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Antibody occurrence of Anti-*Toxoplasma gondii*, *Leishmania* sp. and *Ehrlichia canis* in dogs in Bahia State

Ocorrência de anticorpos Anti-*Toxoplasma gondii, Leishmania spp*. e *Ehrlichia canis* em cães do Estado da Bahia

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Abstract

Toxoplasmosis, leishmaniasis, and ehrlichiosis are zoonoses that have cosmopolitan distribution and cause important clinical changes in dogs and humans. Dogs are important in the epidemiology and transmission of *Leishmania infantum* and *Ehrlichia canis* and serve as indicators of environmental contamination by the transmission of *Toxoplasma gondii*. The aim of this study was to detect antibodies against *T. gondii*, *Leishmania* spp., and *E. canis* in dogs from Bahia and identify the risk factors associated with infections. Blood samples were collected from 353 dogs from several municipalities of Bahia and the sera were tested using reaction of indirect immunofluorescence (RIFI) for *T. gondii* and *Leishmania* spp. and enzyme-linked immunosorbent assay (ELISA) for *E. canis*. The data were tabulated and analyzed using EPI-INFO 7.1.5 program. Of 353 samples analyzed, 144 (40.8%) were positive for *T. gondii* (antibody titer 16-4,096) and 92 (26.1%) were seropositive for *Leishmania* spp. (antibody titer 40-160). The seropositivity for *E. canis* was the highest as compared to that of the other agents. Furthermore, 184 (52.1%) samples were positive for antibodies against *E. canis*. Sixteen (4.5%), 71 (20.1%), and 25 (7.1%) samples were co-infected with *T. gondii* and *Leishmania* spp., *T. gondii* and *E. canis*, and *E. canis* and *Leishmania* spp., respectively, while 20 (5.7%) dogs presented co-seropositivity for all three etiological agents. Risk factors such as age, sex, habitat (stray/not stray), and region of dog's origin were analyzed; age (≥ 1 year) was significantly associated with *T. gondii* ($p = 0.03$) and *E. canis* ($p = 0.02$) infection and stray animals were more exposed to *T. gondii* ($p = 0.03$) and *Leishmania* spp. ($p = 0.01$) infection. The results confirmed the heterogeneous distribution of the three etiological agents in dogs from Bahia and identified animals with co-infections.
Key words: Ehrlichiosis. Risk factor. *Leishmania* infection. Toxoplasmosis. Zoonosis.

Resumo

A toxoplasmose, a leishmaniose e a erliquiose são zoonoses que possuem distribuição cosmopolita e causam importantes alterações clínicas em cães e em seres humanos. Cães são importantes na epidemiologia e transmissão de *Leishmania infantum* e *Ehrlichia canis*, e podem ser indicadores de contaminação ambiental na transmissão de *T. gondii.* Objetivou-se com esse estudo detectar anticorpos IgG anti-*Toxoplasma gondii***,** *Leishmania* spp. e *Ehrlichia canis* em cães do estado da Bahia, bem como identificar fatores de risco associados as infecções. Amostras de sangue de 353 cães foram coletadas em diversos municípios deste estado e os soros foram submetidos à reação de imunofluorescência

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indireta (RIFI) para *T. gondii* e *Leishmania* spp. e ensaio imunoenzimático indireto (ELISA) para *E. canis*. Os dados foram tabulados e analisados utilizando-se o programa EPIINFO 7.1.5. Para o agente etiológico *T. gondii,* das 353 amostras coletadas, 144 (40,8%) foram positivas, com títulos de anticorpos variando de 16 a 4096. Para *Leishmania* spp., 92 (26,1%) amostras mostraram soropositividade, com títulos de anticorpos variando de 40 a 160. A soropositividade para *E. canis* foi a maior em relação aos outros agentes. Dos animais amostrados, 184 (52,1%) tiveram anticorpos anti-*E. canis*, no ELISA. Das 353 amostras analisadas, verificou-se co-soropositividade em 16 (4,5%) animais para *T. gondii* e *Leishmania* spp.; 71 (20,1%) animais foram positivos para *T. gondii* e *E. canis*; 25 (7,1%) para *E. canis e Leishmania* spp.; e 20 (5,7%) cães apresentaram co-soropositividade para os três agentes etiológicos. Foram analisados como fatores de risco a idade, o sexo, se o animal era errante ou não, e a mesorregião de origem dos cães. Dentre os fatores de risco analisados, a idade (≥ 1ano) foi significativa para *T. gondii* (p = 0,03) e *E. canis* (p = 0,02) e animais errantes se apresentaram mais expostos para *T. gondii* (p = 0,03) e *Leishmania* spp. (p = 0,01). Os resultados comprovam a presença dos três agentes etiológicos nos municípios estudados no estado da Bahia, distribuídos de maneira heterogênea e apresentando animais com coinfecções.
Palavras-chave: Erliquiose. Fatores de risco. Leishmaniose. Toxoplasmose. Zoonoses.

Introduction

Toxoplasmosis, leishmaniasis, and ehrlichiosis are important infectious diseases with cosmopolitan distribution. These zoonoses affect the health of dogs (DUBEY; LAPPIN, 2006; PEREZ et al., 2006). Dogs may serve as a reservoir, as observed in *Leishmania* and *Ehrlichia canis* infections, and spread the disease to other animals and humans (COELHO et al., 2013; GIRARDI et al., 2014). For toxoplasmosis, the dog species is important as an indicator of environmental contamination (SALB et al., 2008).

Canine ehrlichiosis is endemic in virtually all regions of Brazil and transmitted to dogs by the tick *Rhipicephalus sanguineus*. During the acute phase, animals present symptoms such as depression, weight loss, anorexia, fever, splenomegaly, lymphadenopathy, vasculitis, polyarthritis, and neurological, muscular, and ocular disorders. After the acute phase, the animal may be healed or enter the subclinical phase. Some animals progress to the chronic phase, which might vary from mild to severe, after the subclinical phase. The chronic phase is usually characterized with complications related to the infection and exacerbation of acute symptoms such as mucous and conjunctival bleeding (DAGNONE et al., 2001; HARRUS; WANER, 2010). The expansion in the population of *R. sanguineus* during recent decades has led to an increase in the occurrence of ehrlichiosis in dogs and emergence of human cases (LABRUNA; PEREIRA, 2001). The tick *R. sanguineus* was described as a human parasite in Uruguay, Venezuela, and Brazil (VENZAL et al., 2003; PEREZ et al., 2006; DANTAS-TORRES et al., 2006). According to Trapp et al. (2006), owners of dogs infested by *R. sanguineus* have 3.2-times higher risk of being parasitized by this tick than owners of dogs not infested by parasites.

Canine visceral leishmaniasis (LVC) is considered as an endemic disease in rural areas of Brazil. In urban environment, domestic dog (*Canis lupus familiaris)* is the main reservoir of *Leishmania infantum* (sin. *L. chagasi*) and may spread the infection in humans. Even asymptomatic dogs are sources of infection for phebotomines and have an active role in the spread of disease (MOREIRA et al., 2007; COELHO et al., 2013). The commonly observed clinical changes in dogs include lymphadenopathy, skin changes, hepatomegaly, splenomegaly, emaciation, and onychogryphosis; however, there is a large percentage of asymptomatic or oligosymptomatic animals (MOREIRA et al., 2007).

The seropositivity to *Toxoplasma gondii* is relatively high in dogs in Brazil, which may

be associated with the consumption of raw or undercooked meat containing tissue cysts or contraction of infection from sporulated oocysts; hence, dog may serve as a sentinel for environmental contamination (DUBEY et al., 2007; DUBEY; JONES, 2008; SALB et al., 2008; CARLOS et al., 2010). Furthermore, dogs may act as mechanical vectors, owing to xenosmophilia or consumption of dog meat by humans (FRENKEL; PARKER, 1996; ETHEREDGE et al., 2004; SALB et al., 2008). Infection by *T. gondii* in dogs is most often asymptomatic and may result in neurological changes in immunosuppressed animals (MORETTI et al., 2002; SILVA et al., 2009). Ferreira et al. (2016) observed higher frequency of neurological signs ($p < 0.0001$) such as seizures, ataxia, paresis, and plegias as compared with ophthalmic, reproductive, and respiratory signs.

It is important to study the severity and spread of diseases caused by *T. gondii*, *Leishmania* spp., and *E. canis*. Evaluation of the endemic character of these agents in dogs from various regions of Brazil and studies involving these three parasites in Bahia may contribute to the greater knowledge of these zoonoses. Here, we verified the occurrence of antibodies against *T. gondii*, *Leishmania* spp., and *E. canis* in dog population from municipalities of Bahia and identified the possible associated risk factors.

Material and Methods

Place and collection of the biological material

The study was carried out in municipalities of the state of Bahia and samples were collected at the Control Center of Zoonoses (CCZ), non-governmental organizations (NGOs) for the protection of animals, and some homes where dogs had access to the street (semi-domestic). The municipalities visited were Dias D'ávila (CCZ), Itabuna (CCZ), Ilhéus (CCZ), Salvador (semi-domestic), Feira de Santana (ONG), Jequié (semi-domestic), Eunápolis (CCZ), Una (domestic), and Brumado (ONG).

The state of Bahia, located in the northeast region of Brazil, includes 14,016,906 inhabitants in an area of 564,733,177 km^2 and 417 municipalities and is divided into seven mesoregions (IBGE, 2010) (Figure 1).

Blood samples were collected from 353 dogs from November 2014 to November 2015. Of these, 18, 81, 27, 37, 84, 70, 25, 17, and 14 samples were from Dias D'ávila, Itabuna, Ilhéus, Salvador, Feira de Santana, Jequié, Eunápolis, Una, and Brumado, respectively. After collection, the samples were packed in styrofoam with gelox and centrifuged to separate serum into a specific 2 mL tube, which was stored at −20°C until use. The sample size was calculated by EPI-INFO 7.1.5 program, considering an expected prevalence of 50% at 5% error and 95% confidence level.

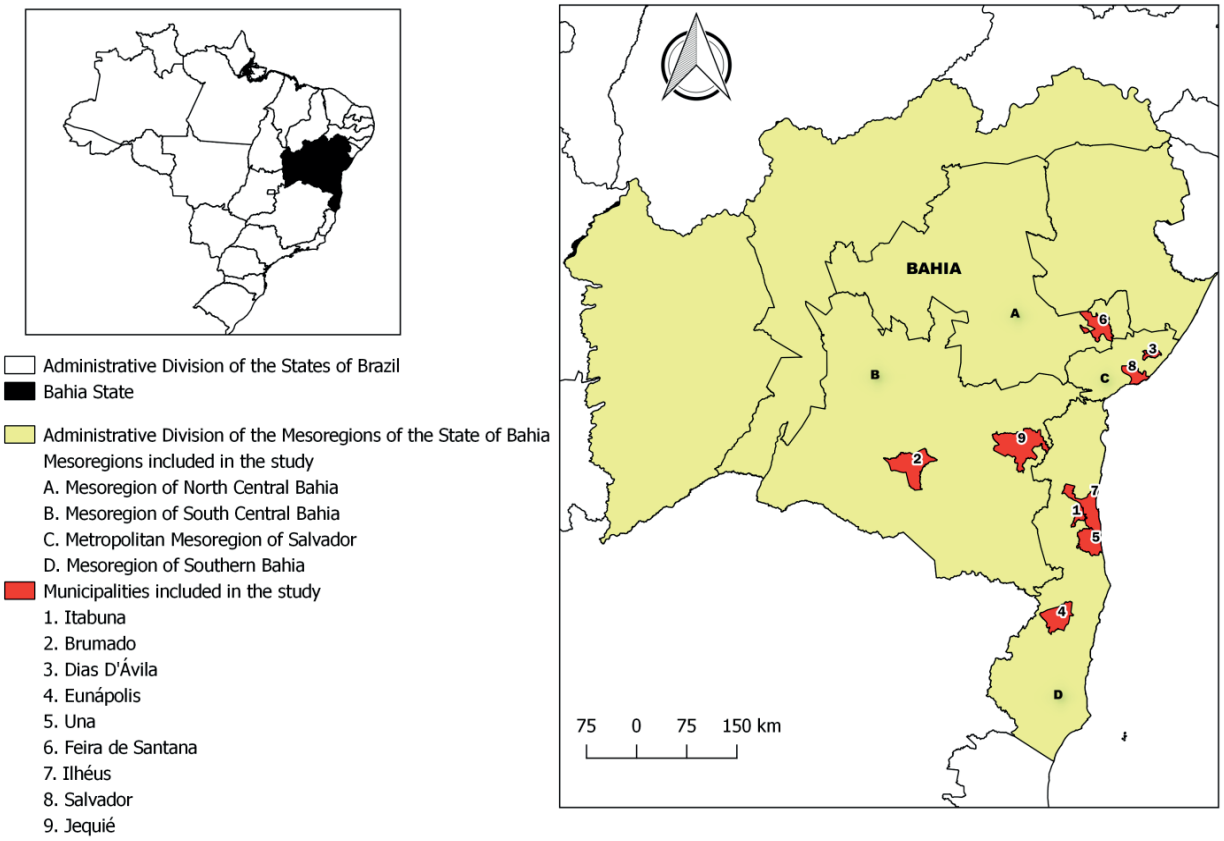
The animals were heterogeneous in age, sex, and breed. The blood was collected by venipuncture (cephalic or jugular). The serum was separated by centrifugation and stored at −20°C until the completion serological examinations. The study was approved by the Ethics Committee on Animal Use (CEUA-UESC 018/2012).

Serological examination

Serology for T. gondii

The serological test for the detection of antibodies against *T. gondii* was performed using Indirect fluorescence antibody test (IFAT) at a cutting point of 1:16. The test was performed using a conjugated canine anti-IgG (anti-dog IgG – F4262, Sigma-Aldrich) at 1:128 dilution and the blades were observed under a microscope with an epifluorescence system. Blades were produced in the Laboratory of Parasitology of the Universidade Estadual de Santa Cruz – BA using antigens from *T. gondii* RH strain. Positive and negative controls were added in all blades.

Figure 1. Map of the state of Bahia; mesoregions of Bahia showing the cities from where samples were collected**.**



Serology for Leishmania spp.

The detection of antibodies against *L. infantum* was performed using IFAT at a cutting point of 1:40. The test was performed with a conjugated canine anti-IgG (anti-dog IgG – F4262, Sigma-Aldrich) at 1:128 dilution. The blades were observed under a microscope with an epifluorescence system. The blades were acquired from Laboratório Imunodot, São Paulo, Brazil. Positive and negative controls derived from the Biomanguinhos Laboratory, Rio de Janeiro, Brazil were added on all blades.

Serology for E. canis

The detection of antibodies against *E. canis* was performed using enzyme-linked immunosorbent assay (ELISA) IMUNOTEST® test for *Ehrlichia canis* of the Laboratório Imunodot, São Paulo, Brazil, as per the manufacturer's instructions. The reading of the reaction was recorded using an ELISA microplate reader (Microplate Reader MRX TC Plus, Dynex Technology) at a wavelength of 405 nm.

Statistical analysis

To identify the risk factors associated with infections, a bivariate analysis was conducted with chi-square and Fisher's exact tests using the statistical program EPI-INFO release 7.1.5. A multivariate logistic regression analysis was performed on the variables obtained from the bivariate analysis with

$p < 0.2$ using EPI-INFO release 7.1.5. Factors analyzed included age, sex, habitat (stray/not stray), and mesoregion of the dog's origin.

Results and Discussion

Of 353 dogs analyzed, 144 (40.8%) were positive for antibodies against *T. gondii*, with their titers ranging from 16 to 4096 (Table 1). In addition, 92 (26.1%) were detected positive for antibodies against *L. infantum* (titer of 40 to 160; Table 2) and 184 (52.1%), for antibodies against *E. canis*. Furthermore, 20 animals were positive for co-infection with the three infectious agents (Figure 2).

All mesoregions analyzed presented animals positive for the three agents at different levels of seropositivity. The mid-center mesoregion of Bahia demonstrated higher seropositivity for *T. gondii* and *Leishmania* spp., while the center-south mesoregion showed the highest positivity for *E. canis* (Table 3); however, no statistical difference was observed between the mesoregions for the three agents. The center-south mesoregion of Bahia showed the highest titer for *Leishmania* spp. (160).

Risk factors such as age, sex, habitat (stray or not stray), and mesoregion of origin were analyzed with bivariate and multivariate analyses (Tables 4 and 5). Among the factors analyzed, an association was observed between age and seropositivity for *T. gondii* and *E. canis* in the multivariate analysis. In addition, an association was observed between stray dogs and infection by *Leishmania* spp. and *T. gondii*. No significant association was reported between the agents studied and sex of animals (Table 4).

Table 1. Titration of positive sera to detect antibodies against *Toxoplasma gondii* by indirect immunofluorescence reaction in dogs from the municipalities of Bahia in 2015.

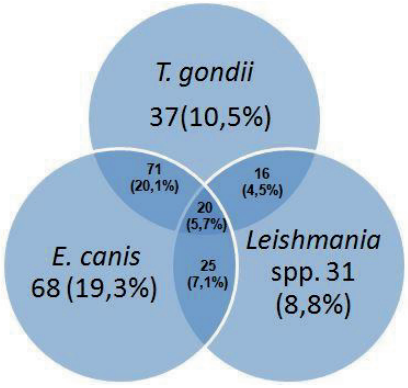
Titration	Number of animals	%
16	3	2,46
32	12	6,56
64	36	25,41
128	27	18,85
256	36	27,05
512	3	2,46
1024	11	5,74
2048	6	4,92
4096	10	6,56
Total	144	100%

In Brazil, the occurrence of dogs seropositive for *T. gondii* varies significantly from 3.1% to 91% (CHAPLIN et al., 1984; GERMANO et al., 1985; AZEVEDO et al., 2005; CARLOS et al., 2010; ZULPO et al., 2012; COELHO et al., 2013). These differences observed may be related to regional geoclimatic characteristics, epidemiological factors (presence of infected cats, age, care for animals, cultural aspects, habitat, and health practices), and sampling methods (LANGONI et al., 2006; CARLOS et al., 2010).

Table 2. Titration of sera (number and percentage) for the detection of antibodies against *Leishmania* spp. by indirect immunofluorescence reaction in dogs from the municipalities of Bahia in 2015.

Titration	Number of animals	%
40	31	33,60
80	55	57,60
160	8	8,80
Total	92	100

Figure 2. Positivity and co-positivity of dogs sampled for the antigens of *Toxoplasma gondii, Leishmania* spp., and *Ehrlichia canis*.



In this study, the predominance of dogs with *T. gondii* was 40.8%. This result was similar to that obtained by Carlos et al. (2010) but lower than that reported by Barbosa et al. (2003). The presence of *T. gondii* infection in dogs indicates an environment contaminated by oocysts or meat contaminated with parasite cysts. These animals living together with humans and feeding on the same meat may serve as sentinels for human infection (DUBEY et al., 2007; SALB et al., 2008; DUBEY; JONES, 2008).

Table 3. Detection of antibodies against *Toxoplasma gondii*, *Leishmania* spp., and *Ehrlichia canis* in dogs and their distribution in Bahia, Brazil, 2015.

Meso-region / Municipality	*Toxoplasma gondii*		*Leishmania* spp.		*Ehrlichia canis*	
	+	%	+	%	+	%
Southern Bahia	58	38,70	39	26	66	44
Itabuna	30	37,00	19	23,40	41	50,60
Ilhéus	8	30,00	6	22,20	8	29,60
Una	10	58,80	2	11,80	9	52,90
Eunápolis	10	40,00	12	48,00	8	32,00
North-Central Bahia	28	43,75	22	34,40	36	56,20
Feira de Santana	28	43,75	22	34,40	36	56,20
South-Central Bahia	35	41,70	23	27,30	51	60,70
Jequié	35	41,70	17	24,30	44	62,90
Brumado	0	0,00	6	42,85	7	50,00
Metropolitan of Salvador	23	41,80	8	14,55	31	56,40
Salvador	12	32,40	2	5,50	16	43,20
Dias D'Ávila	11	61,10	6	33,30	15	83,30

Table 4. Bivariate analysis of factors associated with *Toxoplasma gondii*, *Leishmania* spp., and *Ehrlichia canis* infections in dogs from Bahia, Brazil, 2015.

Variable	Animals		X^2	P value	OR	95%CI
	Positive	Negative				
Toxoplasma gondii						
Age			2,42	0,11	2,46	0,88 – 6,83
≥ 01 year	139	192				
< 01 year	5	17				
Sex			0,25	0,61	1,14	0,74 – 1,74
Male	73	99				
Female	71	110				
Wandering			2,61	0,10	0,68	0,44 – 1,05
Yes	77	131				
No	67	77				
***Leishmania* spp.**						
Age			4,5	0,03	7,96	1,05 – 60,05
≥ 01 year	91	240				
< 01 year	1	21				
Sex			0,006	0,93	1,01	0,62 – 1,62
Male	45	127				
Female	47	134				
Wandering			5,24	0,02	1,85	1,11 – 3,08
Yes	64	144				
No	28	117				
Ehrlichia canis						
Age			4,79	0,02	3,10	1,18- 8,12
≥ 01 year	178	153				
< 01 year	6	16				
Sex			0,001	0,97	1,01	0,66 – 1,54
Male	90	82				
Female	94	87				
Wandering			0,17	0,67	0,89	0,58 – 1,36
Yes	106	102				
No	78	67				

X^2= Chi-square test: probability; OR=Ratio of Possibilities; 95% CI: 95%= Confidence interval.

Logistic regression analysis revealed that dogs over 1 year of age showed the highest probability to acquire *T. gondii* infection, corroborating the results of Carlos et al. (2010).

The prevalence of infection with *Leishmania* spp. was 26.1%, similar to the observation reported by Figueiredo et al. (2014) in Pará (19.7%) but lower than the values reported by Luciano et al. (2009) and Paulan et al. (2013) in São Paulo (62.7% and 37.6%, respectively).

Bahia is an endemic state for canine leishmaniasis; however, the results may have been influenced by the resident dogs in Ilhéus and Una that show no reports of *L. infantum* and *L. braziliensis* infections and those from Salvador where canine case registration is still low (BARBOSA et al., 2003; CARVALHO et al., 2015). However, the potential for the transmission of this zoonosis is high because some cities have borders with endemic regions aside from the intense migration of humans and animals as well as the socio-environmental characteristics favorable

for the expansion of the vector, and, consequently, the disease. In a study performed in Buerarema, a city located in the south mesoregion of Bahia, Leça Junior et al. (2015) showed that the predominance of dogs seropositive for *Leishmania* infection was 50.3%. The presence of this agent in the region may, in the future, influence the transmission of the disease to neighboring cities.

Table 5. Multivariate analysis for risk factors associated with the presence of *Toxoplasma gondii*, *Leishmania* spp., and *Ehrlichia canis* in dogs from Bahia, Brazil, 2015.

Variable	OR	95%CI	P value
Toxoplasma gondii			
Age			
≥ 01 year	2,97	1,05 – 8,42	0,03
< 01 year			
Wandering			
Yes			
No	0,62	0,40 – 0,96	0,03
***Leishmania* spp.**			
Wandering			
Yes	1,85	1,11 – 3,08	0,01
No			
Ehrlichia canis			
Age			
≥ 01 year	3,09	1,18 – 8,11	0,02
< 01 year			

OR = odds ratio; 95%CI = confidence interval.

The potential for the urbanization of the disease is demonstrated by the occurrence of human cases (131) of visceral leishmaniasis in urban centers of major cities in the state of Bahia. These cities include Feira de Santana, Serrinha, Jequié, Juazeiro, Irecê, Camaçari, and Salvador and correspond to 23.73% of cases registered in Bahia. In 2015, the highest incidences were observed in municipalities from the center-north and center-south mesoregion of Bahia, as indicated in the Epidemiological Bulletin of SUVISA/SESAB (2015) and consistent with the results found in this study. We reported dogs with higher seropositivity for *Leishmania* spp. in these mesoregions. The studied municipalities are urbanized areas and found to be positive for leishmaniasis in dogs, thereby confirming the results of Azevedo et al. (1996) and Carvalho et al. (2015) in Bahia.

A predominance of 52.1% was reported for *E. canis* using ELISA serological technique. Trapp et al. (2006) observed a predominance of 23% in Rio Grande do Sul, while Carlos et al. (2011) reported a predominance of 36% in Bahia using the same method. These differences may be related to the variation in the climate of each region and the level of the exposure to ticks for the populations studied.

Brazil is an endemic country for canine ehrlichiosis. This agent can infect humans, highlighting the need to monitor this disease (LABRUNA; PEREIRA, 2001). In this study, the prevalence of ehrlichiosis was 43.2%. Souza et al. (2010) found similar predominance in the city of Salvador. Among the mesoregions studied, the one in South Bahia showed 44% seropositivity, consistent with the results of Carlos et al. (2011) for the same region and Guedes et al. (2015). Both

studies used domestic animals, resulting in a lower predominance of ehrlichiosis. Domestic animals can be treated with acaricides at a greater frequency and may have access to veterinarian treatment. Some factors such as the presence and distribution of the vector *R. sanguineus*, animal behavior, age, habitat, and type of population studied may encourage the development of canine ehrlichiosis (LOULY et al., 2006; AZEVEDO et al., 2011; CARLOS et al., 2011; GUEDES et al., 2015). The high prevalence of *E. canis* in animals from semi-domestic urban areas and stray dogs may be related to the adaptation of *R. sanguineus* to urban environment, particularly in a population of dogs without acaricides treatment. Guedes et al. (2015) indicated that *R. sanguineus* is more frequent in urban areas than rural areas, thereby increasing the exposure of dogs to *E. canis.* The high frequency of dogs seropositive for *E. canis* emphasizes the importance of this agent in the municipalities studied.

Animals over 1 year of age were more prone to *E. canis* infection, thereby increasing the likelihood of infection in dogs, as reported by Azevedo et al. (2011) and Guedes et al. (2015), possibly because younger dogs were less exposed to the vector.

The center-south mesoregion of Bahia presented higher seropositivity for *E. canis* (60.7%; 51/84). This high prevalence may be related to the heat and high humidity in this region, which may encourage the proliferation of the tick.

Co-infection with at least two of these parasites is common in Brazil, indicating that dogs are exposed to different pathogens owing to the tropical climate, which favors the proliferation of vectors and maintenance of oocysts in the environment (DUBEY; LAPPIN, 2006; COELHO et al., 2013; GIRARDI et al., 2014). Paulan et al. (2013) reported 18.27% cases of co-infections of *T. gondii, Leishmania*, and *E. canis* in dogs from Ilha Solteira in São Paulo, higher than that found in this study (5%). The epidemiological significance of co-infection lies in the fact that animals living in areas endemic to these agents are at higher risk. It is important to highlight that a pathogen may work as a facilitator for the establishment of other infections. The presence of antibodies against *Ehrlichia* reveals the previous contact with the agent and may be considered as an immunosuppressive factor, which may trigger reactivation or greater susceptibility to the occurrence of toxoplasmosis and other diseases (BOOZER; MACINTIRE, 2003; DUBEY; LAPPIN, 2006).

Dogs infested with ticks present 53% higher possibility to acquire infection by *L. chagasi* as compared to dogs without infestation. Tick infestations cause anemia, weakness, and increase in the occurrence of co-infection (PAZ et al., 2010). Feitosa et al. (2000) and Mekuzas et al. (2009) reported that the immunosuppression caused by LVC may promote the occurrence of co-infections with other agents such as *E. canis* in endemic areas, thereby concluding that ehrlichiosis may be a contributor to the establishment of leishmaniasis.

Conclusion

This study showed that dogs from many municipalities of Bahia were seropositive for the three etiological agents studied and may even host more than one agent. As co-infections may exacerbate immunosuppression, animals co-infected with multiple agents are more likely to present high parasitemia, which facilitates the vector-borne transmission and permanence of the disease.

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