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Molecular detection of *Anaplasma* species in dogs in Colombia

Detecção molecular de espécies de *Anaplasma* em cães na Colômbia

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Abstract

Anaplasma platys and *A. phagocytophilum* are tick-borne pathogens that parasitize platelets and neutrophils, respectively, of humans and animals. The former is the etiological agent of canine cyclic thrombocytopenia, while the latter is that of canine granulocytic anaplasmosis. This work involved the detection and identification of *Anaplasma* species in blood samples from dogs in Colombia, using molecular techniques. Between December 2008 and April 2009, blood samples were drawn from the cephalic vein of 91 dogs in the central-western region of Colombia (cities of Bogota, Villavicencio and Bucaramanga) and stored in tubes containing EDTA. These samples were used in 16S rRNA-*Anaplasma* spp. nPCR and the preparation of blood smears. One (1.1%) of the 91 sampled dogs showed inclusions suggestive of Anaplasmataceae agents in the cytoplasm of platelets. Based on PCR followed by sequencing and phylogenetic analysis, *A. platys* and *Anaplasma* sp. closed related to *A. phagocytophilum* were detected in two and one dog, respectively. Interestingly, all the samples were negative for specific *msp-2-A. phagocytophilum* real-time qPCR, suggesting the circulation of an *Anaplasma* species phylogenetically related to *A. phagocytophilum* in dogs in the aforementioned region. Hence, *Anaplasma* spp. circulates among dogs in Colombia, albeit with low frequency. To the best of authors' knowledge, this is the first molecular detection of *Anaplasma* spp. in dogs in Colombia.

Keywords: Dogs, *Anaplasma* spp., *Anaplasma platys*, Colombia.

Resumo

Anaplasma platys e *A. phagocytophilum* são patógenos transmitidos por carrapatos que parasitam plaquetas e neutrófilos, respectivamente, de humanos e animais. Enquanto o primeiro é agente etiológico da trombocitopenia cíclica canina, o último é o agente responsável pela anaplasmose granulocítica canina. O presente trabalho objetivou detectar e caracterizar, utiizando técnicas moleculares, a presença de espécies de Anaplasma em amostras de sague de cães na Colômbia. Entre Dezembro de 2008 e Abril de 2009, amostras de sangue colhidas em tubos contendo EDTA da veia cefálica de 91 cães da região centro-oeste da Colômbia (cidades de Bogotá, Villavicencio e Bucaramanga). As amostras de sangue foram utilizadas em reações de nPCR para 16S rRNA-*Anaplasma* spp. e confecção de esfregaços sanguíneos. Um (1,1%) dos 91 cães amostrados mostraram inclusões sugestivas de agentes Anaplasmataceae no citoplasma de plaquetas. Baseada na PCR seguida de sequenciamento e análise filogenética, *A. platys* and *Anaplasma* sp. relacionado a *A. phagocytophilum* foram detectados em dois e um cão, respectivamente. Interessantemente, todas as amostras mostraram-se negativas na PCR em tempo real quantitativa específica para *msp-2-A. phagocytophilum*, sugerindo a circulação de uma espécie de *Anaplasma* sp. filogeneticamente relacionado ao *A. phagocytophilum* em cães na região estudada. Embora em uma baixa freqüência, *Anaplasma* spp. circula entre cães na Colômbia. O presente trabalho representa a primeira detecção de *Anaplasma* spp. em cães na Colômbia.

Palavras-chave: Cães, *Anaplasma* spp., *Anaplasma platys*, Colômbia.

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Introduction

Anaplasma species (Rickettsiales: Anaplasmataceae) are gram-negative obligate intracellular bacteria that can parasitize domestic and wild animals as well as humans (DUMLER et al., 2001; ISMAIL et al., 2010).

Anaplasma phagocytophilum, the etiological agent of human granulocytic anaplasmosis (HGA), equine granulocytic anaplasmosis (EGA), and tick-borne fever in ruminants, parasitizes neutrophils and eosinophils, whose biological cycle is preserved in the environment by ticks of the *Ixodes persulcatus* species complex and vertebrate reservoirs (DUMLER et al., 2001). In the USA, the *Ixodes scapularis* tick is responsible for the transmission of *A. phagocytophilum* in the northeast and mid-west, while the *I. pacificus* tick species is involved in the transmission of the agent on the country's Pacific coast (BARLOUGH et al., 1997; LEVIN & FISH, 2001). In humans, HGA is characterized by fever, headache, myalgia, chills, and malaise (WOLDEHIWET, 2010). Dogs may play a role as reservoirs for *A. phagocytophilum* (OTEO & BROUQUI, 2005). The clinical signs found in *A. phagocytophilum*-infected dogs vary from a subclinical infection to an acute febrile condition accompanied by anorexia and lethargy. Central nervous system dysfunction and lameness have also been recorded in canine anaplasmosis by *A. phagocytophilum* (LESTER et al., 2005).

Anaplasma platys is a bacterial species that infects predominantly canine blood platelets, causing canine cyclic thrombocytopenia, which is characterized by depression, fever, hemorrhage, and thrombocytopenia. The vector suspected of transmitting the pathogen is the brown dog tick, *Rhipicephalus sanguineus* (BEAUFILS et al., 2002).

Several reports have been published about the occurrence of these agents in South America (DAGNONE et al., 2009; MACHADO et al., 2012; SACCHI et al., 2012; SALVAGNI et al., 2010; SANTOS et al., 2009, 2011, 2013; SILVEIRA et al., 2014; ANDRÉ et al., 2012, 2014). In Colombia, although a 20% seroprevalence to *A. phagocytophilum* has been found among rural workers in the country's northern region, the agent has not yet been characterized molecularly in mammals (MÁTTAR & PARRA, 2006). This study involved the detection and identification of *Anaplasma* species in blood samples from dogs in Colombia, using molecular techniques.

Material and Methods

Between December 2008 and April 2009, blood samples were drawn from the cephalic vein of 91 dogs in the central-western region of Colombia and stored in tubes containing EDTA. The dogs were distributed as follows: 21 dogs from the city of Bogotá (used for the detection of explosives in several regions of the country), 31 dogs from the city of Villavicencio (21 from an animal shelter and 10 from the local Zoonosis Control Center), and 39 dogs from the city of Bucaramanga (19 dogs from a local veterinary clinic and 20 from animal shelters). The selection criteria was based on a history of tick infestation. Blood smears were fixed with methanol and stained with Giemsa (Giemsa stain, modified, Sigma-Aldrich, St. Louis, MO, USA).

DNA was extracted from aliquots of 200 µL of whole blood, using a commercial kit (QIAamp DNA Blood Mini kit, QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Each sample of extracted DNA (5 µL) was used as a template in a 25 µL nested PCR reaction based on the sequence of the 16S rRNA (MASSUNG et al., 1998) and *groESL* genes (NICHOLSON et al., 1999). The mixture contained 10X PCR buffer (Life Technologies, Carlsbad, CA, USA), 1.0 mM $MgCl_2$ (Life Technologies, Carlsbad, CA, USA), 0.2 mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies, Carlsbad, CA, USA), 1.5 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA), and 0.5 µM of each primer (Integrated DNA Technologies, Coralville, IA, USA). The sequence of primers based on 16 rRNA and *groESL* gene was respectively, gE3a (5'-CACATGCAAGTCGAACGGATTATTC-3'), gE10R (5'- TTCCGTTAAGAAGGATCTAATCTCC-3') and HS1a (5'-AITGGGCTGGTAITGAAAT-3'), EHR-CS778R (5'- CCICCIGGIACIAIACCTTC-3') in the first reaction, and gE2 (5'- GGCAGTATTAAAAGCAGCTCCAGG-3'), gE9f (5'-AACGGATTATTCTTTATAGCTTGCT-3') and HS43 (5'-AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC-3'), HSVR (5'-CTCAACAGCAGCTCTAGTAGC-3') in the second reaction. One micro liter of DNA amplified in the first reaction was used as DNA template in the second reaction. *Anaplasma platys* DNA positive control was obtained from a naturally infected dog in Brazil (DAGNONE et al., 2009). *Anaplasma phagocytophilum* DNA positive control was kindly supplied by Dr. John Stephen Dumler (University of Maryland, Baltimore, MD, USA). Ultra-pure sterile water (Promega, Wis., USA) was used as negative control. PCR amplifications in the first and second reactions based on 16SrRNA were performed at 94 °C for 5 min, followed by 40 repetitive cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, and ending with a final extension at 72 °C for 5 min in a thermal cycler (T100 Thermal Cycler, Bio-Rad, Hercules, CA, USA). For the PCR amplifications based on *groESL* gene three primary cycles at 94 °C for 1 min, 48 °C for 2 min and 72 °C for 90 sec were followed by 37 repetitive cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 90 sec and ending with a final extension at 72 °C for 5 min in a thermal cycler (T100 Thermal Cycler, Bio-Rad, Hercules, CA, USA). In the second reaction the temperature of annealing was increased to 55 °C.

To avoid PCR contamination, the DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms. The reaction products were purified using a commercial kit (Silica Bead DNA Gel Extraction Kit, Thermo Scientific, Waltham, Mass., USA).

Purified amplified DNA fragments were subjected in-house to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyzer – Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and were used for subsequent phylogenetic analysis. Phylogenetic reconstructions were based on DNA sequence alignment of positive samples. Consensus sequences were obtained through the analysis of the sense and antisense sequences, using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Comparisons were made with sequences deposited in GenBank, using the basic local alignment search tool (BLAST) (ALTSCHUL et al., 1990). The sequences were aligned with sequences published in GenBank using

Clustal/W (THOMPSON et al., 1994) in Bioedit v. 7.0.5.3 (HALL, 1999). Phylogenetic inference was based on maximum likelihood (ML) inference. The ML phylogenies were inferred with RAxMLHPC BlackBox 7.6.3 software (STAMATAKIS et al., 2008) (which includes an estimation of bootstrap node support) through the CIPRES Science Gateway, using a GTR + GAMMA model of evolution and 1000 bootstrapping replicates. The best model of evolution was selected by the program jModelTest2 on XSEDE (DARRIBA et al., 2012) under the Akaike Information Criterion (AIC) (POSADA & BUCKLEY, 2004), through the CIPRES Science Gateway. The trees were visualized in Treegraph 2.0.56-381 beta (STOVER & MULLER, 2010).

Each sample of extracted DNA was also used as a template in 10 µL real-time qPCR assay, according to Drazenovich et al. (2006). The mixtures contained 5 µL of buffer (GoTaq® qPCR Master Mix, Promega, Wis., USA,) a final concentration of 1 µM of each primer ([903f: 5' AGTTTGACTGGAACACACCTGATC-3'] and [1024r: 5' CTCGTAACCAATCTCAAGCTCAAC-3']) and TaqMan-probe (939p FAM -TTAAGGACAACATGCTTGTAGCTATGGAAGGCA-TAMRA) Integrated DNA Technologies, Coralville, IA, USA) and 1µL of DNA sample. qPCR amplifications were performed in a thermal cycler (CFX96 Thermal Cycler, Bio-Rad, Hercules, CA, USA). The amplification conditions were 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were constructed using 10-fold serial dilutions of gBlock gene fragments (Integrated DNA Technologies, Coralville, IA, USA) encoding *msp2-A. phagocytophilum* fragment. The number of copies was determined according to the formula (X g/µL DNA/ [fragment length in bp x 660]) × 6.022 × 10^{23} × copies/µL.

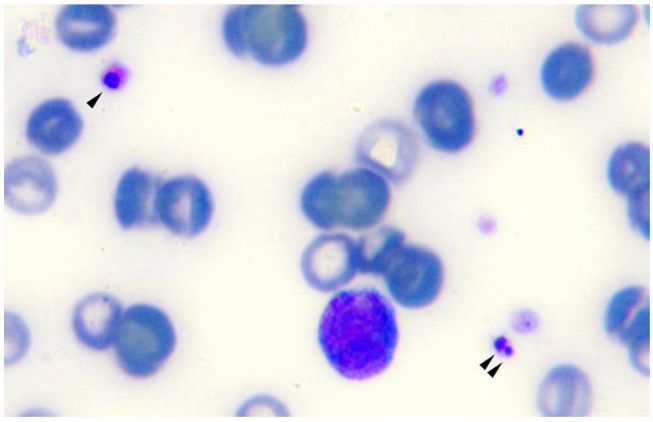

Figure 1. Giemsa-stained blood smear from an *Anaplasma platys*-PCR positive dog, showing inclusions in platelets (arrows). Light microscopy under 1000X magnification.

Results

One (1.1%) of the 91 sampled dogs showed inclusions suggestive of Anaplasmataceae agents in the cytoplasm of platelets (Figure 1). This animal came from an animal shelter in the city of Bucaramanga. Among the 91 dogs, three (3.3%) sampled in Bucaramanga city were positive for *Anaplasma* spp. in the PCR assay based on 16S rRNA gene; two of them showed clinical signs suggestive of hemoparasitosis. All the samples tested were negative at *groESL* nPCR. The BLAST analysis, *A. platys* (99% of identity with *A. platys* detected in dogs in Thailand [EF139459]) and *Anaplasma* sp. (99% of identity with *A. phagocytophilum* isolated in the USA [CP006616]) were identified as the species found in two dogs (one of them showing inclusions in platelets as well as clinical signs [Figure 1]) and one sampled dog (also showing clinical signs), respectively (Figure 2). The phylogenetic tree based on 16S rRNA gene showed that one sequence was located in the same clade than an *A. platys* sequence detected in a domestic cat from Brazil (KC989957) with bootstrap valuie of 83 (Figure 2); the other sequence obtained was positioned in the same clade than an *A. platys* sequence detected in a dog in Rio de Janeiro, Brazil (FJ755157) and *A. platys* strain Gigio

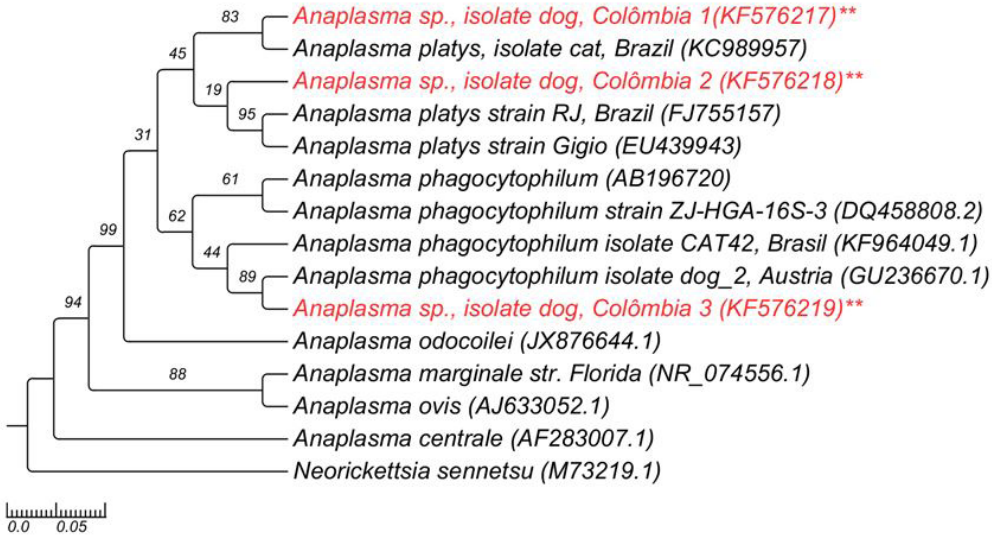

Figure 2. Phylogenetic tree of *Anaplasma* spp. (16SrRNA) based on maximum likelihood, using GTR+G+I as model of evolution. Numbers correspond to bootstrap values over 50. ** Sequences from the present study.

(EU439943), Italy, but without significant clade support (≥50) (Figure 2). The other sequence obtained was grouped with a *A. phagocytophilum* sequence detected in a dog from Austria, with bootstrap value of 89 (Figure 2). The size of the alignment used to perform the phylogenetic analysis was approximately 600pb. *Anaplasma platys* and *Anaplasma* sp. closed related to *A. phagocytophilum* 16S rRNA sequences found in dogs from Colombia were deposited in GenBank under accession numbers KF576217, KF576218, and KF576219, respectively. All the samples tested were negative by the specific *msp-2-A. phagocytophilum* qPCR. The efficiency of qPCR assay was E = 90.0% (slope = −3.588; r2 = 0.995). The qPCR assay followed the Minimum Information for publication of Quantitative real-time PCR Experiments (BUSTIN et al., 2009).

Discussion

This work reports on the first molecular evidence of infection by *Anaplasma* spp. in dogs from Colombia. *Anaplasma platys* was molecularly detected in two (2.2%) out of 91 sampled dogs. One of these dogs (Dog#56) showed clinical symptoms and hematological abnormalities suggestive of tick-borne disease (depression, dehydration, anorexia, pale mucous membranes, vomiting, leucopenia [6500 × 10^3/µL - ref 6700-17000] and severe thrombocytopenia [52000 × 10^3/µL - ref. 150.000-500.000] (data not shown), and had been previously diagnosed with *E. canis* by molecular techniques VARGAS-HERNANDEZ et al., 2012a). The other dog positive for *A. platys* (Dog#72), showing inclusions in platelets was co-infected with *E. canis* and *Hepatozoon canis*, based on previous studies (VARGAS-HERNANDEZ et al., 2012a, b), but showed no clinical symptoms compatible with hemoparasitosis (data not shown). It is worth to mention that, among the dogs with clinical symptoms of anaplasmosis, one of the samples was found to be closely related to *A. phagocytophilum,* based on the phylogenetic analysis. Such dog exhibited hematological findings suggestive of canine anaplasmosis (dehydration, fever, anorexia, hemoconcentration [63%] thrombocytopenia [57000 × 10^3/µL - ref. 150.000-500.000]).

Diagnostics based solely on the presence of inclusions in platelets by blood smear exams shows poor sensitivity because of the low and transient bacteremia presented by this agent (BEAUFILS et al., 2002). The presence of positive PCR and blood smear results associated with clinical symptoms suggests the occurrence of the acute phase of the disease at the time of blood sampling. This underscores the importance of molecular techniques in the differential diagnosis of tick-borne diseases. The presence of positive PCR and blood smear results associated with clinical symptoms suggests the occurrence of the acute phase of the disease at the time of blood sampling.

The molecular prevalence (3.3%) of *Anaplasma* spp. among the dogs sampled in this study was lower than that found for *E. canis* (40.6%) and *Babesia vogeli* (5.5%) in our previous study (VARGAS-HERNÁNDEZ et al., 2012a). The criteria for selection of dogs can influence the low detection of the agent, dogs with clinical signs should be more likely to present positive results in *Anaplasma* nPCRs (DAGNONE et al., 2009). Similar results have been found among dogs without clinical signs from central-western Brazil (SOUSA et al., 2013).

In South America, *A. platys* and has been molecular detected in dogs in Brazil (DAGNONE et al., 2009; SANTOS et al., 2009; SOUSA et al., 2013), Argentina (EIRAS et al., 2013; OSCHEROV et al., 2011), Chile (ABARCA et al., 2007), and Venezuela (HUANG et al., 2005). *Anaplasma* spp. phylogenetically similar to *A. platys* has been detected in marsh deer in Brazil (SACCHI et al., 2012).

One of detected sequences was found to be closely related to *A. phagocytophilum,* based on 16S rRNA phylogenetic analysis. Miranda & Mattar (2015), using a nested PCR and phylogenetic approach based on 16SrRNA gene, also found similar results when analyzed sequences obtained from *Dermacentor nitens* collected from horses in Colombia. However, all the samples from the present study showed negative results at *A. phagocytophilum msp-2* qPCR, which is proven to be a more sensitive and specific protocol for *A. phagocytophilum* detection than nPCR based on 16SrRNA (DRAZENOVICH et al., 2006). These results suggest that there may be possibly a closely related species but not the same as *A. phagocytophilum*-like agent circulating in Colombia. Although *A. phagocytophilum* has already been detected in dogs (SANTOS et al., 2011, 2013) and brown brocket deer (SILVEIRA et al., 2014) in Brazil, previous reports also have found new genotypes of *Anaplasma* spp. phylogenetically similar to *A. phagocytophilum* in wild carnivores kept in captivity in zoos (ANDRÉ et al., 2012), wild birds (MACHADO et al., 2012) and in cats (ANDRÉ et al., 2014) in Brazil, corroborating with the findings of the present study. Besides that, *A. phagocytophilum* antibodies have been found among rural workers in the northern region of the Colombia and among horses in Brazil (SALVAGNI et al., 2010), however, the agent has not yet been molecularly characterized (MÁTTAR & PARRA, 2006).

Although, the tick species implicated in transmission routes of *Anaplasma* spp. in dogs from south America remain unknown, the presence of co-infection with *E. canis, H. canis* and *Anaplasma* spp., found in our previous studies (VARGAS-HERNANDEZ et al., 2012a, b) using the same sampled dogs, reinforce the hypothesis that these pathogens could share the same vector. *Rhipicephalus sanguineus*, *Rhipicephalus (Boophilus) microplus*, *Amblyomma maculatum*, and *Ornithodoros (Alectorobius) puertoricensis* have been found parasitizing dogs in Colombia (ACERO et al., 2011; MIRANDA & MATTAR, 2015; PATERNINA et al., 2009). In Brazil it has been molecularly detected *A. phagocytophilum* in *A. cajennense* and *R. sanguineus* ticks (SANTOS et al., 2013) and Argentina (OSCHEROV et al., 2011).

The real impact of multiple pathogens on the pathogenesis of these infections and the host's response to them remains unknown; hence, studies focusing on this issue are sorely needed. Albeit with low prevalence, *Anaplasma* spp. circulates among dogs in Colombia. Investigations into the zoonotic potential of the *Anaplasma* spp. closely related *A. phagocytophilum* agent detected in this study and the real role of dogs in the epidemiology of human granulocytic anaplasmosis in Colombia are much needed.

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