

Ehrlichia canis and *Rickettsia* spp. in dogs from urban areas in Paraíba state, northeastern Brazil

Ehrlichia canis e *Rickettsia* spp. em cães de áreas urbanas da Paraíba, nordeste do Brasil

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Abstract

The aims of our study was to identify *Ehrlichia canis* and antibodies against *Rickettsia* spp. belonging to the spotted fever group (SFG) in dogs sampled from Paraíba state, northeastern Brazil. Blood and serum samples collected by convenience from dogs in urban areas of five municipalities were analyzed by real-time PCR for the detection of *E. canis* DNA and by immunofluorescence assay test (IFAT) for the identification of antibodies against *Rickettsia rickettsii*, *R. felis*, *R. parkeri*, *R. amblyommii* and *R. rhipicephali* antigens. *E. canis* DNA was detected in 8.9% (64/719) of the blood samples, whereas 5.63% (43/763) of the serum samples were positive for at least one of the *Rickettsia* antigens tested by IFAT. This study showed for the first time the occurrence of *E. canis* and suggested the circulation of SFG *Rickettsia* in dogs in the study region of Paraíba state, northeastern Brazil.

Keywords: *Ehrlichia* spp., *Rickettsia* spp., IFAT, PCR, Brazil.

Resumo

Os objetivos do nosso estudo foram identificar *Ehrlichia canis* e anticorpos contra *Rickettsia* spp. pertencentes ao Grupo da Febre Maculosa (GFM) em cães amostrados no estado da Paraíba, nordeste do Brasil. As amostras de sangue e soro, coletados por conveniência, de cães em áreas urbanas de cinco municípios foram analisadas por PCR em tempo real para a detecção de DNA de *E. canis* e pela Reação de Imunofluorescência Indireta (RIFI) para identificação de anticorpos contra *Rickettsia rickettsii*, *R. felis*, *R. parkeri*, *R. amblyommii* e *R. rhipicephali*. O DNA de *E. canis* foi detectado em 8,9% (64/719) das amostras de sangue, enquanto que 5,63% (43/763) das amostras de soro foram positivas para pelo menos um dos antígenos de *Rickettsia* testados por RIFI. Este estudo mostrou pela primeira vez a ocorrência de *E. canis* e sugere a circulação de *Rickettsia* do GFM em cães na região em estudo do estado da Paraíba, Nordeste do Brasil.

Palavras-chave: *Ehrlichia* spp., *Rickettsia* spp., RIFI, PCR, Brasil.

Introduction

Rickettsioses are vector-borne diseases caused by bacteria of the Rickettsiales order, which include two families of obligate intracellular pathogens, Rickettsiaceae and Anaplasmataceae (DUMLER et al., 2001). Rickettsial infections are widely distributed and are

transmitted by a variety of species of ticks. Thus, the epidemiology of several rickettsial infections is determined by the specific geographic distribution of the tick vector (DANTAS-TORRES, 2007; PAROLA et al., 2013).

Among members of the family Anaplasmataceae, *Ehrlichia canis* is the agent of canine monocytic ehrlichiosis (CME) in Brazil. CME is a multisystem disease manifesting as either acute, sub-clinical or chronic forms, depending on the virulence of the

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E. canis strain and the occurrence of co-infections, especially with other arthropod-borne pathogens, such as *Babesia vogeli* and *Hepatozoon canis* (VIEIRA et al., 2011). The diagnosis of CME is based on the observation of the presence of morulae within leucocytes in blood smears, isolation of the bacterium in cell culture, immunofluorescence assay test (IFAT), and molecular detection (PCR) (ROTONDANO et al., 2012).

Brazilian spotted fever (BSF), caused by the bacterium *Rickettsia rickettsii*, a member of the Spotted Fever Group (SFG), has long been recognized in Brazil, mainly in the southern and southeastern regions (ANGERAMI et al., 2009; PAROLA et al., 2013). More recently, the occurrence of other human pathogenic *Rickettsia* species such as *R. felis*, *R. parkeri* and *Rickettsia* spp. strain Atlantic rainforest has been increasingly reported in Brazil (HORTA et al., 2006; SILVEIRA et al., 2007; SPOLIDORIO et al., 2010). While *R. felis* was detected in *Rhipicephalus sanguineus* ticks in the state of Paraíba, northeastern Brazil (TANIKAWA et al., 2013), there was a recent report of a *R. parkeri*-like strain infecting *Amblyomma nodosum* ticks collected from birds in Paraíba (LUGARINI et al., 2015). The IFAT is the most common test and serological gold standard for the diagnosis of rickettsial infection in humans and animals (DANTAS-TORRES, 2007).

The knowledge of the occurrence of tick borne-agents among canine populations is crucial for early diagnosis, treatment and control of these diseases, preventing their spread to humans and other canine populations. This study aimed to investigate the occurrence of *E. canis* DNA and *Rickettsia* spp. antibodies in urban dogs in the state of Paraíba, northeastern Brazil.

Materials and Methods

Samples and collection area

Blood samples were obtained from 719 domiciled dogs for detection of *E. canis* DNA and sera samples from 763 dogs for the detection of anti-*Rickettsia* spp. antibodies (Table 1). The samples were collected by cephalic or jugular venipuncture into Vacutainer

tubes containing sodium citrate. The animals were clinically healthy and were sampled by convenience of the owner residences at urban areas of the five municipalities in the state of Paraíba: Campina Grande (7°13'S, 35°52'W), Areia (6°57'S, 35°41'W), Uiraúna (6°57'S, 38°24'W), Cajazeiras (6°53'S, 38°33'W) and Sousa (6°45'S, 38°13'W) (Figure 1).

Detection of *Ehrlichia canis* DNA by real-time PCR-DNA extraction and amplification

DNA was extracted from whole blood samples using a commercial DNA extraction kit (Wizard kit for DNA extraction®, Promega) (ROTONDANO et al., 2012). The detection of *E. canis* was performed by real-time PCR to amplify 378 base pairs (bp) of the *dsb* gene encoding a disulfide bond formation protein, using the primers Dsb321 and Dsb671 and a species-specific probe, as previously described (DOYLE et al., 2005). Positive and negative DNA samples were extracted from *E. canis* cultured in DH82 cells and uninfected cultures of DH82 cells, respectively. Controls were included for all PCR assays.

Serological tests to detect antibody against *Rickettsia* spp.

Canine sera were tested by immunofluorescence assay test (IFAT) using crude antigens derived from five Brazilian *Rickettsia* isolates (*R. rickettsii* strain Taiaçu, *R. parkeri* strain At24, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ5, and *R. felis* strain Pedreira) as previously described (LABRUNA et al., 2007).

Briefly, serum samples were serially diluted in phosphate-buffered saline (PBS) in two fold dilutions from 1/64 up to 1/1280, and instilled on glass slides coated with the antigens. A commercial fluorescein, isothiocyanate-labelled rabbit anti-dog IgG (Sigma, St Louis, MO, USA), was used as secondary antibody. In each slide, a known non-reactive (negative control for all tested antigens) and a known reactive serum (positive control for all tested antigens) from

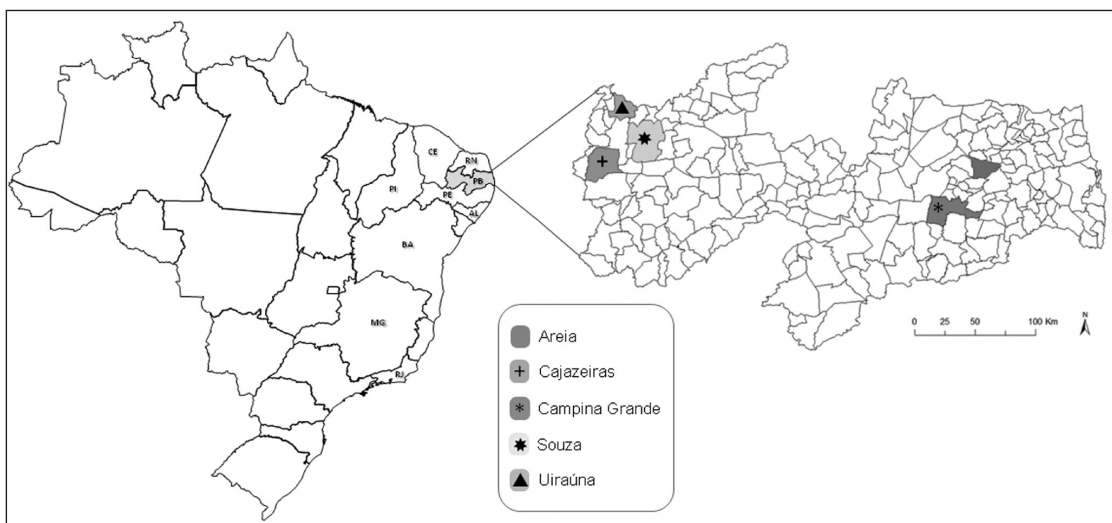


Figure 1. Geographical location of the municipalities included in the study of *Ehrlichia canis* and *Rickettsia* spp. in dogs from urban areas in Paraíba state, northeastern Brazil.

the work of Krawczak et al. (2016) were tested in a 1/64 dilution. For each serum, the endpoint titer reacting with each *Rickettsia* antigen was determined, and the results were categorized as follows per Labruna et al. (2007): serum showing for a *Rickettsia* species titer at least four-fold higher than that observed for any other *Rickettsia* species was considered homologous to the first *Rickettsia* species or to a very closely related genotype.

Statistical analysis

A chi-square test was used to compare the prevalence of the pathogens and the geographic origin of the dogs analyzed. A p value <0.05 was considered statistically significant. All analyses were performed using the SPSS program version 13.0 for Windows.

Ethical considerations

This study was approved by the Ethics Committee of the Federal University of Campina Grande (PB) protocol number 07/2012.

Results

Detection of *E. canis* DNA by real-time PCR

Ehrlichia canis DNA was amplified in 8.9% (64/719) of the dog blood samples analyzed by real-time PCR. The distribution of positive samples and the percentage of positivity per municipality are shown in Table 1.

Table 1. Distribution by municipality of the number of dogs from Paraiba, Brazil, analyzed for *Ehrlichia canis* DNA by real-time PCR and *Rickettsia* spp. by indirect immunofluorescence assay test (IFAT) showing number of positives and percentages.

Municipalities	Number positive/number of analyzed samples, (% positivity)	
	Assay	
	<i>Ehrlichia canis</i> (real-time PCR)	<i>Rickettsia</i> spp. (IFA)
Uiraúna	14/100 (1.94%)	2/100 (2.00%)
Cajazeiras	7/74 (0.97%)	2/97 (2.06%)
Sousa	4/68 (0.55%)	5/89 (5.61%)
Areia	15/101 (2.08%)	4/101 (3.96%)
Campina Grande	24/376 (3.33%)	30/376 (7.97%)
Total	64/719 (8.9%)	43/763 (5.63%)

Table 2. Distribution by municipality of the number of dogs from Paraiba state, Brazil, analyzed for *Rickettsia* spp. by indirect immunofluorescence assay test (IFAT) showing the number of positives and percentages.

Municipalities	Nº Tested dogs	Nº Positive dogs (%)	Number of dogs reactive to each species of <i>Rickettsia</i> (% positivity)					PAIHR
			<i>R. rickettsii</i>	<i>R. parkeri</i>	<i>R. rhipicephali</i>	<i>R. amblyommii</i>	<i>R. felis</i>	
			Uiraúna	100	2 (2)	0 (0)	1 (1)	
Cajazeiras	97	2 (2.06)	1 (1.03)	0 (0)	2 (2.06)	1 (1.03)	0 (0)	0
Sousa	89	5 (5.62)	2 (2.24)	4 (4.49)	1 (1.12)	2 (2.24)	0 (0)	0
Areia	101	4 (3.96)	1 (0.99)	2 (1.98)	2 (1.98)	3 (2.97)	1 (0.99)	2:1 <i>R. amblyommii</i> , 1 <i>R. felis</i>
Campina Grande	376	30 (7.98)	9 (2.39)	13 (3.45)	7 (1.86)	16 (4.25)	2 (0.23)	1 <i>R. rickettsii</i>
Total	763	43 (5.63)	13 (1.7)	20 (2.62)	12 (1.57)	23 (3.01)	3 (0.39)	

PAIHR: probable antigen involved in homologous reaction.

Detection of anti-*Rickettsia* spp. antibodies by IFAT

Among the 763 dog serum samples analyzed by IFAT 5.63% (43/763) were reactive to at least one of the five rickettsial antigens tested, with titers ≥ 64 . The titers of positive sera for the different species ranged from 64 to 512 for *R. rickettsii* and *R. felis*, 128 to 2048 for *R. amblyommii*, and 64 to 1024 for *R. parkeri* and *R. rhipicephali*. Three animals showed homologous reactions: one for *R. amblyommii*, one for *R. felis*, and one for *R. rickettsii*, which was four-fold higher than those for the other antigens tested (Table 2).

There was no significant difference among the municipalities in the frequency of the serological reactivity to different *Rickettsia* species: *R. rickettsii* (p = 0.319), *R. parkeri* (p = 0.089), *R. rhipicephali* (p = 0.501), *R. amblyommii* (p = 0.263) and *R. felis* (p = 0.734).

Only 0.55% (4/719) of the samples were positive for both agents tested in the present study. Four samples from Campina Grande municipality showed antibodies against at least one *Rickettsia* spp. antigens tested and had also DNA of *E. canis*.

Discussion

The present study assessed the occurrence of *E. canis* and exposure to other rickettsial agents in dogs from urban areas in the state of Paraiba in northeastern Brazil. Of the blood samples analyzed by real-time PCR, 8.9% yielded *E. canis* DNA. These results reveal a comprehensive picture of the CME situation in the region since real-time PCR reveals not only the exposure to the pathogen

but also the infection process as ehrlichial DNA was detected. Even though Rotondano et al. (2015) had detected DNA of *E. canis* by real-time PCR in 25% (25/100) of the dogs sampled in Paraíba state, their samples comprised a veterinary hospital population, in contrast to random health dogs in the present study. This fact could be one of the reasons for the higher frequency of infected dogs in the study of Rotondano et al. (2015).

Here, we found 5.63% (43/763) of the canine serum samples reactive to at least one of the *Rickettsia* antigens. According to Piranda et al. (2008) the presence of seropositive animals indicates the circulation of *Rickettsia* of the SFG in an area at least 6-18 months earlier. As common hosts of tick-vectors for BSF, dogs are an important marker for disease in surveillance studies, and the occurrence of serologically positive dogs in a geographical area indicates the threat of human infection (ARAES-SANTOS et al., 2015).

This study provides evidence for exposure to *Rickettsia* spp. in urban dogs in the state of Paraíba. In the serum samples from three animals, it was possible to determine the probable antigen involved in homologous reaction (PAIHR) -*R. amblyommii*, *R. felis* and *R. rickettsii*. Among the other reactive animals, it was not possible to discriminate the infecting agent (Table 2). *R. rickettsii* is a known canine pathogen, whereas the role of pathogenicity by *R. amblyommii* in dogs is limited to serological evidence only. So far, the only tick-transmitted agents involved in human disease in Brazil are *R. rickettsii* and *Rickettsia* sp. strain Atlantic rainforest (ANGERAMI et al., 2009; SPOLIDORIO et al., 2010). The role of *R. amblyommii* in pathogenicity is not yet fully elucidated (BREITSCHWERDT et al., 1988). However, some Rocky Mountain Spotted Fever (RMSF) cases in the U.S. that were attributed to *R. rickettsii* could be due to *R. amblyommii* instead (APPERSON et al., 2008).

In Brazil, two species of ticks are implicated in transmission of *R. rickettsii* to humans and other animals, *Amblyomma sculptum* (published as *A. cajennense*) and *A. aureolatum* (KRAWCZAK et al., 2014; SARAIVA et al., 2014). While *A. aureolatum* is not present in northeastern Brazil (SARAIVA et al., 2014), *A. sculptum* is a very rare or absent tick species in the Caatinga biome of northeastern Brazil (MARTINS et al., 2016). Therefore, it is unlikely that *A. sculptum* acts as an important vector of *R. rickettsii* within this biome.

R. sanguineus is the main ectoparasite in dogs in the dry Agreste and Sertão mesoregions of the Paraíba state (ROTONDANO et al., 2015). Although Tanikawa et al. (2013) had detected *R. felis* DNA in 4,5% (1/22) of *R. sanguineus* collected from Paraíba state, the role of *R. sanguineus* as vector of *R. rickettsii* in Brazil is not totally elucidated, further studies are needed to confirm its role as a vector among dogs in state of Paraíba.

In spite of the fact that *Ctenocephalides felis*, a host for *R. felis*, is the most common flea species infesting dogs in Brazil (OLIVEIRA et al., 2002), only three animals of the present study displayed *R. felis* antibodies and one of them showed antibody against *R. felis* four-fold higher than that observed for any other *Rickettsia* species antigens, being thus considered homologous to *R. felis*. The potential presence of *R. felis* in dogs in the study region has important implications since *R. felis* has already been

considered as a human pathogen (SCHRIEFER et al., 1994; LABRUNA et al., 2007).

A recent study detected two *Rickettsia* species infecting bird ticks in the Atlantic forest of Paraíba state: *Rickettsia* sp. strain NOD (a *R. parkeri*-like agent) in *Amblyomma nodosum*, and *R. amblyommii* in *A. longirostre* (LUGARINI et al., 2015). Since these two ticks species have occasionally been reported infesting dogs in Brazil (MORAES-FILHO et al., 2009; SABATINI et al., 2010), it is possible that they could be involved in the seropositivity of some of the dogs in the present study; however, we have no data on the history of tick infestations of our canine sample.

Conclusions

This study is the first to detect both antibodies to SFG *Rickettsia* and DNA of *E. canis* in dogs from the state of Paraíba in northeastern Brazil. The finding of infection by *E. canis* in the whole blood of the animals analyzed reveals the endemic status of CME in the region. The study detected, for the first time, a positive serological reaction indicating *Rickettsia* spp. antibodies in forty-three samples, and the probable antigen involved in a homologous reaction (*R. amblyommii*, *R. felis* and *R. rickettsii*) was identified in three samples. Taking into account that the dogs can serve as important indicators of tick-borne diseases, further studies to better understand the dynamics of rickettsiae diseases and their vectors in this region are extremely important to know about the real situation the circulation the rickettsias in the Paraíba state, northeastern Brazil.

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