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 anaplasmo granulócita, doença que acomete seres-humanos e várias espécies de animais. O objetivo do presente estudo foi determinar a prevalência de *A. phagocytophilum*-infetidos cães em uma residencial de Belo Horizonte, Minas Gerais, Brasil. A total de 62 cães foram submetidos a testes sorológicos (reação de imunofluorescência indireta - IFI) e moleculares (PCR). Anti-*A. phagocytophilum* antibios foram detectados em 43.8% dos cães. Sete cães (10.9%) foram PCR-positive para o gene *msp4* de *A. phagocytophilum* e sete para o gene *msp2/p44* de *A. phagocytophilum* e quatro para a região 16S rRNA de Anaplasmataceae respectivamente. Esse estudo confirma uma frequência relativamente alta de 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, anaplasma 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, Teglas & 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. platys*) 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 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 laboratorial alterations (*Silveira et al. 2015*). This fact reinforcing the need for a study on the *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 & Pelegriini 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 Araújo & 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 (*Aguirar 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 monocytes. 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/phph/ 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

<table>
<thead>
<tr>
<th>Specitivity</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>AFGAATTAACAGAGATTTGCTTAGG</td>
<td><em>msp4</em></td>
<td>MSP4APS</td>
<td>849</td>
<td>de la Fuente et al. 2005</td>
</tr>
<tr>
<td>First round</td>
<td>TTATGGAAACAACTTGCTGTAG</td>
<td></td>
<td>MSP4AP3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTATGGCCGAGGGCCGAACT</td>
<td></td>
<td>msfpf</td>
<td>381</td>
<td>Bown et al. 2007</td>
</tr>
<tr>
<td>Second round</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTCATCGGGAAATCTGCTTGA</td>
<td></td>
<td>msfp4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>ATGGACTTTTGAGCTGTTCTT</td>
<td>p44F</td>
<td>1082</td>
<td>Lin et al. 2003</td>
<td></td>
</tr>
<tr>
<td>First round</td>
<td>CAATAGTGGTCTGATGAAA</td>
<td></td>
<td>p44R</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCCGAGTGAACTCATCGAAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>GCAGGGTITAGCAAGATAAGAG</td>
<td>p44R</td>
<td>334</td>
<td>Zeidner et al. 2000</td>
<td></td>
</tr>
<tr>
<td>Second round</td>
<td>GCCCAATACCATATAAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Granulocyte/platelet Anaplasma/Ehrlichia</em></td>
<td>CACATGCAGATTGAAGGATATTTC</td>
<td>GE3a</td>
<td>165 rRNA</td>
<td>932</td>
<td>Massung et al. 1999</td>
</tr>
<tr>
<td>First round</td>
<td>TTTCTAGTTAAGGATATTTC</td>
<td></td>
<td>GE10r</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AACGATTATTTCATTACCAGTGCT</td>
<td>GE9f</td>
<td>546</td>
<td>Massung et al. 1998</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGCAGATTAACGACCTTCGAG</td>
<td>GE2</td>
<td>165 rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Monocyte Ehrlichia spp. Lineage</em></td>
<td>ACGGACATTTGCTTAACTTAG</td>
<td>NS16SCH1F</td>
<td>165 rRNA</td>
<td>1195</td>
<td>Kawahara et al. 2009</td>
</tr>
<tr>
<td>First round</td>
<td>ACAATTTTATTGATTAGCTTAAAT</td>
<td>(NS16SCH1R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGGCGATGGCTTGGACTAG</td>
<td>NS16SCH2F</td>
<td>165 rRNA</td>
<td>443</td>
<td>Kawahara et al. 2009</td>
</tr>
<tr>
<td>Second round</td>
<td>CCGTCTGAGGACGATGGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primers used in polymerase chain reactions for the detection of *Anaplasma phagocytophilum* DNA in blood samples from dogs.
GenBank. The msp4 gene sequence of *A. marginale* was employed as the outgroup. Nucleotide sequences were aligned with MUSCLE from MEGA 6.0 package (Tamura et al., 2013). Each alignment was analyzed using the Neighbor-joining method and distance matrices were calculated using the Kimura two-parameter method. Selected percentage bootstrap values (1000 repeats) are presented at the nodes (Fig. 1). Hypothesis that canine *A. phagocytophilum* seroreactivity was associated with biological and management variables were investigated using the Pearson $\chi^2$ and Fisher tests.

### Results

Studied dog population comprised of 27 (43.5%) males and 35 (56.5%) females and the average age was 5.8 ± 1.1 years (range three months to 17 years). The breeds included in the population were mongrels (53.1%), poodles (24.0%), pinchers (8.3%), Yorkshire terriers (3.1%), boxers, cocker spaniels, labradors and German shepherds (8.4%) and others (3.1%). Clinical examination revealed that all dogs were apparently healthy and did not show visible signs of disease. Ticks and fleas collected during examination of the animals were identified as *R. sanguineus sensu lato* (present in 54.4% of dogs) and *C. felis felis* (present in 65.6% of dogs). Anti-*A. phagocytophilum* antibodies were detected at a titration of 1:40 in 43.8% (27/62) of the animals comprising of 15 males and 12 females. Of the infected dogs, 74.0% (20/27) produced positive reactions at a titration of 1:640. Twenty-seven of the animals comprising of 15 males and 12 females were phylogenetically most closely correlated with those obtained from *A. phagocytophilum* isolated from dogs, sheep and *Ixodes ricinus* from European sources (Fig. 1). According to the PCR assays, six of the animals also were positive for *msp2/p44* gene from *A. phagocytophilum*. Sequences displayed 90% to 99% identity to that of the *msp2* sequences from isolates derived from bear and white-footed mouse in USA (DQ519567.1; AF202317.1). Four dogs gave positive results in nPCR analyses for 16S rRNA region of members of Anaplasmataceae that infect granulocytes and platelets. Nucleotide sequences obtained in this study 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; AF741095.1), 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 infection is a matter of public health. Therefore, it is possible that the 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-
Anaplasma phagocytophilum infection in a population of domiciled dogs in an urbanized area

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 asays 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), showing co-infection with Ehrlichia or Babesia and Leishmania in dogs from Minas Gerais (Krawczak et al. 2015).

Currently, our research group is conducting an epidemiological investigation in the study area with the aim of (i) determining the pathogenic and zoonotic potential of the isolates of A. phagocytophilum, and (ii) elucidating the biological or mechanical mechanism of transmission of A. phagocytophilum among the canine population.

CONCLUSION

This study confirms a relatively high frequency of Anaplasma 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.

Acknowledgments.- The authors wish to thank Dr Ulrike G. Munderloh (University of Minnesota, USA) for permission to use the IDEB cell line, Dr. Elida M. L. Rabelo and Dr. Múcio F.B. Ribeiro for assistance with the laboratory work. The study was funded by the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG - Programa PPP, Grant no. CVZ - APQ-04528-10), Programa de Excelência em Pesquisa (PROEP/CRP6R/PRB, Grant no. 401975/2012-6) and the Programa de Apoio à Pesquisa da Fundação Oswaldo Cruz (PAPES VI, Grant no. 407529/2012-8).

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

REFERENCES


