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Characterization of *Anaplasma* spp. infection in dogs from Costa Rica

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ABSTRACT

A cross-sectional study combining serological and molecular techniques for detecting selected *Anaplasma* species was conducted between 2011 and 2012 in dogs and ticks sampled in all provinces of Costa Rica. Global *Anaplasma* spp. seroprevalence was 2.7% (11/408) by indirect immunofluorescence assay. The 16S rRNA PCR confirmed active *A. phagocytophilum* infection only in one dog (0.3%, 1/374); however, the same sample was negative to *groEL* PCR. Out of 122 *Rhipicephalus sanguineus* s.l. ticks analyzed, one (0.8%) was found positive to *A. phagocytophilum* 16S rRNA PCR but negative when tested by *groEL* PCR; this tick was collected from a seronegative and PCR negative dog. Both 16S rRNA sequences were 100% (510/510 bp) identical to *A. phagocytophilum* strains isolated in different countries from different hosts.

The presence of *A. platys* was established in four dogs (1%, 4/374) by both 16SrRNA and *groEL* PCR. Ticks collected from the same dogs tested negative by PCR. The 16S rRNA sequences were 100% identical to the corresponding sequences of *A. platys* strains isolated from dogs in Croatia and Brazil, however *groEL* sequences showed variable similarity levels (99–100%) with different strains of *A. platys* isolated in Chile, Japan and Thailand, pointing out the possible presence of different variants in Central America. Collectively data indicate low prevalence of *A. phagocytophilum* and *A. platys* in dogs from Costa Rica. Furthermore, infections seem to occur without clinical signs but with some hematological changes, and seem to resolve without treatment.

1. Introduction

Anaplasmosis is a tick-borne disease caused by bacteria belonging to the genus *Anaplasma* that infect different vertebrate species, including human. This genus includes obligate intracellular Gram-negative bacteria that mainly infect hematopoietic cells, and have a worldwide distribution (Dumler et al., 2001).

Human granulocytotropic anaplasmosis is caused by *Anaplasma phagocytophilum* (Dumler et al., 2001), which form morulae in the cytoplasm of neutrophilic granulocytes (Goddard, 2008), whereas *Anaplasma platys* is the causative agent of infectious canine cyclic thrombocytopenia (ICCT), and is found in platelets (Ramsey and Tennant, 2012). Ticks involved in the transmission of *A. phagocytophilum* are *Amblyomma* and *Ixodes* spp., while *Rhipicephalus* ticks are the vector of *A. platys* (Mullen and Durden, 2002). These ticks are present in Costa Rica (Álvarez et al., 2005).

The most common clinical signs in dogs infected with *A. phagocytophilum* are non-specific. A few patients show signs of coagulation disorders, such as petechiae, melena, or epistaxis (Rikihisa, 2011; Greene, 2012). Asymptomatic disease often coupled to marked thrombocytopenia (De Farias-Rotondano et al., 2012) is the most common outcome in dogs infected by *A. platys* (McGavin and Zachary, 2006; Ettinger and Feldman, 2010). Reinfection can occur with both agents, and antibody titers decrease observably eight months after bacteremia, approximately (Ettinger and Feldman, 2010; Woldehiwet, 2010).

Diagnosis relies on determining presence or exposure to the agent (Yabsley et al., 2008; De Farias-Rotondano et al., 2012). Serological tests, such as indirect immunofluorescence, are complicated by cross-reaction between different *Anaplasma* species (De la Fuente et al., 2006; Greene, 2012; Zobba et al., 2014). In contrast, polymerase chain reaction (PCR) allows establishing the presence of the pathogen (Parola, 2007; Forbes et al., 2009).

The first two cases of human granulocytotropic ehrlichiosis in Costa Rica were reported in 2007 in two hospitals of the Central Valley, based on clinical symptoms, detection of morulae in peripheral blood granulocytes, and recovery of patients after doxycycline treatment

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(Rojas-Solano and Villalobos-Vindas, 2007; Hernández-de Mezerville and Padilla-Cuadra, 2007). Studies that investigated a blood bank collected from dogs of the Central Valley of Costa Rica, with suspected ehrlichial and ICCT infection, established the presence of *A. platys* in 6.3% (19/300) samples, using a nested 16S rRNA PCR (Ábrego et al., 2009). Another study based on *Ehrlichia* and *Anaplasma* spp. (Rojas et al., 2014) 16S rRNA PCR, carried out in four regions of Costa Rica, revealed 10.0% (14/146) of dogs infected by *A. platys*. Recently, 16S rRNA/*groEL* amplification and sequence analyses provided evidence for *Anaplasma platys*, *Anaplasma phagocytophilum*, and *A. platys*-like infection in *Rhipicephalus sanguineus* s.l. ticks (Campos-Calderón et al., 2016).

In this paper, we investigate the presence of antibodies against *Anaplasma* spp. and DNA of *A. phagocytophilum* and *A. platys* in blood samples of seemingly healthy dogs and in ticks collected from the same animals nationwide, and we molecularly characterize the bacterial strains locally isolated.

2. Material and Methods

2.1. Ethical approval

This article does not contain any studies with human participants. The authors followed all applicable international and institutional guidelines for dogs' blood sampling.

2.2. Study design, sample size and analyzed population

A cross-sectional study was conducted to investigate the presence of *A. phagocytophilum* and *A. platys* in dog blood samples and in ticks collected from the same animals, using serological and molecular assays. The total sample size was estimated to be 369 individuals (5% prevalence, 95% confidence, 2.5% expected error, and a population higher than forty thousand dogs), calculated with Win Episcope 2.0 (Thrusfield et al., 2001). The canine population was analyzed in accordance with the provincial distribution of households. Interviews, clinical records and sampling was performed ultimately from 408 dogs. Population study, sampling methodology and period of study are described in Barrantes-González et al. (2016).

2.3. Interview, clinical examination and sampling

Each owner walking its dog and consenting to participate, was interviewed to obtain information about place of origin, age, breed, household variables (dog residence in the house), and suggestive clinical signs of anaplasmosis (petechiae, melena, epistaxis) and tick infestation in the past, among others. In addition, dog ticks were collected manually of all anatomical sites during 10 min, and a clinical examination was carried out, determining body condition, attitude, capillary refill time, temperature, color of mucous membranes, and suggestive clinical signs of anaplasmosis. Finally, blood samples were collected from each dog, stored at 4 °C until completion of blood analysis and serum separation, and frozen at −20 °C until performing the serological and molecular tests. Ticks were stored in 70% alcohol. in stray dogs living in recreational parks, consent from the municipal council was obtained. Only clinical exam and sampling was performed on these dogs.

2.4. Blood analysis

The microcentrifuge HETTICH® (5 min × 18,600*g*) and hematocrit reader DAMON/IEC was used to determine hematocrit values. Blood smears were stained with Giemsa as described by Cowell et al. (2008), to determine complete blood count (CBC). A total of 374 blood samples of dogs were analyzed.

2.5. Classification of ticks

Taxonomic classification of ticks was performed as described by Barros-Battesti et al. (2006), Nava et al. (2012, 2014). A total of 120 dogs out of 408 dogs were found infested with ticks, 112 dogs with *Rhipicephalus sanguineus* s.l., four with *Amblyomma ovale*, one with *Amblyomma mixtum*, one with *Ixodes boliviensis*, and two dogs with mixed infestation (*R. sanguineus* s.l. - *A. ovale*, and *R. sanguineus* s.l. - *A. mixtum*, respectively). Ticks from each dog were separated in microcentrifuge tubes containing 70% alcohol by species, sex and stage, and stored at room temperature until DNA extraction.

2.6. Indirect immunofluorescense assay (IFA)

The *Ehrlichia canis* and *Anaplasma phagocytophilum* MIF Canine IgG Antibody Kit from Fuller Laboratories® (California, USA) was used as recommended by the manufacturer. Each slide contained two separate antigen spots, with elementary bodies of each antigen (*E. canis* and *A. phagocytophilum*). In the present study, only the results of *A. phagocytophilum* IFA are shown, *E. canis* IFA results were reported previously (Barrantes-González et al., 2016). This assay reported a sensitivity and specificity of 100% for the *A. phagocytophilum* IFA (Naroo Ditech Inc., 2016). To determine the global seroprevalence of *A. phagocytophilum* one serial dilution (1:80) of 408 sera was analyzed. Serum samples that did not show fluorescence to *A. phagocytophilum* antigen in dilutions of 1:80 were considered negative, serum samples that showed fluorescence in dilutions of 1:80 were considered positive (Naroo Ditech Inc., 2016).

2.7. Polymerase chain reaction (PCR) and sequencing

Extraction of DNA from blood samples was performed with the Wizard Genomic kit from Promega®, Wisconsin, USA, whereas extraction of DNA from ticks was performed with the DNeasy Blood and Tissue kit from QIAGEN®, California, USA, as recommended by the manufacturers, additionally tick samples were homogenized and pretreated with proteinase K before DNA extraction. Ticks from each dog were analyzed in groups of the same species. In case of finding individuals of the same species but of different sex or stage, groups of ticks were analyzed according to the following priority: females > nymphs > males > larvae. A total of 374 blood samples and 122 groups of ticks were analyzed by PCR.

Nested PCRs were carried out to amplify fragments of the 16S rRNA gene from *A. phagocytophilum* (Massung et al., 1998) and from *A. platys* (Martin et al., 2005). In the first reactions, primers ge3a/ge10r and 8F/1448R were used for *A. phagocytophilum* and *A. platys*, respectively (Table 1). Each reaction consisted of Dream Taq™ PCR Master Mix 2X (Fermentas®), primers (1 μM), 2 μl of DNA (20 ng), nuclease free water (Fermentas®), in a final volume of 25 μl. The PCR amplification for *A. phagocytophilum* was performed with an initial denaturalization at 95 °C (2 min), followed by 40 cycles of 94 °C (30 s), 55 °C (30 s) and 72 °C (60 s), followed by a final extension at 72 °C (5 min). The amplification profile for *A. platys* PCR consisted in denaturalization at 95 °C (2 min), followed by 40 cycles of 94 °C (60 s), 45 °C (60 s) and 72 °C (120 s); followed by a final extension at 72 °C (5 min). In the nested reaction, Ge9f/Ge2 and EHR16SR/PLATYS primers were used for *A. phagocytophilum* and *A. platys*, respectively (Table 1). The same conditions as described above were used for *A. phagocytophium*, changing only quantity of DNA (1 μl) and number of cycles (30). The nested PCR profile for *A. platys* involved initial denaturalization at 94 °C (1 min), followed by 40 cycles of denaturalization 94 °C (30 s), annealing 55 °C (30 s) and extension at 72 °C (30 s); final extension consisted in 72 °C (5 min).

Positive samples in the 16S rRNA PCR of *A. phagocytophilum* or *A. platys* underwent a nested PCR for amplification of a fragment of *groEL* gene (Alberti et al., 2005). In the first reaction, primers EphplgroEL F/