Original Article

Braz. J. Vet. Parasitol., Jaboticabal, v. 24, n. 1, p. 28-35, jan.-mar. 2015
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612015008

A serological and molecular survey of *Babesia vogeli*, *Ehrlichia canis* and *Rickettsia* spp. among dogs in the state of Maranhão, northeastern Brazil

Detecção sorológica e molecular de *Babesia vogeli*, *Ehrlichia canis* e *Rickettsia* spp. em cães do Estado do Maranhão, Nordeste do Brasil

Andréa Pereira da Costa[1]; Francisco Borges Costa[1]; Marcelo Bahia Labruna[1]; Iara Silveira[1]; Jonas Moraes-Filho[1]; João Fábio Soares[1]; Mariana Granziera Spolidorio[1]; Rita de Maria Seabra Nogueira de Candanedo Guerra[2]*

[1]Laboratório de Doenças Parasitárias, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo – USP, São Paulo, SP, Brasil

[2]Laboratório de Parasitologia, Curso de Medicina Veterinária, Universidade Estadual do Maranhão – UEMA, São Luís, MA, Brasil

Received September 9, 2014
Accepted January 12, 2015

Abstract

This study evaluated exposure and infection by tick-borne agents (*Babesia vogeli, Ehrlichia canis* and *Rickettsia* spp.) in 172 dogs in rural areas and 150 dogs in urban areas of the municipality of Chapadinha, state of Maranhão, northeastern Brazil, using molecular and serological methods. Overall, 16.1% of the sampled dogs (52/322) were seroreactive to *B. vogeli,* with endpoint titers ranging from 40 to 640. For *E. canis,* 14.6% of the dogs (47/322) were seroreactive, with endpoint titers from 80 to 163,840. Antibodies reactive to at least one of the five species of *Rickettsia* were detected in 18.9% of the dogs (61/322), with endpoint titers ranging from 64 to 4,096. High endpoint titers were observed for *Rickettsia amblyommii*. Three (0.9%) and nine (2.8%) canine blood samples were PCR-positive for *Babesia* spp. and *E. canis.* The ticks collected from urban dogs were all *Rhipicephalus sanguineus* sensu lato, whereas the rural dogs were infested by *R. sanguineus* s.l, *Amblyomma cajennense* sensu lato and *Amblyomma ovale.* One *A. ovale* tick was found to be infected by *Rickettsia bellii.* This study provides an epidemiological background for controlling and preventing canine tick-borne diseases in a neglected region of Brazil.

Keywords: Ticks, tick-borne pathogens, hemoparasites.

Resumo

Este estudo avaliou por métodos sorológicos e moleculares a exposição e infecção por agentes transmitidos por carrapatos (*Babesia vogeli, Ehrlichia canis,* and *Rickettsia* spp.) em 172 cães de áreas rurais e 150 cães de áreas urbanas do município de Chapadinha, Estado do Maranhão, Nordeste do Brasil. No geral, 16,1% dos cães amostrados (52/322) apresentaram soros reagentes para *B. vogeli*, com títulos finais variando de 40 a 640. Para *E. canis*, 14,6% cães (47/322) apresentaram soros reagentes com títulos finais de 80 a 163,840. Anticorpos reativos para pelo menos uma das cinco espécies de *Rickettsia* foram detectados em 18,9% dos cães (61/322), com os títulos que variam de 64 a 4096. Foram observados altos títulos para *Rickettsia amblyommii*. Três amostras de sangue canino (0,9%) e 9 (2,8%) foram PCR positivas para *Babesia* spp e *E. canis*. Os carrapatos coletados de cães urbanos eram todos *Rhipicephalus sanguineus* sensulato, e os cães rurais estavam infestados por *R. sanguineus s*.l , *Amblyomma cajennense* sensu lato e *Amblyomma ovale*. Um carrapato *A. ovale* foi encontrado infectado por *Rickettsia bellii*. Este estudo fornece um conhecimento epidemiológico para o controle e prevenção de doenças transmitidas por carrapatos de cães em uma região negligenciada do Brasil.

Palavras-chave: Carrapatos, agentes transmitidos por carrapatos, hemoparasitas.

***Corresponding author:** Rita de Maria Seabra Nogueira de Candanedo Guerra, Laboratório de Parasitologia, Curso de Medicina Veterinária, Universidade Estadual do Maranhão (UEMA), Campus Universitário Paulo VI, S/N, Tiririca, CEP 65055-150, São Luís, MA, Brasil, e-mail address: grita62@hotmail.com.

Introduction

The emergence and reemergence of arthropod-borne diseases have been a challenge for veterinary and human medicine. Arthropods and the infections transmitted by them are expanding their zoogeographic boundaries due to climate changes and increased accessibility to certain environmental niches (SHAW et al., 2001). Vector-borne pathogens such as *Babesia vogeli*, *Ehrlichia canis* and spotted fever group *Rickettsia* cause diseases in dogs in many parts of the world. Dogs infected with these organisms may develop either subclinical infection or clinical signs of pyrexia, lethargy, pallor, hemorrhagic diatheses, icterus and vasculitis. Infection may be fatal in some dogs (HII et al., 2012).

Babesia vogeli, which is transmitted by the tick *Rhipicephalus sanguineus* sensu lato, has been detected in dogs in tropical or subtropical areas of northern, eastern and southern Africa (UILENBERG et al., 1989; LEWIS et al., 1996; OYAMADA et al., 2005), Europe (CRIADO-FORNELIO et al., 2003), Asia (INOKUMA et al., 2006), Australia (HII et al., 2012) and North and South America (BIRKENHEUER et al., 2003; PASSOS et al., 2005). It is considered to be a mildly virulent species, commonly inducing moderate clinical signs in dogs (BECK et al., 2009). Canine infection by *B. vogeli* seems to be widespread in Brazil (COSTA-JÚNIOR et al., 2009; O'DWYER et al., 2009; RAMOS et al., 2010), in accordance with the wide distribution of its vector, *R. sanguineus* s.l., especially in urban and peripheral urban areas (LABRUNA & PEREIRA, 2001).

Ehrlichia canis is a Gram-negative, pleomorphic, obligate intracellular coccus that infects canine monocytes and is also transmitted by *R. sanguineus* (IRWIN, 2001). *E. canis* is more prevalent in tropical and subtropical areas in India, Asia, Central and South America and Africa (SINGLA et al., 2011; BABA et al., 2012; RODRIGUEZ-VIVAS et al., 2005; AGUIAR et al., 2007a; MATJILA et al., 2008). In South America, it has been reported in Brazil (VIEIRA et al., 2011), Venezuela (UNVER et al., 2001), Chile (LÓPEZ et al., 2012) and Peru (VINASCO et al., 2007). In Brazil, canine infection by *E. canis* is widespread, also in accordance with its primary vector, *R. sanguineus* s.l. (VIEIRA et al., 2011).

The genus *Rickettsia* comprises a number of distinct serotypes that have been classified into separate species. Some of them are pathogenic and others are apparently nonpathogenic for humans (YU & WALKER, 2003). They are typically transmitted by arthropod vectors, which include ticks, mites, fleas or lice (BURGDORFER, 1988). The most pathogenic species, *Rickettsia rickettsii,* is the agent of Brazilian spotted fever in southeastern Brazil, where it is transmitted by *Amblyomma* ticks (LABRUNA, 2009). The other tick-associated *Rickettsia* species reported in Brazil are *Rickettsia parkeri*, *Rickettsia amblyommii*, *Rickettsia rhipicephali, Rickettsia monteiroi* and *Rickettsia bellii*. Among these, only *R. parkeri* has unquestionably been associated with human illness (LABRUNA et al. 2011). While several recent studies have revealed circulation of different *Rickettsia* species in some areas of Brazil, no study has been conducted in the state of Maranhão.

Taking into account that dogs are frequently infested by ticks, are companion animals and, depending on their lifestyle, may have access to different environments, they are able to exchange ectoparasites with other animals species and introduce pathogens into certain places, many of which causes zoonoses (SZABÓ et al., 2010). In this context, the present study (cross-sectional) evaluated exposure and infection by *B. vogeli*, *E. canis* and *Rickettsia* spp. in dogs in rural and urban areas in the state of Maranhão, northeastern Brazil, where information on tick-borne diseases is very scarce.

Materials and Methods

Ethics committee. The present study was approved by the Bioethics Committee for Animal Experimentation of the State University of Maranhão, Brazil.

Study area. During March 2010, domestic dogs were sampled in rural and urban areas of three municipalities, Anapurus (03°40'S and 43°06'W), Chapadinha (03°44' S and 43°21'W) and Mata-Roma (03°37'S and 43°06'W), within the Chapadinha region, state of Maranhão, northeastern Brazil. The Chapadinha region is characterized by a humid tropical climate, in which the rainy season predominates from December to May, while the dry season extends from July to November. The annual average temperature is higher than 27°C, with a maximum of 37°C and minimum of 21°C; the mean annual rainfall ranges from 1,200 to 1,400 mm. The area is within the Cerrado biome, with a flora composed of two diverse types of vegetation: deciduous and seasonal forests (SELBACH & LEITE, 2008).

Sampling procedure. Blood samples were collected from 150 dogs living in urban areas, and from 172 dogs living in rural areas of the Chapadinha region. Of these, 123 (50 urban and 73 rural) were in the municipality of Chapadinha, 100 (50 urban and 50 rural) in Anapurus, and 99 (50 urban and 49 rural) in Mata Roma. **Owned and apparently healthy dogs were sampled by convenience directly from their residences**, according to accessibility of the place. All the blood samples were collected from the cephalic or jugular vein. The whole blood and blood serum from each dog were stored separately in microtubes at –20°C until the laboratory analyses were performed. At the time of the blood collection, all the dogs had a healthy appearance. A questionnaire focusing on potential risk factors for tick-borne diseases [age, gender, living place (urban or rural) and hunting practices] was given to each dog owner. At the time of blood collection, the dogs were examined for presence of ticks. Any ticks present were collected and taken to the laboratory for taxonomic identification. The results regarding the ticks that were infesting the dogs of the present study have been reported elsewhere (COSTA et al., 2013).

Serological analyses. Canine serum samples were tested individually by means of the indirect immunofluorescence assay (IFA), using *B. vogeli*-infected canine erythrocytes as the antigen. The Jaboticabal strain of *B. vogeli* was used for this in accordance with the method described by IICA (1987). Serum samples were also tested by means of IFA using *E. canis*-infected DH82 cells as the antigen. The São Paulo strain of *E. canis* was used for this, as previously described (AGUIAR et al., 2007b, 2008). Reactions were performed using fluorescein-conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, MO, USA). Serum was considered to contain antibodies reactive to each agent if it displayed a reaction at the dilution 1:40. Samples that reacted at the screening dilution (1:40)

were then titrated using serial two-fold dilutions to determine endpoint titers.

Antibodies reactive to *Rickettsia* spp. were assayed by simultaneously using five *Rickettsia* isolates from Brazil: *R. bellii* strain Mogi, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ5, *R. rickettsii* strain Taiaçu and *R. parkeri* strain At24, as previously described (LABRUNA et al., 2007). Samples that reacted at the screening dilution (1:64) were then titrated using serial two-fold dilutions to determine endpoint titers. Serum showing a *Rickettsia* species titer at least four times higher than those observed for the other *Rickettsia* species was considered to be homologous to the first *Rickettsia* species or to a very closely related genotype (LABRUNA et al., 2007; PIRANDA et al., 2008).

For all reactions, a nonreactive canine serum specimen (negative control) and a known reactive canine serum specimen (positive control) were included on each slide. These sera were obtained from serum bank of the Laboratory of Parasitic Diseases in the Department of Preventive Veterinary Medicine and Animal Health (VPS) at the Faculty of Veterinary Medicine and Zootechny (FMVZ), University of São Paulo (USP).

Molecular analyses. DNA was extracted from 300 µL of whole blood from each dog using the Wizard® genomic DNA purification kit (Promega corporation, Madison / USA), in accordance with the manufacturer's instructions. DNA samples were tested by means of the polymerase chain reaction (PCR) using the sets of primers described in Table 1. For *Babesia* spp., a single PCR was used (DUARTE et al., 2008); for *Ehrlichia canis,* a nested PCR was performed, with the second reaction specific for *E. canis* (DAWSON et al., 1996; MURPHY et al., 1998). Reactions were performed in a final volume of 25 µl, containing 10 mM of Tris–HCl (pH 8.3), 50 µM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen), 11 pmol of each primer and approximately 100 ηg of canine genomic DNA. The thermocycling conditions for the reactions were 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, with annealing at 56°C (*Babesia* spp.) or 60ºC (first nested reaction) or 55°C (second nested reaction) for 30 s, and extension at 72°C for 1 min; a final extension step at 72°C for 3 min was used. The amplified products were viewed under ultraviolet light after electrophoresis on agarose gel stained with SyBr gold (Invitrogen).

The ticks collected from the dogs were individually subjected to DNA extraction by means of the guanidine isothiocyanate-phenol technique (SANGIONI et al., 2005). They were then tested by means of PCR using the primers CS-78 and CS-323 (Table 1), targeting the citrate synthase gene (*glt*A) of *Rickettsia* spp. (LABRUNA et al., 2004a). The PCR products from the ticks were purified using ExoSap (USB) and were sequenced in an automatic sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 310 Genetic, California, USA), in accordance with the manufacturer's protocol. The partial sequences obtained were subjected to BLAST analyses (ALTSCHUL et al., 1990) to infer the closest similarities to samples in GenBank.

Statistical analysis. The minimum number of sampled dogs in each urban or rural area was statistically calculated considering each canine population, representing one fifth of the human population. Statistical associations of seropositivity to tick-borne pathogens with potential risk factors (independent variables) were tested by means of the chi-square or Fischer`s exact test, when it necessary. The odds ratio (OR) was calculated with 95% confidence limits. All analyses were performed using the Epi Info software, version 6.04d (CDC, Atlanta, GA, USA).

Results

A total of 322 dogs were sampled, comprising 148 males and 122 females; 86 dogs were < 1 year old, 187 dogs were 1-3 years old, and 49 dogs were > 3 years old. In order to increase the robustness of the statistical analyses, canine seroreactivity to *B. vogeli, E. canis* or *Rickettsia* spp. among the three municipalities (Anapurus, Chapadinha, and Mata Roma) were grouped, as presented below.

The serological results from the dogs according to whether they lived in urban and rural areas are shown in Table 2. Significantly, more dogs were seroreactive to *B. vogeli* in the urban areas than in the rural areas ($P = 0.0119$). The chances of dogs becoming seroreactive to *B. vogeli* in urban areas were about twice (OR: 2.27) their chances in rural areas. Regarding to *E. canis*, there was no significant association between urban and rural dogs ($P = 0.4486$). On the other hand, significantly more dogs were seroreactive to *Rickettsia* spp in rural areas, where the chances of dogs becoming seroreactive to *Rickettsia* spp were 18.09 times the chances shown by dogs living in urban areas.

Overall, 16.1% of the sampled dogs (52/322) were seroreactive to *B. vogeli,* with endpoint titers ranging from 40 to 640. For *E. canis,* 14.6% of the dogs (47/322) were seroreactive, with endpoint titers from 80 to 163,840. Antibodies reactive to at least

Table 1. Primer pairs used in the present study for detecting tick-borne agents.

Primer pairs	Target agents (gene)	Primers	Primer sequences (5'-3')	Product size (bp)	Reference
1	*Babesia* spp. (18S rRNA)	BAB1	GTGAACCTTATCACTTAAAGG	590	Duarte et al. (2008)
		BAB4	CAACTCCTCCACGCAATCG		
2	*Ehrlichia* spp. (16S rRNA)	ECC	AGAACGAACGCTGGCGGCAAGC	478	Dawson et al. (1996)
		ECB	CGTATTACCGCGGCTGCTGGCA		
3	*Ehrlichia canis* (16S rRNA)	ECAN5	CAATTATTTATAGCCTCTGGCTATAGGA	398	Murphy et al. (1998)
		HE3	TATAGGTACCGTCATTATCTTCCCTAT		
4	*Rickettsia* spp. (*gltA*)	CS-78	GCAAGTATCGGTGAGGATGTAAT	401	Labruna et al. (2004a)
		CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT		

one of the five *Rickettsia* species were detected in 18.9% of the dogs (61/322), with endpoint titers ranging from 64 to 4,096. All 61 dogs that were seroreactive to *Rickettsia* spp. presented reactions with *R. amblyommii,* for which at least 12 dogs presented endpoint titers that were four times higher than the endpoint titers showed for the other four *Rickettsia* species. These 12 dogs were considered to have been infected by *R. amblyommii* or a very closely related genotype (Table 3). Among the 61 dogs that were reactive to *R. amblyommii,* 54 were reactive to *R. rhipicephali* (highest endpoint titer: 2,048), 30 to *R. parkeri* (highest endpoint titer: 512), 28 to *R. rickettsii* (highest endpoint titer: 1,024) and 19 to *R. bellii* (highest endpoint titer: 512). Ten dogs (3.1%) were seropositive to both *E. canis* and *Rickettsia* sp., while seven dogs (2.2%) were seropositive to both *E. canis* and *B. vogeli*. No dog was seropositive for the three agents or for both *B. vogeli* and *Rickettsia* spp.

The proportions of dogs that were seroreactive to *B. vogeli* and *E. canis* were statistically similar ($P > 0.05$) for dogs of both genders and for different age groups. For *Rickettsia* spp, dogs of different age groups had similar seroreactivity ($P > 0.05$); on the other hand, significantly more female dogs were seroreactive to *Rickettsia* spp. than were male dogs ($P < 0.05$). Hunting practice, reported for 26.4% of the dogs (85/322), was not statistically associated ($P > 0.05$) with seroreactivity to *B. vogeli* or *E. canis*; however, it was significantly associated ($P = 0.00674$) with seroreactivity to *Rickettsia* spp. The chances of hunting dogs becoming seroreactive to *Rickettsia* spp was 2.33 times greater than the chances of dogs with no hunting practice.

Among the 322 blood samples subjected to molecular analyses, three (0.9%) were positive in *Babesia* PCR, comprising two urban dogs in the municipality of Chapadinha and one rural dog in Anapurus. *E. canis*-specific nested PCR was positive in nine dogs (2.8%): four in urban areas and five in rural areas. Because of logistic problems during the study, DNA sequences of these PCR products neither were nor determined.

Parasitism by ticks was found in 59.9% of the dogs (193/322), among which 40.9% (79/193) were urban and 59.1% (114/193) were rural. A total of 929 specimens were collected and identified

Table 2. Results from indirect immunofluorescence assays (IFA) for *Babesia vogeli, Ehrlichia canis* and *Rickettsia* spp. among dogs in urban and rural areas of the Chapadinha region, state of Maranhão, northeastern Brazil.

Antigen	Dogs		Statistical analysis*		
	Locality	N reactive/N tested (%)	OR	CI	*P* value
B. vogeli	Urban	33/150 (22.0)	2.27	1.18-4.39	0.0119
	Rural	19/172 (11.0)			
	Total	52/322 (16.1)			
E. canis	Urban	19/150 (12.7)	0.75	0.38-1.46	0.4486
	Rural	28/172 (16.3)			
	Total	47/322 (14.6)			
Rickettsia spp.	Urban	4/150 (2.7)	18.09	6.07-60.50	<0.000001
	Rural	57/172 (33.1)			
	Total	61/322 (18.9)			

*The proportions of seroreactive dogs for each antigen were compared between dogs in urban and rural areas by means of the chi-square test; CI: confidence interval; OR: odds ratio; N: number.

Table 3. Results from indirect immunofluorescence assays (IFA) for five *Rickettsia* species among dogs in rural areas of the Chapadinha region, state of Maranhão, northeastern Brazil.

Dog serum	Municipality	Endpoint titers for the following rickettsial antigens:					PAIHR*
		R. rickettsii	*R. parkeri*	*R. amblyommii*	*R. rhipicephali*	*R. bellii*	
53	Chapadinha	-	-	128	-	-	*R. amblyommii*
54	Chapadinha	-	-	128	-	-	*R. amblyommii*
55	Chapadinha	-	-	4096	1024	-	*R. amblyommii*
56	Chapadinha	256	128	4096	1024	-	*R. amblyommii*
62	Chapadinha	-	-	512	64	-	*R. amblyommii*
79	Chapadinha	-	-	128	-	-	*R. amblyommii*
84	Chapadinha	-	-	4096	1024	-	*R. amblyommii*
87	Chapadinha	-	-	256	-	-	*R. amblyommii*
88	Chapadinha	-	-	128	-	-	*R. amblyommii*
202	Mata Roma	-	-	128	-	-	*R. amblyommii*
216	Mata Roma	64	128	512	128	64	*R. amblyommii*
225	Mata Roma	256	256	4096	1024	256	*R. amblyommii*

-: not reactive at a serum dilution of 1:64; *It was determined that the reaction was homologous when the endpoint titer for one *Rickettsia* species was at least four times higher than the titers observed for the other *Rickettsia* species. The *Rickettsia* species involved in the highest endpoint titer was considered to be the possible antigen involved in a homologous reaction (PAIHR).

as *Rhipicephalus sanguineus* sensu lato (866 specimens), *Amblyomma cajennense* sensu lato (39) and *Amblyomma ovale* (24). Further details on tick infestations have been reported elsewhere (Costa et al. 2013). A total of 217 *R. sanguineus* s.l., 16 *A. cajennense* s.l., and 20 *A. ovale* specimens were processed by means of PCR. Only one *A. ovale* specimen was positive for the rickettsial *gltA* gene, which generated a DNA sequence 100% equal to *R. bellii* in GenBank (CP000087).

Discussion

This study showed that 16.1%, 14.6% and 18.9% of the dogs in the Chapadinha region were exposed to infection by *B. vogeli, E. canis* and *Rickettsia* spp., respectively, when analyzed by serologic tests. While these agents had been repeatedly reported infecting dogs in other parts of Brazil at a wide range of prevalence values (TRAPP et al., 2006; LABRUNA et al., 2007; MAIA et al., 2007; PINTER et al., 2008; SAITO et al., 2008; FURUTA et al., 2009; GUIMARÃES et al., 2009; SILVA et al., 2010a, b; VIEIRA et al., 2011), data for the state of Maranhão were very scarce or absent. Recently, *B. vogeli* was reported infecting dogs and *R. sanguineus* ticks in another region of Maranhão (SILVA et al., 2012). Conversely, but there had not been any previous reports of *Ehrlichia* spp or *Rickettsia* spp on dogs in this state. On the other hand, infection by *Ehrlichia* sp. on domestic cats was recently reported in in São Luís Island, Maranhão (Braga et al., 2012).

R. sanguineus ticks are the only known vector of *B. vogeli* and *E. canis* (Dantas-Torres, 2008). Costa et al. (2013) reported that infestations by *R. sanguineus* occurred at similar prevalence rates among urban and rural dogs in the present study. This explains the statistically similar prevalence rates for seroreactivity to *E. canis* among urban and rural dogs. On the other hand, the presence of anti-*Babesia canis* antibodies was likely to be more frequent in urban areas than in rural areas in the present study, even though up to 11% of the rural dogs were seroreactive. The relatively higher *B. vogeli* seroprevalence in urban dogs than in rural dogs could be correlated with possibly higher density of *R. sanguineus* ticks in urban areas, where these ticks would find more suitable areas for development of their nidicolous off-host stages (LABRUNA & PEREIRA, 2001; SZABÓ et al., 2001; PASSOS et al., 2005). This density might have less effect on *E. canis* infection because this agent tends to induce lifelong infection (MCCLURE et al., 2010).

Serological results for *Rickettsia* spp. were much higher among rural dogs than among urban dogs, with higher endpoint titers for *R. amblyommii,* which was considered to be the agent possibly infecting these dogs in rural areas. *R. amblyommii* has been reported infecting several *Amblyomma* species in Brazil, including *A. cajennense* s.l. (LABRUNA et al., 2004b, c; OGRZEWALSKA et al., 2008, 2011). The dogs in the present study were found to be infested by *Amblyomma* ticks only in rural areas (COSTA et al., 2013). Therefore, their serological status was possibly due to their infestation by *Amblyomma* ticks (mainly *A. cajennense* s.l.) in rural areas. Similarly, the statistical association of hunting practices with seroreactivity to *Rickettsia* spp. was possibly associated with higher exposure to *Amblyomma* ticks while hunting, since these ticks are primarily associated with wildlife in Brazil (LABRUNA & PEREIRA, 2001).

No *Rickettsia*-infected ticks were found among the 217 *R. sanguineus* s.l. and 16 *A. cajennense* s.l. specimens collected from the dogs. The absence of rickettsial infection among the *R. sanguineus* ticks was corroborated through serological analysis, since this tick was the only species on the urban dogs, which were almost all seronegative for *Rickettsia* spp. Because canine seroreactivity to *R. amblyommii* was strongly associated with rural areas or hunting practices, it is reasonable to speculate that our limited sample of only 16 *A. cajennense* s.l. ticks was too small, thus precluding findings of *R. amblyommii*-infected ticks, given that *R. amblyommii* had already been reported infecting *A. cajennense* s.l. ticks in Brazil, Costa Rica and Panama (LABRUNA et al., 2004b; BERMÚDEZ et al., 2011; HUN et al., 2011). Conversely, one out of the 26 *A. ovale* ticks was shown to be infected by *R. bellii.* Indeed, *R. bellii* is the most common rickettsial agent infecting *Amblyomma* ticks in Brazil, including *A. ovale* (LABRUNA et al., 2011). None of the dogs of our study presented any serological evidence of *R. bellii* infection. It is possible that *R. bellii* is not infective to dogs, since a previous study also found no serological evidence of rickettsial infection among dogs that had been infested specifically by *R. bellii*-infected *Amblyomma* ticks (PINTER et al., 2008).

Regarding the two distinct habitat types (urban and rural areas) sampled in the state of Maranhão, it was shown that canine seroreactivity to *E. canis* was similar in urban and rural areas, whereas seroreactivity to *B. vogeli* was higher among urban dogs, and seroreactivity to *R. amblyommii* was higher among rural dogs. Interestingly, a very similar scenario was recently reported in the state of Pará (a state neighboring Maranhão), where Spolidorio et al. (2013) found anti-*B. vogeli* antibodies in 59.6% of the urban dogs, and in 29.1% of the rural dogs ($P < 0.05$). For *E. canis*, the seroprevalence was similar among urban dogs (15.7%) and rural dogs (16.6%); for *Rickettsia* spp, rural dogs presented significantly higher ($P < 0.05$) prevalence (40.3%) than urban dogs (21.1%), with highest endpoint titers for *R. amblyommii*. Similarly, in the state of Mato Grosso, in the central-western region of Brazil, Melo et al. (2011) reported similar seroreactivity to *E. canis* among urban dogs (74.3%) and rural dogs (67.5%) ($P > 0.05$), whereas seroreactivity to *Rickettsia* spp. was significantly higher among rural dogs (75.6%) than urban dogs (19.3%) ($P < 0.05$), also with highest endpoint titers for *R. amblyommii*. Indeed, this common scenario reported for dogs in three different Brazilian states is primarily related to the tick species found on the dogs, with predominance of *R. sanguineus* in urban areas and presence of *Amblyomma* species in rural areas. This scenario should be considered to be the epidemiological background for controlling and preventing canine tick-borne diseases in these neglected regions of Brazil.

Acknowledgements

We acknowledge the Coordination Office for Advancement of Higher-Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) for a scholarship and the Research and Scientific and Technological Development Support Foundation of Maranhão (Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão,

FAPEMA) for financial support. The cooperation of the dog owners in offering their valuable time and their animals for sampling is highly appreciated.

References

Aguiar DM, Cavalcante GT, Pinter A, Gennari SM, Camargo LM, Labruna MB. Prevalence of *Ehrlichia canis* (Rickettsiales: Anaplasmataceae) in dogs and *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks from Brazil. *J Med Entomol* 2007a; 44(1): 126-132. http://dx.doi.org/10.1093/jmedent/41.5.126. PMid:17294930

Aguiar DM, Saito TB, Hagiwara MK, Machado RZ, Labruna MB. Diagnóstico sorológico de erliquiose canina com antígeno brasileiro de *Ehrlichia canis. Cienc Rural* 2007b; 37(3): 796-802. http://dx.doi.org/10.1590/S0103-84782007000300030.

Aguiar DM, Hagiwara MK, Labruna MB. *In vitro* isolation and molecular characterization of an *Ehrlichia canis* strain from São Paulo, Brazil. *Braz J Microbiol* 2008; 39(3): 489-493. http://dx.doi.org/10.1590/S1517-83822008000300014. PMid:24031251

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215(3): 403-410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2. PMid:2231712

Baba K, Itamoto K, Amimoto A, Kitagawa K, Hiraoka H, Mizuno T, et al. *Ehrlichia canis* infection in two dogs that emigrated from endemic areas. *J Vet Med Sci* 2012; 74(6): 775-778. http://dx.doi.org/10.1292/jvms.11-0401. PMid:22214859

Beck R, Vojta L, Mrljak V, Marinculić A, Beck A, Zivicnjak T, et al. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int J Parasitol* 2009; 39(7): 843-848. http://dx.doi.org/10.1016/j.ijpara.2008.12.005. PMid:19367832

Bermúdez CS, Zaldívar AY, Spolidorio MG, Moraes-Filho J, Miranda RJ, Caballero CM, et al. Rickettsial infection in domestic mammals and their ectoparasites in El Valle de Antón, Coclé, Panamá. *Vet Parasitol* 2011; 177(1-2): 134-138. http://dx.doi.org/10.1016/j.vetpar.2010.11.020. PMid:21144663

Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *J Clin Microbiol* 2003; 41(9): 4172-4177. http://dx.doi.org/10.1128/JCM.41.9.4172-4177.2003. PMid:12958243

Braga MS, André MR, Freschi CR, Teixeira MCA, Machado RZ. Molecular and serological detection of *Ehrlichia* spp. in cats on São Luís Island, Maranhão, Brazil. *Rev Bras Parasitol Vet* 2012; 21(1): 37-41. http://dx.doi.org/10.1590/S1984-29612012000100008. PMid:22534943

Burgdorfer W. Ecological and epidemiological considerations of Rocky Mountain spotted fever and scrub typhus. In: Walker DH. *Biological of Rickettsial Diseases*. Boca Raton: CRC Press; 1988. p. 33-50. vol. II.

Costa AP, Silva AB, Costa FB, Xavier GS, Martins TF, Labruna MB, et al. A survey of ectoparasites infesting urban and rural dogs of Maranhão state, Brazil. *J Med Entomol* 2013; 50(3): 674-678. http://dx.doi.org/10.1603/ME12243. PMid:23802466

Costa-Júnior LM, Ribeiro MFB, Rembeck K, Rabelo EML, Zahler-Rinder M, Hirzmann J, et al. Canine babesiosis caused by *Babesia canis vogeli* in rural areas of the State of Minas Gerais, Brazil and factors associated with its seroprevalence. *Res Vet Sci* 2009; 86(2): 257-260. http://dx.doi.org/10.1016/j.rvsc.2008.07.002. PMid:18723199

Criado-Fornelio A, Martinez-Marcos A, Buling-Saraña A, Barba-Carretero JC. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe: Part I. Epizootiological aspects. *Vet Parasitol* 2003; 113(3-4): 189-201. http://dx.doi.org/10.1016/S0304-4017(03)00078-5. PMid:12719133

Dantas-Torres F. Canine vector-borne diseases in Brazil. *Parasit Vectors* 2008; 1(1): 25. http://dx.doi.org/10.1186/1756-3305-1-25. PMid:18691408

Dawson JE, Biggie KL, Warner CK, Cookson K, Jenkins S, Levine JF, et al. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. *Am J Vet Res* 1996; 57(8): 1175-1179. PMid:8836370.

Duarte SC, Linhares GFC, Romanowsky TN, Silveira OJ No, Borges LMF. Assessment of primers designed for the subspecies-specific discrimination among *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* by PCR assay. *Vet Parasitol* 2008; 152(1-2): 16-20. http://dx.doi.org/10.1016/j.vetpar.2007.12.013. PMid:18242863

Furuta PI, Oliveira TMFS, Teixeira MC, Rocha AG, Machado RZ, Tinucci-Costa MG. Comparison between a soluble antigen-based ELISA and IFAT in detecting antibodies against *Babesia canis* in dogs. *Rev Bras Parasitol Vet* 2009; 18(3): 41-45. http://dx.doi.org/10.4322/rbpv.01803007. PMid:19772774

Guimarães AM, Rocha CMBM, Oliveira TMFS, Rosado IR, Morais LG, Santos RRD. Fatores associados à soropositividade para *Babesia*, *Toxoplasma*, *Neospora* e *Leishmania* em cães atendidos em nove clínicas veterinárias do município de Lavras, MG. *Rev Bras Parasitol Vet* 2009;18(Suppl 1): 49-53. http://dx.doi.org/10.4322/rbpv.018e1009. PMid:20040191

Hii SF, Kopp SR, Thompson MF, O'Leary CA, Rees RL, Traub RJ. Canine vector-borne disease pathogens in dogs from south-east Queensland and north-east Northern Territory. *Aust Vet J* 2012; 90(4): 130-135. http://dx.doi.org/10.1111/j.1751-0813.2012.00898.x. PMid:22443327

Hun L, Troyo A, Taylor L, Barbieri AM, Labruna MB. First report of the isolation and molecular characterization of *Rickettsia amblyommii* and *Rickettsia felis* in Central America. *Vector Borne Zoonotic Dis* 2011; 11(10): 1395-1397. http://dx.doi.org/10.1089/vbz.2011.0641. PMid:21612539

Inokuma H, Oyamada M, Davoust B, Boni M, Dereure J, Bucheton B, et al. Epidemiological survey of *Ehrlichia canis* and related species infection in dogs in eastern Sudan. *Ann N Y Acad Sci* 2006; 1078(1): 461-463. http://dx.doi.org/10.1196/annals.1374.085. PMid:17114754

Instituto Interamericano de Cooperacion para la Agricultura – IICA. *Tecnicas para El diagnostico de babesiosis y anaplasmosis bovinas*. San José; 1987.

Irwin PJ. The first report of canine ehrlichiosis in Australia. *Aust Vet J* 2001; 79(8): 552-553. http://dx.doi.org/10.1111/j.1751-0813.2001.tb10746.x. PMid:11599816

Labruna MB, Pereira MC. Carrapato em cães no Brasil. *Clin Vet (Milano)* 2001; 30: 24-31.

Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, et al. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. *J Clin Microbiol* 2004a; 42(1): 90-98. http://dx.doi.org/10.1128/JCM.42.1.90-98.2004. PMid:14715737

Labruna MB, Whitworth T, Bouyer DH, McBride J, Camargo LMA, Camargo EP, et al. *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma* ticks from the state of Rondônia, western Amazon, Brazil. *J Med Entomol* 2004b; 41(6): 1073-1081. http://dx.doi.org/10.1603/0022-2585-41.6.1073. PMid:15605647

Labruna MB, McBride JW, Bouyer DH, Camargo LMA, Camargo EP, Walker DH. Molecular evidence for a spotted fever group *Rickettsia* species

in the tick *Amblyomma longirostre* in Brazil. *J Med Entomol* 2004c; 41(3): 533-537. http://dx.doi.org/10.1603/0022-2585-41.3.533. PMid:15185961

Labruna MB, Horta MC, Aguiar DM, Cavalcante GT, Pinter A, Gennari SM, et al. Prevalence of *Rickettsia* infection in dogs from the urban and rural areas of Monte Negro municipality, western Amazon, Brazil. *Vector Borne Zoonotic Dis* 2007; 7(2): 249-255. http://dx.doi.org/10.1089/vbz.2006.0621. PMid:17627445

Labruna MB. Ecology of *Rickettsia* in South America. *Ann N Y Acad Sci* 2009; 1166(1): 156-166. http://dx.doi.org/10.1111/j.1749-6632.2009.04516.x. PMid:19538276

Labruna MB, Mattar S, Nava S, Bermudez S, Venzal JM, Dolz G, et al. Rickettsioses in Latin America, Caribbean, Spain and Portugal. *Rev MVZ Córdoba* 2011; 16(2): 2435-2457.

Lewis BD, Penzhorn BL, Lopez-Rebollar LM, De Waal DT. Isolation of a South African vector-specific strain of *Babesia canis*. *Vet Parasitol* 1996; 63(1-2): 9-16. http://dx.doi.org/10.1016/0304-4017(95)00881-0. PMid:8792576

López J, Abarca K, Mundaca MI, Caballero C, Valiente-Echeverría F. Identificación molecular de *Ehrlichia canis* en un canino de la ciudad de Arica, Chile. *Rev Chilena Infectol* 2012; 29(5): 527-530. http://dx.doi.org/10.4067/S0716-10182012000600008. PMid:23282495

Maia MG, Costa RT, Haddad JP, Passos LMF, Ribeiro MFB. Epidemiological aspects of canine babesiosis in the semiarid area of the state of Minas Gerais, Brazil. *Prev Vet Med* 2007; 79(2-4): 155-162. http://dx.doi.org/10.1016/j.prevetmed.2006.11.013. PMid:17210194

Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol* 2008; 155(1-2): 152-157. http://dx.doi.org/10.1016/j.vetpar.2008.04.012. PMid:18502588

McClure JC, Crothers ML, Schaefer JJ, Stanley PD, Needham GR, Ewing SA, et al. Efficacy of a doxycycline treatment regimen initiated during three different phases of experimental ehrlichiosis. *Antimicrob Agents Chemother* 2010; 54(12): 5012-5020. http://dx.doi.org/10.1128/AAC.01622-09. PMid:20921310

Melo AL, Martins TF, Horta MC, Moraes-Filho J, Pacheco RC, Labruna MB, et al. Seroprevalence and risk factors to *Ehrlichia* spp. and *Rickettsia* spp. in dogs from the Pantanal Region of Mato Grosso State, Brazil. *Ticks Tick Borne Dis* 2011; 2(4): 213-218. http://dx.doi.org/10.1016/j.ttbdis.2011.09.007. PMid:22108015

Murphy GL, Ewing SA, Whitworth LC, Fox JC, Kocan AA. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. *Vet Parasitol* 1998; 79(4): 325-339. http://dx.doi.org/10.1016/S0304-4017(98)00179-4. PMid:9831955

O'Dwyer LH, Lopes VV, Rubini AS, Paduan KS, Ribolla PE. Babesia spp. infection in dogs from rural areas of São Paulo State, Brazil. *Rev Bras Parasitol Vet* 2009; 18(2): 23-26. http://dx.doi.org/10.4322/rbpv.01802005. PMid:19602312

Ogrzewalska M, Pacheco RC, Uezu A, Ferreira F, Labruna MB. Ticks (Acari: Ixodidae) infesting wild birds in an Atlantic forest area in the state of São Paulo, Brazil, with isolation of *Rickettsia* from the tick *Amblyomma longirostre*. *J Med Entomol* 2008; 45(4): 770-774. PMid:18714882.

Ogrzewalska M, Uezu A, Labruna MB. Ticks (Acari: Ixodidae) infesting wild birds in the Atlantic Forest in northeastern Brazil, with notes on rickettsial infection in ticks. *Parasitol Res* 2011; 108(3): 665-670. http://dx.doi.org/10.1007/s00436-010-2111-8. PMid:20953629

Oyamada M, Davoust B, Boni M, Dereure J, Bucheton B, Hammad A, et al. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. *Clin Diagn Lab Immunol* 2005; 12(11): 1343-1346. PMid:16275954.

Passos LMF, Geiger SM, Ribeiro MFB, Pfister K, Zahler-Rinder M. First molecular detection of *Babesia vogeli* in dogs from Brazil. *Vet Parasitol* 2005; 127(1): 81-85. http://dx.doi.org/10.1016/j.vetpar.2004.07.028. PMid:15619377

Pinter A, Horta MC, Pacheco RC, Moraes-Filho J, Labruna MB. Serosurvey of *Rickettsia* spp. in dogs and humans from an endemic area for Brazilian spotted fever in the State of São Paulo, Brazil. *Cad Saude Publica* 2008; 24(2): 247-252. http://dx.doi.org/10.1590/S0102-311X2008000200003. PMid:18278271

Piranda EM, Faccini JL, Pinter A, Saito TB, Pacheco RC, Hagiwara MK, et al. Experimental infection of dogs with a Brazilian strain of *Rickettsia rickettsii*: clinical and laboratory findings. *Mem Inst Oswaldo Cruz* 2008; 103(7): 696-701. http://dx.doi.org/10.1590/S0074-02762008000700012. PMid:19057821

Ramos R, Ramos C, Araújo F, Oliveira R, Souza I, Pimentel D, et al. Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil). *Parasitol Res* 2010; 107(5): 1115-1120. http://dx.doi.org/10.1007/s00436-010-1979-7. PMid:20680344

Rodriguez-Vivas RI, Albornoz RE, Bolio GM. *Ehrlichia canis* in dogs in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors. *Vet Parasitol* 2005; 127(1): 75-79. http://dx.doi.org/10.1016/j.vetpar.2004.08.022. PMid:15619376

Saito TB, Cunha-Filho NA, Pacheco RC, Ferreira F, Pappen FG, Farias NA, et al. Canine infection by rickettsiae and ehrlichiae in southern Brazil. *Am J Trop Med Hyg* 2008; 79(1): 102-108. PMid:18606772.

Sangioni LA, Horta MC, Vianna MC, Gennari SM, Soares RM, Galvão MA, et al. Rickettsial infection in animals and Brazilian spotted fever endemicity. *Emerg Infect Dis* 2005; 11(2): 265-270. http://dx.doi.org/10.3201/eid1102.040656. PMid:15752445

Selbach JF, Leite JRSA. *Meio ambiente no Baixo Parnaíba: olhos no mundo, pés na região*. São Luis: EDUFMA; 2008.

Shaw SE, Day MJ, Birtles RJ, Breitschwerdt EB. Tick-borne infectious diseases of dogs. *Trends Parasitol* 2001; 17(2): 74-80. http://dx.doi.org/10.1016/S1471-4922(00)01856-0. PMid:11228013

Silva AB, Costa AP, Sá JC, Costa FB, Santos ACG, Guerra RMSNC. Detecção molecular de *Babesia canis vogeli* em cães e em *Rhipicephalus sanguineus* na mesorregião do oeste maranhense, nordeste brasileiro. *Cienc Anim Bras* 2012; 13(3): 388-395.

Silva JN, Almeida AB, Boa Sorte EC, Freitas AG, Santos LGF, Aguiar DM, et al. Soroprevalência de anticorpos anti-*Ehrlichia canis* em cães de Cuiabá, Mato Grosso. *Rev Bras Parasitol Vet* 2010a; 19(2): 108-111. http://dx.doi.org/10.4322/rbpv.01902008. PMid:20624348

Silva ME, Ribeiro RR, Costa JO, Moraes-Filho J, Pacheco RC, Labruna MB. Prevalência de anticorpos anti-*Rickettsia* spp. em cães da cidade de Belo Horizonte, MG. *Arq Bras Med Vet Zootec* 2010b; 62(4): 1007-1010. http://dx.doi.org/10.1590/S0102-09352010000400036.

Singla LD, Singh H, Kaur P, Singh ND, Singh NK, Juyal PD. Serodetection of *Ehrlichia canis* infection in dogs from Ludhiana district of Punjab, India. *J Parasit Dis* 2011; 35(2): 195-198. http://dx.doi.org/10.1007/s12639-011-0055-2. PMid:23024503

Spolidorio MG, Minervino AH, Valadas SY, Soares HS, Neves KA, Labruna MB, et al. Serosurvey for tick-borne diseases in dogs from the Eastern Amazon, Brazil. *Rev Bras Parasitol Vet* 2013; 22(2): 214-219. http://dx.doi.org/10.1590/S1984-29612013005000023. PMid:23802236

Szabó MP, Cunha TM, Pinter A, Vicentini F. Ticks (Acari: Ixodidae) associated with domestic dogs in Franca region, São Paulo, Brazil. *Exp Appl Acarol* 2001; 25(10-11): 909-916. http://dx.doi.org/10.1023/A:1020433003028. PMid:12455880

Szabó MP, de Souza LG, Olegário MM, Ferreira FA, Pajuaba AA No. Ticks (Acari: Ixodidae) on dogs from Uberlândia, Minas Gerais, Brazil. *Transbound Emerg Dis* 2010; 57(1-2): 72-74. http://dx.doi.org/10.1111/j.1865-1682.2010.01111.x. PMid:20537111

Trapp SM, Dagnone AS, Vidotto O, Freire RL, Amude AM, Morais HS. Seroepidemiology of canine babesiosis and ehrlichiosis in a hospital population. *Vet Parasitol* 2006; 140(3-4): 223-230. http://dx.doi.org/10.1016/j.vetpar.2006.03.030. PMid:16647817

Uilenberg G, Franssen FF, Perié NM, Spanjer AA. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. *Vet Q* 1989; 11(1): 33-40. http://dx.doi.org/10.1080/01652176.1989.9694194. PMid:2655263

Unver A, Perez M, Orellana N, Huang H, Rikihisa Y. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. *J Clin Microbiol* 2001; 39(8): 2788-2793. http://dx.doi.org/10.1128/JCM.39.8.2788-2793.2001. PMid:11473993

Vieira RF, Biondo AW, Guimarães AM, Santos AP, Santos RP, Dutra LH, et al. Ehrlichiosis in Brazil. *Rev Bras Parasitol Vet* 2011; 20(1): 1-12. http://dx.doi.org/10.1590/S1984-29612011000100002. PMid:21439224

Vinasco J, Li O, Alvarado A, Diaz D, Hoyos L, Tabachi L, et al. Molecular evidence of a new strain of *Ehrlichia canis* from South America. *J Clin Microbiol* 2007; 45(8): 2716-2719. http://dx.doi.org/10.1128/JCM.01102-07. PMid:17596368

Yu XJ, Walker DH. The Order Rickettsiales. In: Dworkin M. *The Prokaryotes: an evolving electronic resource for the microbiology community*. 3rd ed. New York: Springer-Velag; 2003. [cited 2013 Jan 20]. Available from: http://link.springer.ny.com/link/service/books/10125.