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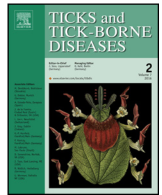

Anaplasma platys in dogs from Uruguay

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ABSTRACT

Anaplasmataceae family members include vector-borne bacteria of veterinary importance that may also affect humans. *Ehrlichia canis* and *Anaplasma platys* are the main members of this family detected in dogs worldwide. In Uruguay there are not many published studies on tick-borne pathogens affecting dogs, the only haemoparasite molecularly confirmed in dogs, is the piroplasm *Rangelia vitalii*. The aim of the present work was to detect the presence of *A. platys* and *E. canis* in dogs and dogs-associated ticks of two localities in Northwestern Uruguay. Blood samples from dogs with and without clinical signs associated with vector-borne diseases, and *Rhipicephalus sanguineus* obtained from these dogs were analyzed by PCR for Anaplasmataceae. Positive dogs were further analyzed by PCR for *Ehrlichia* spp. and *A. platys*. All the ticks were found negative. No dog was detected infected with *E. canis,* while eight dogs (4.2%) were found to be infected with *A. platys*. Phylogenetic analysis of *groESL* operon sequence for *A. platys* revealed no differences with sequences described for *A. platys* in neighbor countries and from other regions of the world. This is the first report of the presence of *A. platys* in Uruguay.

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1. Introduction

Vector-borne pathogens *Ehrlichia* spp. and *Anaplasma* spp. are obligate intracellular gram-negative bacteria of the family Anaplasmataceae, order Rickettsiales. Both bacterial genera are mainly transmitted by ticks, and were found in peripheral blood of a wide range of mammals (Sainz et al., 2015; Vieira et al., 2011). Ehrlichiosis and anaplasmosis are considered emerging diseases in humans and animals. *Ehrlichia canis* and *Anaplasma platys* are the main members of Anaplasmataceae family detected in dogs.

Ehrlichia canis is the etiological agent of canine monocytic ehrlichiosis (CME), and the intracellular morulae of *E. canis* can be found in mononuclear cells from infected animals (Allison and Little, 2013). CME is considered one of the most important infectious diseases among dogs. Clinical signs vary from asymptomatic infection to severe illness. *Ehrlichia canis* is transmitted mainly by *Rhipicephalus sanguineus*, and was found in dogs worldwide (Bowman et al., 2009; Clarke et al., 2014; Sainz et al., 2015; Ybañez et al., 2012). In South America, *E. canis* was reported in dogs from Argentina, Brazil, Chile, Colombia, Peru and Venezuela (Dantas-Torres, 2008; Eiras et al., 2013; López et al., 2012; Suksawat et al., 2001; Vargas-Hernández et al., 2012; Vinasco et al., 2007). Furthermore, it was found in cats (Braga et al., 2014) and wild canids (Cardoso et al., 2015). *Ehrlichia canis* was also reported in humans from Venezuela with and without clinical signs compatible with human monocytic ehrlichiosis (Perez et al., 2006).

Anaplasma platys (formerly known as *Ehrlichia platys*) cause canine infectious cyclic thrombocytopenia. *Anaplasma platys* is a platelet-specific microorganism whose morular forms resemble those described in leukocytes of dogs infected with *E. canis* (Harvey et al., 1978). Dogs are the main host of *A. platys* but natural infections by *A. platys* have also been reported in cats, foxes, red deer, wild boars and a goat (Chochlakis et al., 2009; Hegarty et al., 2015; Lima et al., 2010; Pereira et al., 2016; Qurollo et al., 2014). Moreover, a recent study suggests that *A. platys* may be vertically transmitted from the pregnant bitch to the offspring (Latrofa et al., 2016).

The pathogen are found in dogs with mild and severe thrombocytopenia, but also in asymptomatic ones. Furthermore, co-infection with other vector-borne pathogens influences the severity of *A. platys* infection (de Caprariis et al., 2011; Eiras et al., 2013). There is molecular evidence of the presence of *A. platys*

Abbreviations: *A*, *Anaplasma*; *E*, *Ehrlichia*; CME, canine monocytic ehrlichiosis; *N*, *Neorickettsia*.

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in dogs worldwide. In South America, infected dogs have been reported in Argentina, Brazil, Chile and Venezuela (Abarca et al., 2007; Cicuttin et al., 2014a,b; Eiras et al., 2013; Lasta et al., 2013; Santos et al., 2009; Suksawat et al., 2001). Human cases of infection with *A. platys* were confirmed by polymerase chain reaction (PCR) in Venezuela and USA (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014), which supports the role of *A. platys* as a zoonotic agent.

The tick *R. sanguineus* is presumed to be the main vector for *A. platys* (Inokuma et al., 2000; Sanogo et al., 2003). However, the only study attempting to confirm *R. sanguineus* as vector of *A. platys* was unsuccessful (Simpson et al., 1991). Some authors have argued that this unsuccessful attempt could be the result of low sensitivity of the detection method, or the tick strain/species used (Ramos et al., 2014; Sanogo et al., 2003). The hypothesis that *R. sanguineus* is the main vector of *A. platys* is yet to be confirmed. *Anaplasma platys* was found in adults and nymphs of *R. sanguineus* collected from negative dogs suggesting that a transstadial transmission may be occurring (Ramos et al., 2014).

In Uruguay there are few studies published on tick-borne pathogens affecting dogs. The only haemoparasite molecularly confirmed in dogs, is the piroplasm *Rangelia vitalii*, the etiological agent of canine rangeliosis (Soares et al., 2015). The aim of the present work was to detect by PCR the presence of *A. platys* and *E. canis,* members of Anaplasmataceae family, in dogs and dogs-associated ticks from two localities in Northwestern Uruguay. We have found for the first time in Uruguay molecular evidence of dogs infected with *A. platys*.

2. Material and methods

2.1. Sample collection

The study was carried out between September 2014 and November 2015. We assayed dogs-associated ticks and blood samples obtained from dogs of urban, suburban and rural areas from two localities in Northwestern Uruguay: Salto (31°23′18″S, 57°57′38″W), Salto Department, and Bella Unión (30°15′36″S, 57°35′57″W), Artigas Department. We selected dogs with and without clinical signs compatible with vector-borne diseases. Ticks retrieved from dogs were stored in tubes with 95% ethanol, and identified using keys provided by Onofrio et al. (2006) and Walker et al. (2000). Blood samples were collected in EDTA-tubes and stored at −20 °C until processing.

2.2. DNA extraction and PCR amplification

For molecular analysis, DNA was extracted from 200 μl of whole blood of each sample and from ticks obtained from dogs. Adult ticks and nymphs collected from the same dog were pooled. Ticks were dissected with sterile scalpel blades and forceps, rinsed with sterile water to remove ethanol, and were crushed with a homogenization pestle. DNA from ticks and blood was extracted using Pure Link™ Genomic DNA Kit (Invitrogen™, USA) according to the instructions specified by the manufacturer.

Molecular screening of Anaplasmataceae family members was done by PCR targeting the 16S ribosomal RNA (rRNA) gene, as previously described by Parola et al. (2000), using primers EHR16SD (GGTACCYACAGAAGAAGTCC) and EHR16SR (TAGCACTCATCGTTTACAGC) that amplify a fragment of 345 bp. *Anaplasma marginale* DNA was used as a positive control. Samples with a positive result to 16S rRNA gene were further tested with specific primers for *A. platys* and for *Ehrlichia* spp. Molecular screening of *Ehrlichia* spp. was done by hemi-nested PCR targeting the disulfide bond formation protein gene (*dsb*), as previously described by Almeida et al. (2013). The first reaction was performed using primers Dsb-330 (GATGATGTTTGAAGATATSAAACAAAT) and Dsb-720 (CTATTTTACTTCTTAAAGTTGATAWATC) that amplify a fragment of 401 bp, and the second reaction with the primers Dsb-380 (ATTTTTAGRGATTTTCCAATACTTGG) and Dsb-720 that amplify a fragment of 349 bp of the *dsb* gene. *Ehrlichia canis* DNA was used as a positive control. For *A. platys* amplification we used the primers PLATYS (GATTTTTGTCGTAGCTTGCTATG) and EHR16SR, as described by Inokuma et al. (2000), that amplify a fragment of 678 bp of 16S rRNA gene, and primers PLA-HS475F (AAGGCGAAAGAAGCAGTCTTA) and PLA-HS1198R (CATAGTCTGAAGTGGAGGAC), as described by Inokuma et al. (2002), that amplify a fragment of 724 bp of the heat shock operon *groESL*. All PCR reactions were performed including water as a negative control. PCR products were analyzed by 1.5% agarose gel electrophoresis. Amplicons were purified and submitted to the Institut Pasteur de Montevideo (Uruguay) for sequencing studies.

2.3. Phylogenetic analysis

The alignments and phylogenetic analysis were performed with 16S rRNA and *groESL* nucleotide sequences using MEGA 5.2.2. (Tamura et al., 2011). The sequences obtained in this study were aligned with sequences retrieved from GenBank, and regions with gaps were removed from the alignment. Phylogenetic trees were reconstructed by the Maximum-Likelihood (ML) algorithms using PhyML 3.0 software (Guindon et al., 2010). The support of the internal branching of the ML trees was assessed using the bootstrapping method (500 bootstrap replicates).

3. Results

During the study, we assayed blood samples obtained from 191 dogs (38 from private veterinary clinics of Salto, 28 from a dog shelter in Salto; and 125 from poor periurban neighborhoods in Salto and Bella Unión, 58 and 67 respectively). Thirty-two (16.8%) dogs presented clinical signs compatible with vector-borne diseases such as anemia, depression, petechiae, weakness, fever and lethargy.

Out of 191 dogs tested, 8 (4.2%) blood samples, 3 from Salto and 5 from Bella Unión, all of them asymptomatic, were positive for the 16S rRNA PCR of the Anaplasmataceae. Further *Ehrlichia* spp. and *A. platys* specific PCR assays revealed that the 8 dogs were positive only for *A. platys.* Analysis of *A. platys* 16S rRNA PCR amplification showed that the eight sequences were 100% identical to each other and with *A. platys* 16S rRNA sequences from Philippines and Malaysia (GenBank accession numbers: KP006397.1 and KU500914.1) among others around the world. Furthermore, the 8 samples were also *A. platys groESL* PCR positives. Two of these positive samples, one from each location, were randomly selected for sequencing. Both sequences resulted 100% identical to one another and with *A. platys groESL* sequences from Salta-Argentina and Thailand (GenBank accession numbers: KR909453.1 and KU765205.1) among other sequences. Representative *A. platys* 16S rRNA and *groESL* partial sequences (658-bp and 614-bp, respectively) obtained in this study were deposited in Genbank (accession numbers: KX792011 and KX792012). Phylogenetic analysis inferred from *groESL* operon partial sequences showed that the 614-bp sequence obtained from both samples clustered with sequences of *A. platys* from Chile, Argentina, Venezuela, Congo and Japan (Fig. 1).

Also, 153 *R. sanguineus* (80 females, 71 males and 2 nymphs) collected from 28 dogs were analyzed. Ticks collected from the same dog (adults and nymphs) were treated in pools of 2–10 ticks, obtaining a total of 30 DNA samples. None of the pools of ticks analyzed were PCR positive for Anaplasmataceae, neither 4 samples (24 ticks,

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