

Serosurvey of *Borrelia* in dogs, horses, and humans exposed to ticks in a rural settlement of southern Brazil

Soroprevalência e fatores associados a *Borrelia* em cães, equinos e humanos expostos a carrapatos em um assentamento rural do sul do Brasil

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Abstract

The aims of the present study were to serosurvey dogs, horses, and humans highly exposed to tick bites for anti-*Borrelia burgdorferi* s.l. antibodies, identify tick species present, and determine risk factors associated with seropositivity in a rural settlement of Paraná State, southern Brazil. Eighty-seven residents were sampled, along with their 83 dogs and 18 horses, and individual questionnaires were administered. Immunofluorescence antibody test (IFAT) was performed on serum samples and positive samples were subjected to western blot (WB) analysis. Anti-*B. burgdorferi* antibodies were found in 4/87 (4.6%) humans, 26/83 (31.3%) dogs, and 7/18 (38.9%) horses by IFAT, with 4/4 humans also positive by WB. Ticks identified were mostly from dogs and included 45/67 *Rhipicephalus sanguineus*, 21/67 *Amblyomma ovale*, and 1/67 *A. cajennense* sensu lato. All (34/34) horse ticks were identified as *A. cajennense* s.l.. No significant association was found when age, gender, or presence of ticks was correlated to seropositivity to *Borrelia* sp. In conclusion, although anti-*Borrelia* antibodies have been found in dogs, horses and their owners from the rural settlement, the lack of isolation, molecular characterization, absence of competent vectors and the low specificity of the commercial WB kit used herein may have impaired risk factor analysis.

Keywords: Immunofluorescence antibody test (IFAT), Lyme disease, serology, tick-borne disease, western blot.

Resumo

Os objetivos do presente estudo foram realizar um levantamento sorológico de cães, cavalos e humanos altamente expostos a picadas de carrapatos para anticorpos anti-*B. burgdorferi* s.l., identificar as espécies de carrapatos presentes, e determinar os fatores de risco associados a soropositividade em um assentamento rural do Estado do Paraná, sul do Brasil. Oitenta e sete residentes foram amostrados junto com seus respectivos 83 cães e 118 cavalos e questionários individuais foram aplicados. O teste de imunofluorescência indireta (IFI) foi realizado nas amostras sorológicas e as positivas foram submetidas a análise por *western blot* (WB). Anticorpos anti-*B. burgdorferi* foram detectados em 4/87 (4,6%) humanos, 26/83 (31,3%) cães e 7/18 (38,9%) cavalos pela IFI, com 4/4 humanos também positivos pelo WB. Os carrapatos identificados foram em sua maioria de cães e incluíram 45/67 *Rhipicephalus sanguineus*, 21/67 *Amblyomma ovale* e 1/67 *A. cajennense* sensu lato. Todos (34/34) carrapatos dos cavalos foram identificados como *A. cajennense* s.l.. Não foram observadas diferenças estatísticas entre idade, sexo ou presença de carrapatos e soropositividade para *Borrelia* sp. Em conclusão, embora anticorpos anti-*Borrelia* tenham sido encontrados em cães, equinos e seus proprietários do assentamento rural, a ausência de isolamento, caracterização molecular, ausência de vetores competentes e baixa especificidade do kit comercial de WB utilizado podem ter limitado a análise de fatores de risco.

Palavras-chave: Teste de imunofluorescência (IFI), doença de Lyme, sorologia, doenças transmitidas por carrapatos, western blot.

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Introduction

Lyme disease (LD) is a tick-borne disease caused by several bacteria in the *Borrelia burgdorferi* sensu lato (s.l.) complex (STANEK & REITER, 2011). *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, and *B. garinii* have been identified as the major etiological agents of LD in Europe (STANEK et al., 2012). In South America, *Borrelia* spp. closely related to *B. americana* were found in *Ixodes* ticks from Uruguay (BARBIERI et al., 2013) and Argentina (NAVA et al., 2014). In southern Brazil, *B. burgdorferi* s.s. was reported to be found in *Dermacentor nitens* ticks removed from horses (GONÇALVES et al., 2014).

Because of differences in the clinical syndrome and vectors present in the region, LD in Brazil is also known as LD-like syndrome or Baggio-Yoshinari syndrome (BYS) (MANTOVANI et al., 2007). Although several human cases have been described throughout the country (YOSHINARI et al., 2003, 2007; CARRANZA-TAMAYO et al., 2012; ROSA et al., 2014), *Borrelia* spp. have not yet been isolated or characterized from such cases and *Borrelia* infection in humans remains to be confirmed in Brazil (YOSHINARI et al., 2010).

Domestic animals in Brazil have shown evidence of anti-*B. burgdorferi* s.l. antibodies, with the antibody seroprevalence in dogs ranging from less than 1% up to 51% (LABARTHE et al., 2003; ALVES et al., 2004; SPOLIDORIO et al., 2010; VIEIRA et al., 2013a, b) and, in horses, from 4.2% to 26.7% (GALO et al., 2009; SPOLIDORIO et al., 2010; VIEIRA et al., 2013a). Since studies in Brazil have focused either on suspected human cases or antibody prevalence in animals, the zoonotic etiology of *Borrelia* infections has not yet been confirmed in Brazil.

In Brazil, rural settlements are commonly characterized by inadequate sanitary care with people sharing the same environment with domestic and wild animals, in climate and environmental conditions favorable for the ticks. The present study aimed to survey humans, along with their dogs and horses, for anti-*B. burgdorferi* s.l. antibodies, to identify tick species present, and to determine risk factors associated with seropositivity in a rural settlement of Paraná State, southern Brazil.

Materials and Methods

Ethical principles

The present study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare (protocol no. 82/2006) and by the Ethics Committee on Human Research (protocol no.124/2007) at the Universidade Estadual de Londrina.

Study design

The rural settlement of formerly landless people was characterized by inadequate infrastructure, low incomes, limited resources, and poor sanitary conditions, situated in Alvorada do Sul county (22° 54' 34.4" S 51° 13' 49.1" W), Paraná State, Southern Brazil. The settlement was subdivided into 60 homesteads with

approximately 12 hectares each. Humans, dogs, and horses shared the same environment, with continuous exposure to common ticks.

Samples were collected house-to-house between November 2006 and January 2007, at the end of spring and beginning of summer in the southern hemisphere. At the time of sampling, residents responded to an epidemiological questionnaire that included animal species, number, breed, age, and gender, and known presence or previous contact with ticks. The ages of dogs were stratified into groups of < 1 year, 1-5 years, and > 5 years. The ages of horses were stratified into groups of ≤ 5 years and > 5 years. Resident age, gender, and known contact with ticks were also included in the questionnaire.

Collection of ticks

A total of 101 tick specimens were collected from dogs and horses during the study. Ticks from each host were removed using tweezers and placed in 70% ethanol in labeled tubes. Ticks were later identified according to standard taxonomic keys (ARAGÃO & FONSECA, 1961; GUIMARÃES et al., 2001; MARTINS et al., 2010; ONOFRIO et al., 2009).

Sampling

Dog (n = 83) and horse (n = 18) blood samples (up to 10 mL) were collected by veterinarians through jugular venipuncture, and human (n = 87) blood samples were collected in the same visit by nurses through brachial venipuncture. All samples were collected in tubes without anti-coagulant, kept at room temperature (25 °C) until the clot visibly retracted, and then centrifuged at 1500 g for 5 min. Serum was separated and kept at -20 °C until testing.

Immunofluorescence antibody test (IFAT)

Sera from dogs, horses, and humans were screened for anti-*B. burgdorferi* s.s. antibodies by indirect immunofluorescence antibody test (IFAT) using *B. burgdorferi* s.s. strain B31 for the antigen. The reaction was performed as previously described (COLLARES-PEREIRA et al., 2000), with fluorescein isothiocyanate-conjugated rabbit anti-dog IgG (Sigma-Aldrich, St. Louis, MO), rabbit anti-horse IgG (Sigma-Aldrich), and rabbit anti-human IgG (Sigma-Aldrich) used for testing of dog, horse, and human sera, respectively. Samples were considered positive when fluorescence was observed at dilutions of 1:64 or higher in dogs and horses or 1:128 or higher in humans. Antibody titers (endpoint titers) were defined as the reciprocal of the highest dilution of serum in which fluorescence was visualized. Positive and negative controls were provided by the Leptospirosis and Lyme Disease Group at the Universidade Nova de Lisboa, Portugal.

Western blot (WB)

Human sera that were positive by IFAT were subjected to western blot (WB) using a commercial kit (Anti-*Borrelia* Euroline-RN-AT IgG, Euroimmun, Lübeck, Germany) that includes highly

purified recombinant antigens of *B. burgdorferi* s.s. (Bb), *B. afzelii* (Ba), and *B. garinii* (Bg). This test was designed to detect the following antigens: recombinant VlsE from Ba, Bg, and Bb; lipids from Ba and Bb; p83 from Ba, p41 from Bg, p39 from Bg, OspC from Bg, and new recombinant antigens p58, p21, p20, p19, and p18. Procedures were performed according to the manufacturer's instructions and samples were considered positive when \geq two reactive bands were present.

Dog sera that were positive by IFAT were subjected to WB using a commercial kit (*recomBlot Borrelia canis* IgG, Mikrogen, Germany), which was designed to detect anti-*B. burgdorferi* s.s., anti-*B. garinii*, anti-*B. afzelii*, and anti-*B. bavariensis* antibodies. The test contains highly purified recombinant *B. burgdorferi* antigens (OspA, OspC, p100, VlsE, p39, p18, and p41). All procedures were performed according to the manufacturer's instructions, and samples were considered positive when \geq two reactive bands were present.

Statistical analysis

The Chi-square or Fisher's exact test was applied to determine the individual risk factors associated with antibody seropositivity to *B. burgdorferi*. Odds ratios (OR), 95% confidence intervals, and *p* values were calculated separately for each variable. Results were considered significant at $p < 0.05$. Data were gathered and analyzed using freely available software (Epi Info version 3.5.3, Centers of Disease Control, Atlanta, GA, USA).

Results

Anti-*B. burgdorferi* antibodies were found in 4/87 (4.6%; 95% CI: 1.8-11.2) human residents by IFAT, with antibody titers ranging from 128 to 256. Commercial WB revealed that 4/4 (100%) positive human sera reacted to antigen p41 Bg and new recombinant antigens p21, p20, and p19; 2/4 (50%) reacted to p83 Ba and p39 Bg; 1/4 (25%) reacted to p39 Bg; and 1/4 (25%) reacted to the new recombinant antigen p18.

A total of 26/83 (31.3%; 95% CI: 22.3-41.9) dogs were determined seropositive for anti-*B. burgdorferi* antibodies by IFAT, with antibody titers ranging from 64 to 128. In addition, 15/26 (57.7%) dog sera were confirmed positive by commercial WB, with 12/15 (80%) sera reacting to p100, 9/15 (60%) to p41, 4/15 (26.7%) to p39, 3/15 (20%) to OspC. Results of the 15 positive dogs on WB are summarized in Table 1. No significant association was found between seropositivity to *B. burgdorferi* and dog age (Fisher's exact test: $p > 0.05$), gender (Chi-square: $p = 0.163$), or presence of ticks (Chi-square: $p = 0.865$). The seroprevalence of *B. burgdorferi* in dogs and risk factors for infection are presented in Table 2.

An overall 7/18 (38.9%; 95% CI: 21.6-63.9) horses were determined seropositive for anti-*B. burgdorferi* antibodies by IFAT, with antibody titers ranging from 64 to 256. No significant association was found between seropositivity to *B. burgdorferi* and horse age (Chi-square: $p = 0.864$), gender (Chi-square: $p = 0.705$), or presence of ticks (Chi-square: $p = 0.4404$).

Table 1. Results of immunofluorescence antibody testing (IFAT) and western blotting for anti-*Borrelia* sp. antibodies in dogs from a rural settlement, Paraná State, southern Brazil.

Dogs	Antibody titer (IFAT)	Western Blot
12	64	p100, VlsE, p41
21	128	p39
22	64	p41, OspC,
23	64	p41
24	64	p100, p41
26	128	p100
29	64	p100, p41
31	128	p100, p39, OspC
45	64	p100, p41
46	64	p100, p41
53	64	p100, p41
60	128	p100, p39, OspC
66	128	p100
75	128	p100, p39
90	64	p100, p41

Table 2. Seroprevalence of anti-*Borrelia burgdorferi* antibodies and risk factors for *Borrelia* infection in dogs from a rural settlement, Paraná State, southern Brazil.

Variable	+/N (%)	OR	95% CI	<i>p</i> -value
Presence of Ticks				
Yes	19/65 (29.2%)	2.06	0.53-7.96	0.225
No	3/18 (17.7%)			
Age (Years)				
<1	4/23 (17.4%)			
1-5	15/48 (31.3%)	2.15	0.62-7.45	0.217
>5	3/12 (25%)	1.58	0.29-8.61	0.453
Gender				
Male	14/62 (22.60)	0.47	0.16-1.37	0.163
Female	08/21 (38.10)			

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

Of the ticks collected, 67/101 (66.3%; 95% CI: 56.6-74.8) were from dogs and 34/101 (33.7%; 95% CI: 25.2-43.3) were from horses. Three tick species were identified on dogs: *Rhipicephalus sanguineus* (n = 45, 67.1%), *Amblyomma ovale* (n = 21, 31.4%) and *A. cajennense* sensu lato (n = 1, 1.5%). From horses, all 34/34 (100%) ticks were identified as *A. cajennense* sensu lato.

Discussion

Anti-*B. burgdorferi* antibodies were found in 31.3% of dogs, 38.9% of horses, and 4.6% of residents from the settlement. Antibody positivity to this organism has been previously described in subjects from rural areas of Brazil (GONÇALVES et al., 2013). However, to the author's knowledge, no survey or study to date has both assessed the anti-*B. burgdorferi* antibody prevalence in domestic animals and their corresponding owners and determined the risk factors associated with seropositivity.

Human sera that were positive for anti-*Borrelia* antibodies by IFAT were confirmed positive by WB, as recommended by the U.S. Centers for Disease Control and Prevention (CDC, 2011). Interestingly, although the LD agent in Brazil may exhibit a different pattern of reactivity by WB when *B. burgdorferi* s.s. from the Northern Hemisphere is used (MANTOVANI et al., 2007; YOSHINARI et al., 2010), human sera from this study showed IgG reactivity by WB to *B. garinii*, *B. afzelii*, and *B. burgdorferi* antigens. The overlapping occurrence of these three *Borrelia* species has been only reported in the Palearctic region and associated to ticks belonging to the *Ixodes ricinus* complex (ESTRADA-PENÁ et al., 2011). Thus, the authors have not excluded the possibility that the anti-*Borrelia* IgG detected in the present study is a result of cross-reactivity with bacteria not belonging to the genus *Borrelia* or even with the BYS agent.

Antibody seropositivity to *Borrelia* spp. was found by WB in 18% of dogs in the present study, which is a higher prevalence than has been reported previously in tick endemic areas (JOPPERT et al., 2001; O'DWYER et al., 2004). Previous studies have shown cross-reactivity with antigens of other spirochetes, such as *Leptospira* antigens p41 and OspC (BRUCKBAUER et al., 1992; LESCHNIK et al., 2010), and it is important to note the possibility that dog sera in this study (untested for anti-*Leptospira* antibodies) may have tested positive by WB as result of cross-reactivity. However, since 80% dogs' sera also demonstrated antibodies against p100 by WB (Table 1), an antigen for which no cross-reactivity has been reported, the antibodies detected here are likely a result of *Borrelia* infection. In addition, the present study corroborates previous studies (O'DWYER et al., 2004) in which no association was established between seropositivity to *Borrelia* spp. and age, gender, or presence of ticks in dogs.

An overall 38.9% of horses were seropositive for anti-*Borrelia* spp. antibodies by IFAT. This seroprevalence was higher than previously reported by ELISA testing, with seroprevalences of 26.7% in urban cart horses in northern Brazil (GALO et al., 2009) and 28.4% in farm horses in southeastern Brazil (MADUREIRA et al., 2007). Results suggest a much higher exposure of horses to ticks and *Borrelia* spp. at this rural settlement, which may differ from dog and human exposure since prevalence in dogs and owner were in agreement with previous studies.

Our research hypothesis is that tick-borne infections are common in Brazil, because of a favorable climate and high prevalence and wide distribution of ticks, and that these diseases are underestimated. Moreover, we hypothesize that two major groups of humans are at highest risk of *Borrelia* infection: immune-compromised and those that are highly exposed to ticks. Based on the results of the present study and a previous study by our group on *Ehrlichia* spp. in subjects highly exposed to ticks (VIEIRA et al., 2013a), the authors emphasize that physicians should consider tick-borne diseases in inhabitants from rural settlements in Brazil.

In conclusion, anti-*Borrelia* antibodies were found in dogs, horses and their owners in a rural settlement from southern Brazil. However, the lack of isolation, molecular characterization, absence of competent vectors and the low specificity of the commercial WB kit used herein may have impaired risk factor analysis.

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