Important frequency of Anaplasma phagocytophilum infection in a population of domiciled dogs in an urbanized area in south-eastern Brazil

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ABSTRACT.- Silveira J.A.G., Reis I.A., Estevam L.G.T.M., Pinto M.C.C., Zweygarth E., Passos L.M.F. & Paz G.F. 2017. Important frequency of Anaplasma phagocytophilum infection in a population of domiciled dogs in an urbanized area in south-eastern Brazil. Pesquisa Veterinária Brasileira 37(9):958-962. Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG 31270-901, Brazil. E-mail: juliaags@yahoo.com.br

Anaplasma phagocytophilum is responsible for granulocytic anaplasmosis in humans and various animal species. The aim of the present study was to determine the prevalence of A. phagocytophilum-infected dogs in a residential area of Belo Horizonte, Minas Gerais state, Brazil. A total of 62 dogs were submitted to serological (indirect fluorescent-antibody - IFI) and molecular (PCR) tests. Anti-A. phagocytophilum antibodies were detected in 43.8% of the dogs. Seven dogs (10.9%) were PCR-positive for the msp4 gene, six and four of these were positive for the for the msp2/p44 gene of A. phagocytophilum and 16S rRNA region of granulocytic Anaplasmataceae respectively. This study confirms a relatively high frequency of A. phagocytophilum infection in a population of domiciled dogs in an urbanized area in south-eastern Brazil and highlights the need for further studies on the role of Rhipicephalus sanguineus sensu lato ticks in the transmission of this bacterium to dogs in urban Brazilian areas.

INDEX TERMS: Anaplasma phagocytophilum, dogs, canine anaplasmosis, epidemiology, IFAT, PCR.

RESUMO.- [Importante frequência da infecção por Anaplasma phagocytophilum em uma população de cães domiciliados em área urbanizada no sudeste do Brasil.] Anaplasma phagocytophilum é responsável pela anaplasmosse granulocítica, doença que acomete seres-humanos e várias espécies de animais. O objetivo do presente estudo foi determinar a prevalência de cães acometidos por A. phagocytophilum em uma área residencial de Belo Horizonte, MG, Brasil. Sessenta e dois cães foram submetidos a testes sorológicos (reação de imunofluorescência indireta - IFAT) e moleculares (PCR). Anticorpos anti-A. phagocytophilum foram detectados em 43.8% dos cães. Sete cães (10.9%) foram positivos no PCR para o gene msp4 de A. phagocytophilum e seis e quatro deles foram positivos para o gene msp2/p44 Anaplasma e região 16S rRNA de Anaplasmataceae respectivamente. Esse estudo confirma a frequência relativamente alta da infecção por A. phagocytophilum em uma população de cães domiciliados em área urbanizada no sudeste do Brasil e destaca a necessidade de pesquisas para determinar o papel do carrapato Rhipicephalus sanguineus sensu lato na transmissão desse microrganismo para cães de áreas urbanas brasileiras.

TERMOS DE INDEXAÇÃO: Anaplasma phagocytophilum, cães, anaplasmosse canina, epidemiologia, IFAT, PCR.
**INTRODUCTION**

*Anaplasma phagocytophilum* is an obligate intracellular gram-negative bacterium responsible for human granulocytic anaplasmosis (HGA) (*Dumler et al. 2001*). HGA are widespread in North America, Europe and Asia (*Ohashi et al. 2005, Teqgas & Foley 2006, Zhang et al. 2013*). Serology is used mainly for screening, but the specificity of the method is low and cross-reactions with other members of the family Anaplasmataceae (mainly *A. platts*) have been reported (*Carrade et al. 2009*).

Molecular methods are very specific, particularly when the tests include nucleotide sequencing (*Ebani et al. 2013*). The features of granulocytic anaplasmosis in dogs include malaise, lethargy, fever, anorexia, weakness, indisposition, nervous tension, lymphadenomegaly, hepatomegaly and splenomegaly (*Dumler et al. 2001*) and the occurrence of anaplasmosis in dogs has been geographically associated with HGA (Human Granulocytic Anaplasmosis) (*Madewell & Gribble 1982*).

In Brazil, the bacterium has been detected by molecular methods in dogs (*Santos et al. 2011, 2013, Silveira et al. 2015*), in carnivorous birds (*Machado et al. 2012*) and in brown brocket deer (*Mazama gouazoubira*) (*Silveira et al. 2014*); and in horses (*Salvagni et al. 2010*) and Brazilian marsh deer (*Blastocerus dichotomus*) (*Sacchi et al. 2012*) by serological methods. In Minas Gerais state observation of *A. phagocytophilum* has been increasing in animals (*Silveira et al. 2014, 2015*) and recently, the present study group detected a dog with *Ehrlichia canis* and *A. phagocytophilum* co-infection in the city of Belo Horizonte. Lethargy and skin lesions were the clinical signs observed and abnormal hematological parameters such as severe thrombocytopenia were the most important laboratory alterations (*Silveira et al. 2015*). This fact reinforcing the need for a study on a larger number of animals, especially dogs that live in close proximity with humans, as this agent is responsible for an important zoonosis in other countries. To answer this question, the present study aimed to determine the frequency of *A. phagocytophilum* infection in dogs using IFAT and PCR in an urbanized area in south-eastern Brazil.

**MATERIALS AND METHODS**

The study was approved by the Ethics Committee for Animal Research of the Fundação Oswaldo Cruz (Fiocruz) under protocol number LW-76/12. Written informed consent was obtained from dog owners prior to the commencement of the study. The research was conducted between August 2011 and May 2012 in a region to the northeast of Belo Horizonte (latitude: 19°55’15” S; longitude: 43°56’16” W), Minas Gerais, Brazil. Socioeconomic status of area was defined as lower middle class (*Buss & Pelegrini 2007*). That is endemic for canine vector-borne diseases (unpublished data supplied by Secretaria Municipal de Saúde, Belo Horizonte). Canine population comprised 62 domiciled dogs, corresponding to 80% of the canine population of the area, and distributed within 43 households, 27 of which had only one dog, 12 had two dogs and four had three dogs. During the inspection procedures, 50 samples of fleas and ticks were collected and specimens were identified according to Aragão & Fonseca (1961) and Linardi & Guimarães (2000). Blood samples were collected and serum samples were used for IFAT, while whole blood samples were employed for molecular analysis. The test was performed with an antigen prepared from embryonic tick cells (IDEB) infected with *A. phagocytophilum* that had been isolated from a dog in Germany. The antigen was produced following the methodology described previously (*Aguir et al. 2007*) and positive samples were further diluted until 1:640. Slides were examined under a fluorescence microscope (Olympus Corporation, Tokyo, Japan). DNA was extracted from whole blood using a Wizard Genomic DNA Purification Kit (*Promega, Madison, WI, USA*). PCR was performed using a set of primers for the *msp4* gene coding for an *A. phagocytophilum* surface protein. Samples from the *msp4*-positive dogs were submitted to further PCR analyses in which the target was the *msp2/p44* gene from *A. phagocytophilum* and 16S rRNA region of members of the Anaplasmataceae family that infects granulocytes and platelets and monococytes. All PCR assays were performed according to *Silveira et al. (2014), Zeidner et al. (2000) and Lin et al. (2003)* (Table 1). Purified positive samples were sequenced and analyzed at URL http://asparagin.cenargen.embrapa.br/pphp/ and using MEGA 6.0 software (*Tamura et al. 2013*). Identity of each sequence was confirmed by comparison with sequences available in GenBank using BLAST software. Phylogenetic tree was constructed using the nucleotide sequences of the *msp4* gene obtained in this study and selected

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Table 1. Primers used in polymerase chain reactions for the detection of *Anaplasma phagocytophilum* DNA in blood samples from dogs

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primers (5' - 3')</th>
<th>Target</th>
<th>Name</th>
<th>Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>ATGAATTTACAGAGAATTGCTTAGGG</td>
<td>msp4</td>
<td>MS4AP5</td>
<td>849</td>
<td>de la Fuente et al. 2005</td>
</tr>
<tr>
<td>First round</td>
<td>TAGTTGAAGCAGCTTGTAGG</td>
<td>msp4</td>
<td>MS4AP3</td>
<td>849</td>
<td>de la Fuente et al. 2005</td>
</tr>
<tr>
<td>Second round</td>
<td>ATGTTGGYGGNGCYAGACT</td>
<td>msp4f</td>
<td>381</td>
<td>Bowman et al. 2007</td>
<td></td>
</tr>
<tr>
<td>Granulocyte/platelet <em>Anaplasma/Ehrlichia</em></td>
<td>GACGGTTTAGAAGAATAGAAG</td>
<td>msp2/p44</td>
<td>p44F</td>
<td>1082</td>
<td>Lin et al. 2003</td>
</tr>
<tr>
<td>First round</td>
<td>CACGTTTAGAAGAATAGAAG</td>
<td>msp2/p44</td>
<td>p44R</td>
<td>1082</td>
<td>Lin et al. 2003</td>
</tr>
<tr>
<td>Second round</td>
<td>GCGAGTAAATCATATAGCC</td>
<td>msp2-3F</td>
<td>334</td>
<td>Zeidner et al. 2000</td>
<td></td>
</tr>
<tr>
<td>Monocyte <em>Ehrlichia</em> spp. Lineage</td>
<td>ACGGAGATTTAGTATAGCCTTA</td>
<td>16S rRNA</td>
<td>932</td>
<td>Massung et al. 1998</td>
<td></td>
</tr>
<tr>
<td>First round</td>
<td>TACCATCGGAAGGAGATATTTC</td>
<td>GE3a</td>
<td>16S rRNA</td>
<td>932</td>
<td>Massung et al. 1998</td>
</tr>
<tr>
<td>Second round</td>
<td>TCTGGTGAAGAGATATTTC</td>
<td>GE10r</td>
<td>932</td>
<td>Massung et al. 1998</td>
<td></td>
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<td></td>
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<td>932</td>
<td>Massung et al. 1998</td>
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<td></td>
<td>932</td>
<td>Massung et al. 1998</td>
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</tr>
</tbody>
</table>

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played 98 to 100% similarity with the GenBank sequences with accession numbers KF445223 - KF445234, KF445232, KF445230, KF445229, KF445226, KF445227. Sequences obtained in this study and sequences of *A. phagocytophilum* were deposited in GenBank under the accession numbers KF790911 and KF790913. Sequences of four samples exhibited 97 to 99% similarity with sequences from isolates derived from dogs in Tunisia and the USA (EU781707.1; AY741095.1), and that of one sample presented 99% similarity with isolates derived from a human patient suffering from granulocytic anaplasmosis in the USA (AF093789.1; AF093788.1).

**DISCUSSION**

Infection with *A. phagocytophilum* is a matter of public health, although there is no evidence of human infection in Brazil, the increased occurrence of the agent in domestic animals has been demonstrated. Present investigation showed that the frequency of seropositive dogs was 42.8%, a value that is similar to seroprevalences of 55 and 50% reported for dogs in North America and Europe, respectively (Beall et al. 2008, Barutzki et al. 2006). These findings indicate that the animals are frequently exposed to infection and that study area may be endemic for *A. phagocytophilum*. However, even though the prevalence of dogs presenting anti-*A. phagocytophilum* antibodies was high, none of the animals exhibited clinical signs of anaplasmosis. It is possible that cross-reaction between species of Anaplasmataceae, rather than exposure to *A. phagocytophilum*, was responsible for the positive serology (Carrade et al. 2009). Moreover, although canine granulocytic anaplasmosis is a self-limiting infection, the antibodies can be detected by IFA for various months (Egenvall et al. 1997). Therefore, it is possible that a seropositive IFAT may not necessarily reflect an actual infection by *A. phagocytophilum*. IgG antibodies can be detected approximately eight days after exposure to the infecting agent, and diagnosis via PCR during this interval is important since the visualization of bacterial morulae in blood smears is not always possible. High antibody titers may persist for up to 12 months after the resolution of clinical signs (Poitout et al. 2005), a 4-fold increase in IgG titer is required to indicate a recent infection. Of the seven PCR positive samples, only two were seropositive according to IFA test at a titration of 1:40, suggesting that these animals were recently infected and that their antibody levels were, as yet, insufficient for seroconversion. This may explain the observation in some of the study dogs of seroreactivity at the 1:640 titer but with lack of clinical signs. Clearly, in areas where occurrences of *A. phagocytophilum* infection are rare, as is the case in Brazil, diagnosis of granulocytic ana-
plasmosis requires the use of multiple techniques (Carrade et al. 2009). It has been reported that A. phagocytophilum isolates vary with respect to pathogenicity and that some isolates display zoonotic potential (Overzier et al. 2013). Moreover, in the present study, nucleotide sequence of one of the dogs presented 99% similarity with isolates derived from a human patient in USA. Since A. phagocytophilum is widely distributed in the studied area, as indicated by high frequency of residences (62.8%) housing infected dogs, there is a distinct possibility that the agent could be transmitted to pet owners. The only ticks found on the study dogs were R. sanguineus sensu lato and A. phagocytophilum infection was described in these ticks from domesticated dogs in Rio de Janeiro, Brazil (Santos et al. 2013). In the same area of the study, dogs were positive to serological assays for Leishmania (ELISA - 4.2%, IFAT - 12.5%, rK39 RDT - 14.6%, DPP- 20.8%), Ehrlichia (IFAT - 23.9%) and Babesia (IFAT - 31.2%). No significant association was identified between the results of tests for detecting Babesia or Ehrlichia and those for detecting Leishmania (p-value>0.05), and those for detecting Anaplasma and Neorickettsia, because dogs may act as sentinels for human exposure, recent detection of A. phagocytophilum and possibility of transmission to humans. Moreover, in the present study, nucleotide sequence of one isolate display zoonotic potential (Overzier et al. 2013).

**CONCLUSION**

This study confirms a relatively high frequency of Anaplasm phagocytophilum infection in a population of domiciled dogs in an urbanized area in south-eastern Brazil and highlights the need for further studies on the role of Rhipicephalus sanguineus sensu lato ticks in transmission of this bacterium to dogs in urban areas. Considering the importance of this zoonotic agent, and because dogs may act as sentinels for human exposure, recent detection of A. phagocytophilum themselves, the likely vectors of the pathogen and possibility of transmission to humans.

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**Conflict of interest statement.** The authors declare that they have no competing interests.

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