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Important frequency of *Anaplasma phagocytophilum* infection in a population of domiciled dogs in an urbanized area in south-eastern Brazil[1]

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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 anaplasmose 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. phagocytophlium* 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*, seis para o gene *msp2/p44 A. phagocytophilum* e quatro para a região 16S rRNA de Anaplasmataceae granulocíticas. 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, anaplasmose canina, epidemiologia, IFAT, PCR.

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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 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 laboratorial alterations (Silveira et al. 2015). This fact reinforcing the need for a study on a larger number of animals, especially dogs that live in closely 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 (IDE8) infected with *A. phagocytophilum* that had been isolated from a dog in Germany. The antigen was produced following the methodology described previously (Aguiar 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 1. Primers used in polymerase chain reactions for the detection of *Anaplasma phagocytophilum* DNA in blood samples from dogs

Specificity	Primers (5'- 3')	Target	Name	Size (bp)	References
Anaplasma phagocytophilum					
First round	ATGAATTACAGAGAATTGCTTGTAGG	*msp4*	MSP4AP5	849	de la Fuente et al. 2005
	TTAATTGAAAGCAAATCTTGCTCCTATG		MSP4AP3		
Second round	CTATTGGYGGNGCYAGAGT	*msp4*	msp4f	381	Bown et al. 2007
	GTTCATCGAAAATTCCGTGGTA		msp4r		
Anaplasma phagocytophilum	ATTGGACTTTTGAGCTGTCTT	*msp2/p44*	p44F	1082	Lin et al. 2003
	CAATAGTYTTAGCTAGTAACC		p44R		
Anaplasma phagocytophilum	CCAGCGTTTAGCAAGATAAGAG	*msp2/p44*	msp2-3F	334	Zeidner et al. 2000
	GCCCAGTAACATCATAAGC		msp2-3R		
Granulocyte/platelet *Anaplasma/Ehrlichia*					
First round	CACATGCAAGTCGAACGGATTATTC	GE3a	16S rRNA	932	Massung et al. 1998
	TTCCGTTAAGAAGGATCTAATCTCC	GE10r			
Second round	AACGGATTATTCTTTATAGCTTGCT	GE9f		546	Massung et al. 1998
	GGCAGTATTAAAAGCAGCTCCAGG	GE2	16S rRNA		
Monocyte *Ehrlichia* spp. Lineage					
First round	ACGGACAATTGCTTATAGCCTT	NS16SCH1F	16S rRNA	1195	Kawahara et al. 2009
	ACAACTTTTATGGATTAGCTAAAT	NS16SCH1R			
Second round	GGGCACGTAGGTGGACTAG	NS16SCH2F	16S rRNA	443	Kawahara et al. 2009
	CCTGTTAGGAGGGATACGAC	NS16SCH2R			

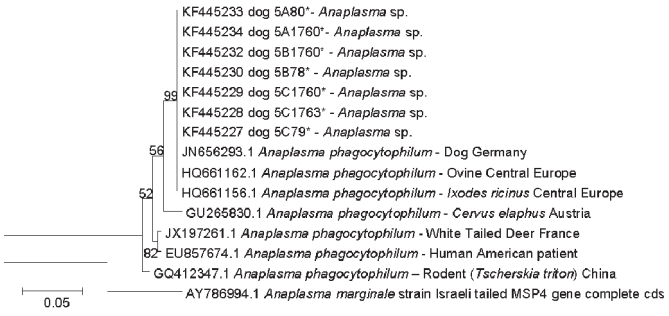

Fig.1. Phylogenetic clustering of the partial msp4 gene of *Anaplasma phagocytophilum*: The tree was obtained using the Neighbor-joining method and the software package MEGA 6.0 after alignment of consensus sequences of the msp4 genes obtained in this study and sequences of *A. phagocytophilum* from various sources available in GenBank (accession numbers provided). Distance matrices were calculated using the Kimura two-parameter method. Selected percentage bootstrap values (1000 repeats) are presented at the nodes. *Anaplasma marginale* was used as outgroup. * Samples from this study.

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 in MEGA 6.0 software. 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 χ^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%), pinschers (8.3%), Yorkshire terriers (3.1%), boxers, cocker spaniels, labradors and German sheppards (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 43 households studied (62.8%) possessed at least one *A. phagocytophilum*-seropositive dog. Frequency of seropositive animals in households with only one dog was 55.5%, where the frequency in households with multiple dogs was 75%. Analyses by nPCR with *msp4* primers revealed that seven dogs (10.9%) were positive for the presence of *A. phagocytophilum* DNA. Nucleotide sequences determined in these seven samples were deposited in GenBank with accession numbers KF445227 - KF445234, and displayed 98 to 100% similarity with the GenBank sequences HQ661162.1, HQ661156.1 and CP006618.1 as shown by BLASTN analysis. Sequences obtained in the present study 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; 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), 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 *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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