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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.

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.