E. canis* infection in dogs (Vieira et al., 2011).

Conclusion

The present study demonstrated the clinical, hematological, parasitological and molecular detection of *A. phagocytophilum* in Brazil for the first time. The strain of the present study was 99% identical to the sequences obtained from a human in Slovenia. In Brazil, this pathogen is increasingly detected in animals using molecular methods and determining the pathogenic and zoonotic potential of the isolates of *A. phagocytophilum* is essential. This finding highlights the increasing importance of this agent in South America.

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