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Serosurvey of tick-borne pathogens in dogs from urban and rural areas from Parana State, Brazil

Avaliação sorológica de patógenos transmitidos por carrapatos em cães urbanos e rurais do estado do Paraná, Brasil

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Abstract

Considering the zoonotic potential of tick-borne disease (TBD) agents and the fact that dogs may act as sentinels for human infection, the aim of the present study was to determine the seroprevalence of TBD agents and risk factors for exposure in two different canine populations from Parana State, Southern Brazil. A total of 138 dog serum samples from urban (UA) (n=68) and rural (RA) (n=70) areas were tested with commercial ELISA rapid test for *Anaplasma phagocytophilum*, *Ehrlichia canis* and *Borrelia burgdorferi* antibodies and indirect immunofluorescence assay (IFAT) for *Babesia vogeli*. An overall of 92/138 (66.7%) dogs, being 62/68 (91.2%) from UA and 30/70 (42.9%) from RA, were seropositive for at least one TBD agent. From the total number of dogs, sixty-two were positive for *E. canis* (44.9%), 19 (13.8%) for *A. phagocytophilum*, and 64 (46.4%) for *B. vogeli*. Anti-*B. burgdorferi* antibodies were not detected. Dogs from UA showed a higher percentage of tick infestation (p = 0.0135) and were highly associated with seropositivity to *E. canis* (p = 0.000005), *A. phagocytophilum* (p = 0.0001), and *B. vogeli* (p = 0.0012). In summary, the findings indicate that dogs from urban areas present higher potential risk exposure to TBD pathogens than those from rural areas.

Keywords: *Ehrlichia canis*, *Babesia vogeli*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, serology, Parana.

Resumo

Considerando o potencial zoonótico das doenças transmitidas por carrapatos (DTCs) e que os cães podem atuar como sentinelas para infecções em humanos, os objetivos deste estudo foram determinar a soroprevalência de agentes das DTCs e fatores de risco para a exposição em duas diferentes populações caninas do Estado do Paraná, região Sul do Brasil. Um total de 138 amostras de soro de cães de área urbana (AU) (n = 68) e rural (AR) (n = 70) foram testadas utilizando um teste de ELISA comercial rápido para detecção de anticorpos contra *Anaplasma phagocytophilum*, *Ehrlichia canis* e *Borrelia burgdorferi* e imunofluorescência indireta (IFI) para *Babesia vogeli*. Um total de 92/138 (66,7%) cães, sendo 62/68 (91,2%) da AU e 30/70 (42,9%) da AR, foram soropositivos para pelo menos um agente. Do número total de amostras, sessenta e duas (44,9%) foram positivas para *E. canis*, 19 (13,8%) para *A. phagocytophilum* e 64 (46,4%) para *B. canis vogeli*. Anticorpos anti-*B. burgdorferi* não foram detectados. Os cães da AU apresentaram o maior percentual de infestação por carrapatos (p = 0,0135) e foram altamente associados com a positividade para *E. canis* (p = 0,000005), *A. phagocytophilum* (p = 0,0001) e *B. vogeli* (p = 0,0012). Em resumo, nossos achados indicam que cães de áreas urbanas têm um maior risco potencial de exposição a agentes patogênicos das DTCs comparados aos das áreas rurais.

Palavras-chave: *Ehrlichia canis*, *Babesia vogeli*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, sorologia, Paraná.

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Introduction

Ehrlichiosis, anaplasmosis, babesiosis, and borreliosis are important tick-borne diseases (TBDs) affecting dogs worldwide (LABARTHE et al., 2003; CARLOS et al., 2007; AMUSATEGUI et al., 2008; BOWMAN et al., 2009; WONG et al., 2011). While ticks of the genus *Amblyomma* are commonly found in rural areas, *Rhipicephalus sanguineus*, the brown dog tick, is the most common species infesting domestic dogs in urban areas in Brazil (LABRUNA et al., 2001). It is involved in the transmission of *Ehrlichia canis* and *Babesia vogeli* (DANTAS-TORRES, 2008). *E. canis* is the causative agent of canine monocytic ehrlichiosis (CME), widespread in most regions of the country, with seroprevalence varying from 0.7% to 86.2%, depending on the population studied, geographic area and diagnostic test used (VIEIRA et al., 2011). *B. vogeli* is the most common reported agent of canine babesiosis and seroprevalence data, ranging from 18.8% to 73.3% (TRAPP et al., 2006; MAIA et al., 2007; FURUTA et al., 2009; SPOLIDORIO et al., 2010).

It has been suggested that *R. sanguineus*, widely found across the country, may be a potential vector for *Anaplasma platys* to dogs in Brazil (DANTAS-TORRES, 2008). The identification of this organism can be achieved by the observation of *A. platys* inclusions in platelets during the examination of stained peripheral blood smears and by molecular methods (DAGNONE et al., 2009). Prevalence of *A. platys* infection ranges from 18.8% to 48.8% (DAGNONE et al., 2009; SANTOS et al., 2009; RAMOS et al., 2010); however, the seroprevalence remains to be fully established, since there is no developed serological diagnostic assay for this agent to date. Serological assays, such as ELISA, immunofluorescence antibody test (IFAT) and dot-ELISA, originally developed for the detection of anti-*A. phagocytophilum* antibodies, have been used as an alternative, due to the cross-reaction between *A. phagocytophilum* and *A. platys* antibodies (CHANDRASHEKAR et al., 2010).

A. phagocytophilum (a combination of organisms previously known as *E. equi*, *E. phagocytophila* and human granulocytic ehrlichiosis [HGE] agent) is the causative agent of granulocytic anaplasmosis and has been reported infecting dogs, cats, horses, ruminants and humans worldwide (WOLDEHIWET, 2010). *Ixodes scapularis*, *I. pacificus* and *I. ricinus* ticks act as the main vectors for this agent (AMUSATEGUI et al., 2008; BOWMAN et al., 2009) and although these ticks are not present in Brazil, other *Ixodes* and *Amblyomma* species are frequently found (FIGUEIREDO et al., 1999; ABEL et al., 2000). Recently, *A. phagocytophilum* was detected, by molecular methods, infecting dogs in Brazil (SANTOS et al., 2011).

Ticks of the *Ixodes* genus are also involved in the transmission of *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme disease (BURGDORFER et al., 1989). In Brazil, it is suspected that both *Amblyomma cajennense* and *Rhipicephalus* (*Boophilus*) *microplus* are involved in the transmission of *B. burgdorferi* (s.l.) (BARROS-BATTESTI et al., 2000; YOSHINARI et al., 2003). There are serological evidences of *B. burgdorferi* (s.l.) infection in Brazilian dogs, with prevalence data ranging from 0.04% to 53.5% (LABARTHE et al., 2003; CARLOS et al., 2007; SPOLIDORIO et al., 2010). Nevertheless, it is important to mention that *B. burgdorferi* (s.l.) has never been isolated or molecularly detected in this country, and thus, the role of these ticks on the transmission of Lyme disease remains to be established.

Considering the zoonotic potential of these agents and that dogs may act as sentinels for tick-borne infections to humans (AMUSATEGUI et al., 2008), the aims of the present study was to determine the seroprevalence of *A. phagocytophilum*, *E. canis*, *B. burgdorferi* and *B. vogeli*, the identification of tick species found in dogs, and the evaluation of the risk factors for exposure to the organisms, in two different canine populations from Paraná State, Southern Brazil.

Materials and Methods

1. Ethical principles

The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare of the "Universidade Estadual de Londrina" – UEL (number 34/2011).

2. Study area

The study was carried out in rural and urban areas located in the central-northern region of the State of Parana. The urban area (UA) is located in a region of 'Jardim California', a neighborhood located in the eastern side of Londrina (23° 08' 47" S and 51° 19' 11" W). The area is situated at 610 m above sea level; presents a subtropical climate with rainfall throughout the year, but concentrated during summer. The average annual temperature is around 20 °C (INMET, 2011). Dogs have free access to an abandoned square, covered by a mixed overgrowth pasture composed by grass and undesired plants (bushes, scrubs); dogs are also in constant contact with horses. This square is also used for neighbors' leisure, and present high amount of ticks.

The rural area (RA) is a settlement situated in the 'Alvorada do Sul' county (22° 54' 34.4" S and 51° 13' 49.1" W). The area is located within the rural perimeter of Alvorada do Sul, 16 km far from downtown, 380 m above sea level. The region presents subtropical climate with rainfall throughout the year, but with a tendency of concentrating rain during the summer months with an average temperature of 25 °C (INMET, 2011). The area is subdivided in 60 land properties with approximate area of 12 ha each, totalizing 786 ha. The rural settlement lacks basic sanitation and the main activity of dwellers is the cultivation of grains and vegetables. The area also comprises 20% of native forest, presenting diverse fauna, with a varied population of wild animals. In this region, a large number of ticks can be found throughout the year.

Animals

A total of 138 dogs of different ages, breeds, and both sexes from urban and rural areas of the central-northern region of the State of Parana were used. A total of 68 dogs from UA and 70 dogs from RA were sampled during an active surveillance program for infectious diseases.

3. Collection of ticks

A total of 126 adult ticks were collected from dogs, being 59 from the UA and 67 from the RA. Ticks were removed using tweezers and placed in tubes containing 70% ethanol solution and identified according to morphological keys (ARAGÃO; FONSECA, 1961; GUIMARÃES et al., 2001).

4. Study design

Owners responded to a previous validated epidemiological questionnaire during the sampling, addressing breed, age, gender and presence of ticks. Age was classified in groups: ≤ 1 and > 1 year old.

5. Sampling

Dog blood samples (10 mL) were collected by venipuncture of jugular vein into tubes without anti-coagulant and kept at room temperature (25 °C) until visible clot retraction, centrifuged at 1500 g for 5 minutes. Sera were separated and kept at –20 °C until testing.

6. Detection of antibodies against A. phagocytophilum, E. canis and B. burgdorferi (s.l.)

All 138 dogs serum samples were tested for *A. phagocytophilum*, *B. burgdorferi* (s.l.), and *E. canis* using a commercial ELISA rapid test (SNAP® 4Dx®, IDEXX Laboratories Inc., Westbrook, ME, USA), according to the manufacturer's instructions.

7. Detection of antibodies against B. vogeli

Antibodies anti-*B. vogeli* were detected by indirect immunofluorescence antibody test (IFAT) using antigens obtained from a splenectomized dog inoculated with *B. vogeli*, as previously described (TRAPP et al., 2006), with modifications. Briefly, IFAT was performed with 10 µL of serum samples incubated at 37 °C for 30 minutes in slides previously seeded with *B. vogeli* and washed three times for 5 minutes in phosphate buffered saline (PBS, pH 7.2). Ten microliters of fluorescein isothiocyanate-conjugated rabbit anti-dog IgG (Sigma-Aldrich, St. Louis, MO) at 1:1000 dilution in 0.01% Evans blue was applied onto the slide. Slides were then incubated at 37 °C for 30 minutes, washed three times for 5 minutes, allowed to air dry and, subsequently, examined in microscope with fluorescent light source. Serum samples with fluorescent protozoa at dilution ≥ 1:80 were considered positive. Titers were determined to the largest dilution in which fluorescence was visualized around the protozoa.

8. Statistical analysis

Either Chi-square or Fisher's exact test was used to determine the difference between whether individual factors were associated with seropositivity to *A. phagocytophilum*, *B. burgdorferi* (s. l.), *E. canis* and/or *B. canis*. Odds ratio (OR), 95% confidence interval and p values were calculated separately for each variable. Results were considered statistically significant when $p < 0.05$. Data were compiled and analyzed using Epi Info™ Software (version 3.5.3).

Results

From the total of 138 dogs sampled, 90 (65.2%) were males and 48 (34.8%) females. Breeds included Cocker Spaniel (2), Pit Bull (1), Poodle (2), and mixed breed (133) dogs. A total of 92/138 (66.7%; 95% CI: 58.1-74.5%) dogs were seropositive for different TBD agents: 62/68 (91.2%; 95% CI: 81.8-96.7%) from UA and 30/70 (42.9%; 95% CI: 31.1-53.3%) from RA. Dogs from the UA were 13.7 times more likely to be seropositive for at least one TBD agent than those dwelling RA (95% CI: 5.26-36.07%; p = 0.0001).

Using the commercial ELISA rapid test, it was possible to verify that 62/138 (44.9%; 95% CI: 36.5-53.6%) dogs were seropositive for *E. canis*. Seroprevalence in UA and RA were 44/68 (64.7%; 95% CI: 52.2-75.9%) and 18/70 (25.7%; 95% CI: 16.0-37.6%), respectively. Antibodies against *A. phagocytophilum* were found in 19/138 (13.8%; 95% CI: 8.5-20.7%) dogs: 17/68 (25%; 95% CI: 15.3-37%) from UA and 2/70 (2.9%; 95% CI: 0.3-9.9%) from RA. *B. burgdorferi* antibodies were not detected in dogs from both locations (Table 1).

Anti-*B. vogeli* antibodies were detected in 64/138 (46.4%; 95% CI: 37.9–55.1%) dogs by IFAT. Seroprevalence in UA and RA were 41/68 (60.3%; 95% CI: 47.7-72%) and 23/70 (32.9%; 95% CI: 22.1-45.1%), respectively (Table 1). Antibodies titers ranged from 80 to 20480 in dogs from UA and from 80 to 2560 in dogs from RA. Additionally, 37/138 (26.8%; 95% CI: 20.1-34.7%) dogs were seropositive for *E. canis* and *B. vogeli*, 8/138 (5.8%; 95% CI: 2.9-11%) for *A. phagocytophilum* and *B. vogeli*, and 15/138 (10.8%; 95% CI: 6.7-17.1%) dogs were seropositive for *A. phagocytophilum* and *E. canis*.

Dogs from the UA showed higher percentage of tick infestation – 48/68 (70.6%), compared to the RA – 35/70 (50%), (p = 0.0135). From the total of the 126 ticks collected from the UA and RA, 58/59 (98.3%) and 45/67 (67.1%), respectively, were identified as *Rhipicephalus sanguineus*. Nonetheless, only 1/59 (1.7%) and 1/67 (1.5%) were identified as *Amblyomma cajennense*, in UA and RA, respectively. Twenty-one out of 67 (31.3%) ticks from RA were identified as *Amblyomma ovale.*

Dogs living in the urban area were highly associated with seropositivity to *E. canis* (p = 0.000005), *A. phagocytophilum* (p = 0.0001), and *B. vogeli* (p = 0.0012). Dogs > 1 year old were highly associated with seropositivity to *E. canis* (p = 0.00001). No significant association was found between age or gender and seropositivity to *A. phagocytophilum* and *B. vogeli*. Results for the seroprevalence of *A. phagocytophilum* and *E. canis* in dogs from rural and urban areas within each variable studied are shown in Table 1.

Discussion

In Brazil, data on the distribution of arthropods and canine TBDs are still incomplete. In this study, 91.2% of the dogs from

Table 1. Serological prevalence of *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Babesia vogeli* in dogs within each variable studied, State of Parana, southern Brazil.

Variable	*Anaplasma phagocytophilum*				*Ehrlichia canis*				*Babesia vogeli*			
	+/N (%)	OR	95% CI	*P*-value	+/N (%)	OR	95% CI	P-value	+/N (%)	OR	95% CI	*P*-value
Place												
Urban Area	17/68 (25)	11.33	2.50-51.27	0.0001	44/68 (64.7)	5.29	2.54-11.00	0.000005	41/68 (60.3)	3.10	1.54-6.22	0.0012
Rural Area	2/70 (2.9)				18/70 (25.7)				23/70 (32.9)			
Presence of ticks												
Yes	15/83 (18.1)	2.81	0.88-8.98	0.0714	37/83 (44.6)	0.96	0.48-1.91	0.9192	40/83 (48.2)	1.20	0.60-2.38	0.5992
No	4/55 (7.3)				25/55 (45.5)				24/55 (43.6)			
Age (years)												
>1	14/86 (16.3)	1.83	0.61-5.41	0.2709	51/86 (59.3)	5.43	2.46-11	0.00001	45/86 (52.3)	1.91	0.94-3.86	0.0715
≤1	5/52 (9.6)				11/52 (21.2)				19/52 (36.5)			
Gender												
Male	11/90 (12.2)	0.69	0.26-1.87	0.4704	42/90 (46.7)	1.03	0.51-2.09	0.9255	38/90 (42.2)	0.73	0.36-1.47	0.3816
Female	8/48 (16.1)				22/48 (45.8)				24/48 (50)			

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval.

the urban area (UA) were seropositive for at least one tick-borne disease agent, and were 13.7 times more likely to be seropositive than those living in rural areas (RA). Besides, previous studies have shown a wide variation in seroprevalence for different TBD agents, such as *E. canis*, *B. vogeli* and *B. burgdorferi*, in different regions of Brazil (LABARTHE et al., 2003; CARLOS et al., 2007, SPOLIDORIO et al., 2010), but analyses were performed individually for each agent, and thus, there is no data to compare the overall seroprevalence found.

The higher seroprevalence found in the present study in dogs from UA was mainly due to seropositivity to *E. canis* (64.7%) and *B. vogeli* (60.3%), both agents transmitted by *R. sanguineus* ticks. Animals living in urban areas have a higher chance of becoming infested with *R. sanguineus* ticks than dogs dwelling rural areas (LABRUNA et al., 2001). This fact was supported by the present study, where 70% of the dogs from UA were infested by ticks, with 98.3% of these identified as *R. sanguineus*. High infestation rates of *R. sanguineus* have been shown to increase the risk of attack to humans (USPENSKY; IOFFE-USPENSKY, 2002). A previous study, also performed in the municipality of Londrina, showed that owners of tick-infested dogs were 3.2 times more likely to have removed ticks from themselves (TRAPP et al., 2006). Thus, there is a potential risk of human infection by TBD agents in the UA studied.

In this study, 60.3% of the dogs from UA were seropositive for *B. vogeli*. Lower seroprevalence data (35.7%) were obtained in a hospital population of dogs in the same study area (TRAPP et al., 2006). A previous study showed no difference in seroprevalence for *B. canis* between rural (46.5%) and urban (42.9%) areas in the State of Espirito Santo (SPOLIDORIO et al., 2010). In the present study, dogs from UA were 3.1 times more likely to be seropositive to *B. vogeli* than those from RA (p = 0.0012). However, association between age, sex, gender and seropositivity to *B. vogeli* was not observed (Table 1). The difference in seroprevalence found in other studies may have been due to climatic variation, population studied, diagnostic test used, and the cut-off used in the IFAT to *B. vogeli*.

Dogs from UA were 5.29 times more likely to be seropositive to *E. canis* than dogs from RA (p < 0.05). Besides, higher percentage of tick infestation have been found in dogs from UA (70.6%) than those from RA (50%) (p = 0.0135), which may explain the seroprevalence difference; association between seropositivity to *E. canis* and the presence of ticks was not observed (p = 0.9192). However, the relatively small number of samples analyzed has limited statistical power and thus, differences might have been missed. Serological surveys of *E. canis* in dogs have found seroprevalence data ranging from 4.8% to 38% and from 24.7% to 65.6% in urban and rural areas, respectively, using different methods, such as IFAT and ELISA (VIEIRA et al., 2011). The commercial ELISA rapid test used in this study utilizes synthetic peptides from p30 and p30-1 outer membrane proteins of *E. canis* as antigen, and detects anti-*E. canis* and anti-*E. chaffeensis* antibodies (O'CONNOR et al., 2006; SNAP® 4Dx® product insert and IDEXX Laboratories, unpublished data). In a previous study, the assay was able to identify only 30% of the serum samples from dogs with low-titer (80 to 160) on *E. canis*-IFA, but identified all dogs with titers > 320 (O'CONNOR et al., 2006). Thus, seroprevalence for *E. canis* in dogs from the studied areas may be higher, since dogs with low titers may not have been recognized when a point-of-care ELISA

assay was used, as previously described in dogs from an animal shelter in central Spain (COUTO et al., 2010).

Antibodies against *A. phagocytophilum* were found in 13.8% of the dogs using the commercial ELISA. Different results were found in dogs from the United States, France and Central Spain, which reported seroprevalence data of 4.8%, 2.72%, and 19% (BOWMAN et al., 2009; PANTCHEV et al., 2009; COUTO et al., 2010), respectively. Although *A. phagocytophilum* infection was reported in dogs from the State of Rio de Janeiro, southeastern Brazil, by a quantitative real-time PCR followed by sequencing of the *msp2* gene (SANTOS et al., 2011), no serological data regarding this agent have been reported for Brazilian dogs to date. It is worth mentioning that the results found in the present study may be due to the cross-reactivity between *A. phagocytophilum* and *A. platys* in the assay used (CHANDRASHEKAR et al., 2010; SNAP® 4Dx® product insert and IDEXX Laboratories, unpublished data). Using the same commercial ELISA rapid test in serum samples from 16 PCR-positive to *A. platys* dogs, in acute stage of disease, cross-reaction between these two bacteria was not found (FERREIRA et al., 2008). Unfortunately, PCR was not performed in the present study and it was not possible to establish whether antibodies detected were due to *A. phagocytophilum* or *A. platys*. Further studies should be conducted in order to elucidate the cross-reactivity between *A. phagocytophilum* and *A. platys*.

Conclusion

From two different populations, it was possible to determine seroprevalence data of two major tick-borne disease agents, *E. canis* and *B. vogeli*, which cause severe clinical illness in dogs in Brazil. Moreover, the findings of this study indicate a higher potential risk for dogs exposure to TBD pathogens in urban than in rural areas.

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