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Borrelia burgdorferi ANTIBODIES IN DOGS FROM COTIA COUNTY, SÃO PAULO STATE, BRAZIL

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SUMMARY

Dogs sera samples collected from Cotia County, São Paulo were tested using indirect immunoenzymatic test (ELISA) in order to study Lyme disease serology in dogs. ELISA method was standardized and G39/40 North American strain of *Borrelia burgdorferi* was used as antigen. Positive results were confirmed employing the Western blotting technique. Because of the possibility of cross-reactions, sera were also tested for different serological strains of *Leptospira interrogans* and *L. biflexa* using microscopic sera agglutination test. Twenty-three of 237 (9.7%) serum samples were positive in the ELISA; 20 of them (86.9%) were confirmed by the Western blotting, what suggests that Cotia may be a risk area for Lyme disease. Although 4 samples (1.7%) were positive for Lyme disease and leptospirosis, no correlation was found between the results (X^2 = 0.725; p = 0.394) what suggests absence of serological cross reactivity.

KEYWORDS: Lyme disease; *Borrelia burgdorferi*; Dogs; ELISA; Brazil.

INTRODUCTION

Lyme disease is a multisystemic zoonotic disease caused by a tick-borne spirochete *Borrelia burgdorferi*[16]. The disease in humans typically begins with a skin rash named *erythema migrans*, and is often associated with flu-like symptoms that may be followed weeks to months later by cardiac, neurological, and joint disease[22].

Many *Borrelia* strains have been isolated from *Ixodes* sp. ticks, reservoir animals and Lyme disease patients in many parts of the world. *B. burgdorferi* sensu latu, that was first thought to be a homogeneous species, is classified into new genospecies: *B. burgdorferi* sensu strictu isolated in North America and Europe, *B. garinii* and *B. afzelli* in Europe, *B. japonica* in Japan, and *B. andersonii* in North America[2].

Borrelia burgdorferi has been isolated and identified in many different animal species[10], yet clinical manifestations of the disease have been reported in dogs, horse and cattle[6,7,12]. Affected dogs may exhibit a variety of clinical signals including fever, lethargy, lymphoadenopathy, kidney disorders, heart block and recurrent polyarthritis, the latter being the most frequent manifestation in seropositive animals[12]. Due to a lack of pathognomonic clinical signals, serology has been widely used for the diagnosis of canine borreliosis, and has been a helpful tool for epidemiological surveys. Indirect fluorescent antibody test (IFA) and enzyme immunosorbent assay (ELISA) are used in humans and dogs to test for exposure to *Borrelia burgdorferi*, and for confirmation, Western blotting assay[8].

In areas where Lyme disease is endemic, the prevalence of antibodies to *B. burgdorferi* in dogs is higher than in co-resident human population[9]. This probably is because dogs are more exposed to the vectors, which remain longer on the host making easier the transmission of the spirochetes. For this reason, dogs are considered as sentinel animals, as a means of determining the prevalence of Lyme disease in a geographic area.

In Brazil, there are reports of clinical cases in humans patients,[24,26,27,28] and Cotia County in São Paulo State was the first region where disease was identified in brothers, and the diagnosis was confirmed by the presence of *B. burgdorferi* antibodies revealed by ELISA and Western blotting tests. Seroepidemiological study performed in this area[25] revealed that about 7.5% of the people were seropositive, similar prevalence observed at risk areas in North America and Europe[15].

Despite of the known existence of human infection, no data was available for dogs disease in the same area, so to fulfil this gap, the seroprevalence of *B. burgdorferi* in dogs was studied, using indirect ELISA technique, and confirmation of positive samples by the Western blotting method.

MATERIAL AND METHODS

Sera: During anti-rabies vaccination campaign in Cotia County (São Paulo, Brazil), 237 sera samples from dogs of any age, gender and breed,

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owned near the woods were collected, from August 18th to September 1st, 1992. Informations related to previous or recent contact with ticks were obtained for all dogs.

ELISA METHODS

Antigen: For antigen preparation it was used *Borrelia burgdorferi* G39/40 strain of North American origin, maintained at Laboratório de Investigação em Reumatologia da Faculdade de Medicina da Universidade de São Paulo. The preparation of whole sonicated spirochetes suspension was made as previously described[3]. Briefly, microorganisms were grown in Kelly's modified medium for approximately one week at 33 °C. At late-log-phase growth, organisms were pelleted by centrifugation at 12000 g for 20 min at 4 °C, washed with a solution of cold PBS 0.001*M* $MgCl_2$ $6H_2O$ PBS (pH 7.4) and sonicated on ice for 3 min with 15 sec intervals with a cell sonicator (Sonic Dismembrator Model 300, Dynatech Lab, Inc).

The suspension was filtered (0.45 μm diameter pore) and the protein concentration was determined by the method of Folin. The antigen preparation was stored in aliquots at -70 °C until use.

Positive control serum: To obtain a positive control serum, a dog without serum antibodies against *Borrelia burgdorferi* (Canine *B. burgdorferi* antibody test kit, Cite) and *L. interrogans* (Microscopic sera agglutination test) was inoculated twice, with a 7 days interval, with a heat killed (56 °C – 30 min) *B. burgdorferi* suspension at a dose of 0.1 mg/Kg of body weight.

The antigen suspension was prepared as above described but was not submitted to sonication, and 0.5 ml of the suspension was homogenizated with 0.5 ml of complete Freund's adjuvant and inoculated subcutaneously.

Serum samples were collected 4, 7, 10, 14, 21, 36, 44, 58 and 64 days after the first inoculation and tested for the presence of antibodies to *B. burgdorferi* using ELISA and Western blotting methods. Sample collected on day 36 was chosen as the standard positive control. Antibodies against *L. interrogans* were tested by microscopic sera agglutination assay.

Negative control sera: Sera from eight dogs, free for ticks and negative for *Borrelia* (Canine *B. burgdorferi* antibody test kit, Cite) and *Leptospira* antibodies (microscopic agglutination test) were used as negative controls.

ELISA: The ELISA was performed by standard method[23] with modifications. Briefly, the microtitrations plates (EIA/RIA plate, Costar) were coated with 200 μl of a 0.015 mg/ml solution of the spirochete proteins diluted in 0.05*M* sodium carbonate (pH 9.6) and incubated overnight at 4 °C. The plates were then washed three times with PBS containing 0.05% Tween 20 and blocked with PBS Tween 20 and 5% skimmed milk (pH 7.4). The wash procedure was repeated and test samples and control sera were diluted in blocking solution and plated in duplicate (200 μl/well). The samples and negative control sera were diluted 1/400 and the positive control serum was diluted using serial two ratio dilutions starting on 1/400. After incubation at room temperature for 1 hour, the wash procedure was repeated and the conjugate was added (alkaline phosphatase conjugated rabbit serum anti-dog IgG, Sigma) diluted 1/1000 in blocking solution. After incubation and wash procedure was added the substrate p-nitrophenyl sodium phosphate substrate (Sigma) diluted at 1 mg/ml in glycine buffer (pH 10.5). The plates were read at 405 nm in a Titertek Multiscan MCC/340 (Flow Laboratories), when the first dilution of the positive control serum reached the optical density value near 1.0.

Cut off value was obtained by considering the mean optical density plus three standard deviations of the eight negative controls sera. Optical densities higher than cut off values were considered positive and the titers were estimated by regression curve.

Western blotting (WB): Electrophoresis and immunoblotting were done as previously described with modifications[14]. Briefly, 750 μg of spirochete proteins, reduced with SDS, was electrophoresed (Mini-Protean II System, Bio Rad) on a 10% acrylamide gel. After running, gel proteins were transferred to nitrocellulose paper over night (Mini-Trans Blot System, Bio Rad), and the paper cut in strips, which were washed in distilled water. One strip containing *Borrelia burgdorferi* antigens and other with molecular weight markers were separated and stained with a solution 1:1 of colloidal gold (Bio Rad). The others strips were blocked with 0.1% TBS Tween 20 and 5% skimmed milk for 1 hour at room temperature and then washed five times with 0.1% TBS Tween 20. The sera samples to be tested (positive and negative controls, and test sera), were diluted 1:100 in blocking solution and added to incubate with strips for 1 hour at room temperature. After washing, the strips were incubated for 1 hour with alkaline phosphatase-conjugated rabbit serum anti-dog IgG (Sigma Chemical) diluted at 1/1000 with blocking solution. Washing was repeated and substrate consisting of NBT/BCIP diluted in bicarbonate buffer pH 9.8 was added. The reaction was blocked when positive control developed color.

Microscopic sera agglutination reaction: Study on antibodies to the main sorovars of *Leptospira interrogans and L. biflexa* was performed in the Laboratório de Zoonoses do Departamento de Medicina Veterinária Preventiva e Saúde Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, using the microscopic sera agglutination technique[11].

Statistical analysis: The chi-square test was used in order to evaluate the correlation between positive results for Lyme disease and positive results for leptospirosis and to verify the presence of significant differences between distribution of positive animals, using the indirect ELISA test, according to gender, age and history of contact with ticks.

RESULTS

A total of 23 (9.7%) of the 237 serum samples analysed by ELISA had antibodies to *Borrelia burgdorferi* at a dilution of 1/400 or higher (Table 1). Although a higher frequency (69.6%) was found on males when compared to females (30.4%) no difference among them could be find ($p = 0.417$). Also, no difference on the frequency of reagents was observed related to age of dogs ($p = 0.256$) (Table 2). On the other hand, positive results were found mainly in dogs with history of previous contact with ticks ($p = 0.034$) (Table 3).

When ELISA positive sera were submitted to Western blotting, all samples except three (86.9%) presented reactivity to more than five bands,

Table 1
Distribution of dogs reagent to *B. burgdorferi* antigens using ELISA test, according with gender

	Gender		
ELISA	Males	Females	Total
Positive	16 (69.6%)	7 (30.4%)	23 (100.0%)
Negative	125 (58.4%)	89 (41.6%)	214 (100.0%)
Total	141 (59.5%)	96 (40.5%)	237 (100.0%)

Table 2
Distribution of dogs reagent to *Borrelia burgdorferi* antigens using the ELISA test, according with the age

	Age			
ELISA	6 to 11 months	1 to 5 years	6 to 10 years	Total
Positive	2 (8.7%)	16 (69.6%)	5 (21.7%)	23 (100.0%)
Negative	24 (11.2%)	168 (78.5%)	22 (10.3%)	214 (100.0%)
Total	26 (11.0%)	184 (77.6%)	27 (11.4%)	237 (100.0%)

Table 3
Distribution of dogs reagent to *Borrelia burgdorferi* antigens using the ELISA test, according to history of previous contact with ticks

	History of contact with ticks			
ELISA	Contact	No contact	Unknown	Total
Positive	16 (69.6%)	7 (30.4%)	0	23 (100.0%)
Negative	90 (42.1%)	115 (53.7%)	9 (4.2%)	214 (100.0%)
Total	106 (44.7%)	122 (51.5%)	9 (3.8%)	237 (100.0%)

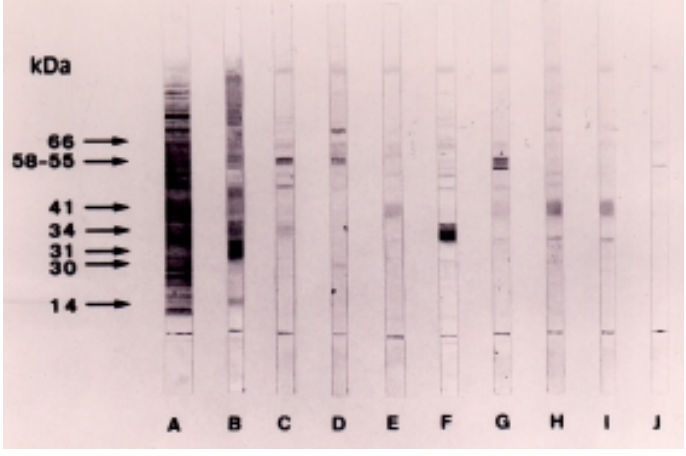

Fig. 1 - Western blotting results for sera of dogs from Cotia. A- *Borrelia burgdorferi* antigens stained with colloidal gold; B- positive standard serum; C to I- positive test samples; J- negative sample.

according to GREENE *et al*., 1991[13] standardization confirming the presence of *Borrelia burgdorferi* antibodies (Fig. 1).

The microscopic seroagglutination test for leptospira antibodies, showed that 24 of 237 sera (10.1%) had positive reaction, with titer ranging from 1/100 and 1/400 for several serovars of *Leptospira interrogans*. Only four samples (1.7%) of the 237 gave positive serology for Lyme disease and leptospirosis. No correlation (p = 0.394) between both diseases regarding cross reactivity could be found.

DISCUSSION

Lyme disease is an emerging disease in Brazil, and seems to be endemic in some areas where ticks and wild animals are present, close to human residents.

Cotia county is located near from São Paulo city and has a vast wooded area named Atlantic Forest. Epidemiological studies performed at this region, where first cases of Lyme disease in humans had been described[25], revealed presence of potential vectors[4] such as ticks of species *Ixodes loricatus, I. didelphidis* and *Amblyomma cajennense*. Spirochete like organisms were identified in the blood culture of wild animals and ticks, however these microorganisms did not grow longer in BSK medium[1].

Seroepidemiological surveillance done at Cotia county revealed a prevalence of 7.5% of positive results for *Borrelia burgdorferi* antibodies, suggesting that residents of the area had previous contact with spirochetes[25] .

The prevalence found here in dogs (9.7%), slightly higher than the human's one, confirmed the possibility of transmission of *B. burgdorferi* sensu latu to dogs, although no clinical case of Lyme disease had been observed. The low prevalence of reagents, when compared to some endemic areas in USA[5], where as high as 53% of dogs were found to be positive for *B. burgdorferi*, might be due to a low rate of infected ticks. Also, in areas where Lyme disease is endemic, the seroprevalence for Lyme disease in dogs is higher than the one found in co-resident human population[9], and low prevalence in dogs was found around the world, where human cases of disease are rarely described.

The incidence of *B. burgdorferi* infection in dogs[20] is closely related to the ticks density in a given area, the rate of tick infection and outdoor or indoor habits of dogs. Also, to the sensitivity and specificity of the surveillance method employed[17].

A decrease in the sensitivity of the method may have occurred because of the antigen employed. It is known that there are antigenic differences among *B. burgdorferi* sensu latu strains isolated around the world[19]. In this study G39/40 strain of *B. burgdoferi* of north American origin was used, because native isolate was not isolated in Brazil yet.

During introduction of ELISA test for *B. burgdorferi* antibodies search in dogs, some difficulties were found. Because of not existence of positive control serum, a dog previously known to be free of *Borrelia* sp antibodies tested by commercial ELISA was inoculated with killed microorganism plus Freund's adjuvant twice. The use of alive spirochetes was avoided to prevent any risk of bacterial dissemination to the

environment, despite of employment of a non virulent strain of *Borrelia burgdorferi*.

The successful production of specific antibodies was confirmed by ELISA and WB methods, and curiously the immunoblotting showed different humoral reactivity between the serum obtained from sensitized dog and positive test samples from the region of Cotia. This data suggested the presence of a different etiological agent in Brazil.

As WB is a more specific method for *Borrelia burgdorferi* antibodies detection, it was used to confirm the results of ELISA test of samples and controls. It was considered positive WB, when the assay showed reactivity to at least 5 bands[13]. Twenty of 23 (86.9%) positive test samples at ELISA were confirmed by Western blotting assay, revealing good correlation between these methods. The remaining three samples and eight negative controls sera were considered negative for *B. burgdorferi* antibodies by WB analysis.

The possibility of cross reaction between *Borrelia burgdorferi* and others spirochetes is likely[18]. At the present work four sera (1.7%) of the dogs had presented simultaneous antibodies against both infections, but no correlation could be found.

SOARES in 1998[21] studied presence of IgG class antibodies to *B. burgdorferi* and *Babesia canis* in 150 dogs sera from Itaguaí, Rio de Janeiro, Brazil, and using ELISA test standardized at same way, obtained similar results, when it was found 30 positive samples (20.0%) for borrelia antigens, and did not find serological cross-reactivity between these diseases.

Interestingly, low titers of antibodies to known borrelias such us *B. burgdorferi*, *B. garinii* and *B. afzelli* are obtained with sera of Brazilian patients with Lyme disease, becoming difficult the diagnosis of this illness in our country[26,27,28]. WB pattern is also quite different, suggesting the presence of another etiological agent. Spirochete like microorganisms are seen in peripheral blood and cerebrospinal fluid of patients with borreliosis, however are uncultivable and not identified by PCR assays[1,28]. The present work done in dogs from risk area for Lyme disease, confirms data obtained in humans, supporting the idea of existence of a slightly different clinical entity in Brazil, which has been named as Lyme like disease.

RESUMO

Pesquisa de anticorpos anti- *Borrelia burgdorferi* em cães da região de Cotia, São Paulo, Brasil

Com a finalidade de estudar a ocorrência da doença de Lyme em cães no Brasil, o teste imunoenzimático (ELISA) indireto, utilizado para o diagnóstico da doença no homem, foi padronizado para a espécie canina. Utilizou-se como antígeno a cepa americana de *Borrelia burgdorferi* G 39/40. Os soros de cães procedentes da região de Cotia, área de risco para ocorrência da doença de Lyme, foram testados e os resultados positivos foram confirmados através da técnica de "Western blotting". Para investigação de possíveis reações cruzadas, os soros foram também testados para diferentes variantes sorológicas de *Leptospira interrogans* e *L. biflexa* pela técnica de soroaglutinação microscópica. Dos 237 soros testados pela técnica de ELISA, 23 (9,7%) foram positivos, sendo que 20 (86,9%) destes soros foram também positivos pela técnica de "Western blotting", sugerindo que a região de Cotia seja área de risco para doença de Lyme. Embora 4 (1,7%) dos soros testados apresentassem positividade para doença de Lyme e para leptospirose concomitantemente, não foi observada associação entre estes resultados (X^2 = 0,725 e p = 0,394), sugerindo ausência de reatividade sorológica cruzada entre as duas enfermidades.

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