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Original article

Molecular characterization of *Rickettsia massiliae* and *Anaplasma platys* infecting *Rhipicephalus sanguineus* ticks and domestic dogs, Buenos Aires (Argentina)

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ABSTRACT

Rickettsioses, ehrlichioses and anaplasmoses are emerging diseases that are mainly transmitted by arthropods and that affect humans and animals. The aim of the present study was to use molecular techniques to detect and characterize those pathogens in dogs and ticks from Buenos Aires city. We studied 207 *Rhipicephalus sanguineus* ticks and 52 canine blood samples from poor neighborhoods of Buenos Aires city. The samples were molecularly screened for the genera *Rickettsia*, *Ehrlichia*, and *Anaplasma* by PCR and sequencing. DNA of *Rickettsia massiliae* (3.4%) and *Anaplasma platys* (13.5%) was detected in ticks and blood samples, respectively. For characterization, the positive samples were subjected to amplification of a fragment of the 190-kDa outer membrane protein gene (spotted fever group rickettsiae) and a fragment of the *groESL* gene (specific for *A. platys*). A phylogenetic tree was constructed using the neighbor-joining method, revealing that the sequences were closely related to those of strains from other geographic regions. The results indicate that human and animal pathogens are abundant in dogs and their ticks in Buenos Aires city and portray the potentially high risk of human exposure to infection with these agents, especially in poor neighborhoods, where there is close contact with animals in an environment of poor health conditions.

Introduction

Rickettsioses, ehrlichioses and anaplasmoses are infectious diseases caused by Gram-negative obligate intracellular bacteria from the order Rickettsiales (Dumler et al., 2001). These bacteria are primarily transmitted by arthropods, and are considered to be important emerging pathogens for both humans and animals (Parola and Labruna, 2009).

Rickettsia massiliae is commonly associated with ticks of the genus *Rhipicephalus* in different regions (Parola et al., 2008). Pathogenicity associated with *R. massiliae* remained unknown for many years, until it was first confirmed as a human pathogen in 2005 following a retrospective study conducted on archived samples obtained from a patient with rickettsiosis in the 1980s (Vitale et al., 2006). Few cases of human disease were confirmed by this pathogen, although it has been implicated in numerous European cases by serological methods (Vitale et al., 2006; Parola et al., 2008; García-García et al., 2010; Renvoisé et al., 2012). *Rickettsia massiliae* may also be a health threat to domestic dogs, causing clinical signs that are similar to those of other canine rickettsioses (Beeler et al., 2011).

Anaplasma platys is distributed worldwide and is transmitted by ticks from the *Rhipicephalus sanguineus* complex. This bacterium is the causative agent of canine infectious cyclic thrombocytopenia, which is usually a mild disease, though virulence may vary from region to region (de la Fuente et al., 2006; Abarca et al., 2007; Santos et al., 2009). According to available knowledge, the role of *A. platys* as a zoonotic pathogen remains inconclusive (Tamí and Tamí, 2004; Abarca et al., 2007; Ramos et al., 2009).

In Argentina, 3 species of rickettsia (*Rickettsia rickettsii*, *R. parkeri*, and *R. massiliae*) and one of ehrlichia (*Ehrlichia chaffeensis*) have been described as being associated with clinical conditions in humans. Additionally, *R. amblyommii*, *R. bellii*, *R. felis*, *E. canis* and

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Table 1
Primers used in this study.

Organism	Name	Target	Sequence (5′–3′)	Ref
Rickettsia spp.	RCK/23-5-F	23S-5S intergenic spacer	GATAGGTCRGRTGTGGAAGCAC	Jado et al. (2006)
	RCK/23-5-R	23S-5S intergenic spacer	TCGGGAYGGGATCGTGTGTTTC	Jado et al. (2006)
Rickettsia spp. (spotted fever group)	Rr190.70p	*ompA*	ATGGCGAATATTTCTCCAAAA	Regnery et al. (1991)
	Rr190.602n	*ompA*	AGTGCAGCATTCGCTCCCCCT	Regnery et al. (1991)
Anaplasma-taceae family	EHR16SD	16S rRNA	GGTACCYACAGAAGAAGTCC	Parola et al. (2000)
	EHR16SR	16S rRNA	TAGCACTCATCGTTTACAGC	Parola et al. (2000)
Anaplasma platys	PLA-HS475F	*groESL*	AAGGCGAAAGAAGCAGTCTTA	Inokuma et al. (2002)
	PLA-HS1198R	*groESL*	CATAGTCTGAAGTGGAGGAC	Inokuma et al. (2002)

A. platys were detected in several regions of the country (Venzal and Nava, 2011; Cicuttin et al., 2012; Eiras et al., 2013).

The aim of this study was to investigate the presence of *Rickettsia*, *Ehrlichia*, and *Anaplasma* species in ticks and domestic dogs from poor neighborhoods in Buenos Aires city, as well as to characterize the positive samples.

Materials and methods

From November 2009 to February 2011, ticks and whole blood samples were collected from domestic dogs (*Canis familiaris*) in poor neighborhoods in Buenos Aires city. These areas are characterized by the abundance of free-roaming dogs, high levels of parasitism by ticks, and close human–animal coexistence.

Clinically healthy dogs were selected randomly from a subpopulation of animals included in a surgical neutering program. With the consent of the owners, blood samples were collected by jugular or cephalic venipuncture with EDTA (ethylenediaminetetraacetic acid) anticoagulant. Ticks were collected manually from each animal and identified by using previously described taxonomic keys (Boero, 1957). All samples were stored at −70 °C until processing.

Nymphs were grouped in pools of 3–6 specimens by dog, and adults were processed individually. Each individual or pool was resuspended in Tris–EDTA buffer, sectioned with scalpel, and macerated. DNA extraction was performed by using the guanidine thiocyanate method (Casas et al., 1995). DNA from whole-blood samples was extracted by using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA), according to the manufacturer's instructions. Nuclease-free water was used as a negative control for the extraction.

The initial screening for *Rickettsia* was performed by PCR that amplifies a fragment of the 23S-5S rRNA intergenic spacer (Jado et al., 2006). The size of the amplicon ranges from 329 bp in *R. typhi* to 519 bp in *R. helvetica*. PCR-positive samples were further confirmed by amplifying an approximately 532-bp fragment of the 190-kDa outer membrane protein gene (*ompA*) (Regnery et al., 1991). *Rickettsia parkeri* was used as a positive control. To detect *Ehrlichia* and *Anaplasma*, a 345-bp fragment of the 16S rRNA was amplified (Parola et al., 2000), and positives were confirmed by an *A. platys groESL* gene-specific PCR protocol (724 bp) (Inokuma et al., 2002). *Anaplasma bovis* was used as a positive control for 16S rRNA PCR. No positive control was performed in *A. platys groESL* PCR to avoid any source of sample contamination. Nuclease-free water was used as a negative control. PCRs were performed according to methods previously described by authors cited in Table 1.

PCR products were purified by using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen-Life Technologies, Carlsbad, CA, USA) and sequenced with a 3500 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA) at the Servicio de Neurovirosis, Instituto Nacional de Enfermedades Infecciosas (ANLIS Dr. Carlos G. Malbrán, CABA, Argentina). Sequences obtained were first analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast). A phylogenetic analysis was further performed using MEGA version 5 (Tamura et al., 2011). The DNA sequences obtained (23S-5S rRNA intergenic spacer, *ompA*, 16S rRNA, and *groESL*) were aligned with sequences available from GenBank. For each analyzed gene, a dendogram was constructed by using neighbor-joining (NJ) with Kimura 2-parameter model. The confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

Nucleotide sequence accession numbers

Representative sequences obtained in this study have been deposited in the GenBank database under the following accession numbers: KC525896 (23S-5S intergenic spacer fragment of *R. massiliae*), KC525893 (ompA gene fragment of *R. massiliae*), KC525894 (16S rRNA fragment of *A. platys*), and KC525895 (*groESL* gene fragment of *A. platys*).

Results

A total of 52 domestic dogs was sampled, from which 207 ticks (128 nymphs, 60 females, and 19 males) were collected. All ticks were identified as belonging to the *Rh. sanguineus* complex.

PCR amplification of the 23S-5S intergenic space of *Rickettsia* spp. was positive for 6 pools of nymphs and 1 male tick, whereas it was negative for all dogs (Table 2). Positive ticks were collected from 4 dogs. Sequencing of positive products was successful in 5/6 pools of nymphs and 1 male tick. The sequences were 100% identical to each other and to the corresponding partial sequence 23S-5S rRNA of *R. massiliae* strain AZT80 (CP003319) and to *Rickettsia* sp. Bar29 (AY125014) and 99.7% to *R. massiliae* MTU5 (CP000683). Three of the 7 positive samples also tested positive for the *ompA*

Table 2
Summary of results obtained.

	Total	*Rickettsia* spp.				F. Anaplasmataceae		*A. platys*	
	n	23S-5S		*ompA*		16S rRNA		*groESL*	
		n	%	*n*	%	*n*	%	*n*	%
Rh. sanguineus	207	7	3.4[a]	3/7	42.8	0	0	–	–
Dogs	52	0	0	–	–	7	13.5	7/7	100

[a] Minimum infection rate.