

ORIGINAL ARTICLE

An enzyme-linked immunosorbent assay (ELISA) for serological detection of antibodies against *Borrelia* in calves in Brazil

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ABSTRACT: Bacteria of the genus *Borrelia* are described as pathogenic agents of several human and animal diseases. An infection nowadays is usually detected via determination of circulating antibodies against *Borrelia*-specific antigens in body fluids. Thus, the aim of this study was to detect circulating antibodies against *Borrelia* sp. by enzyme-linked immunosorbent assay (ELISA) in cattle. Seven hundred twenty serum samples from 20 female bovines living in tick-infested areas were collected during three years. Statistical analyses were done by qui-square or Fischer's exact tests, with a confidence level of 95%. For homologous antibodies response to *Borrelia* sp., the iELISA test showed 93.33% sensitivity and 86.66% specificity. The prevalence of antibodies against *Borrelia* sp. were 15%, 23.8%, 48.6% and 65.8% at birth, first, second and third year, respectively. The rate of animal showing antibodies to *Borrelia* sp. indicated the presence of the tickborne spirochaetal agent in the cattle population in the studied region. Therefore, an ELISA could be used to confirm the exposure of cattle to *Borrelia* sp.

Key words: cattle, diagnostics, serologic testing, spirochaetes.

Un ensayo de inmunoabsorción ligado a enzimas (ELISA) para la detección serológica de anticuerpos contra *Borrelia* sp. en terneros en Brasil

RESUMEN: Las bacterias del género *Borrelia* se describen como agentes patógenos de varias enfermedades humanas y animales. Actualmente la infección se detecta, por lo general, mediante la determinación de anticuerpos circulantes contra antígenos específicos de *Borrelia* en los fluidos corporales. El objetivo del presente trabajo fue detectar, anticuerpos circulantes en bovinos, contra *Borrelia* sp. mediante un ensayo ELISA. Se colectaron durante tres años 700 muestras de suero de 20 bovinos hembras, que viven en zonas infestadas de garrapatas. Los análisis estadísticos se realizaron mediante Chi cuadrado y la prueba de Fischer, con un nivel de confianza del 95%, para una respuesta de anticuerpos homólogos a *Borrelia* sp. La prueba iELISA mostró 93,33% de sensibilidad y 86,66% de especificidad. La prevalencia de anticuerpos contra *Borrelia* sp. fue 15%, 23,8%, 48,6% y 65,8% en el nacimiento, primero, segundo y tercer años, respectivamente. La cantidad de animales que tenía anticuerpos contra *Borrelia* sp. indica la presencia de esta espiroqueta transmitida por garrapatas en la población bovina en la región estudiada, por lo que se plantea que el ELISA se puede utilizar para confirmar la exposición de bovinos a *Borrelia* sp.

Palabras clave: ganado, diagnósticos, pruebas serológicas, espiroquetas.

INTRODUCTION

Lyme borreliosis is the most common tick-borne disease in Europe and the USA and can lead to multi-organ infections in humans and animals (1). This zoonosis is caused by spirochaetes belonging to the

Borrelia burgdorferi sensu lato complex, which comprises worldwide at least 18 genospecies (2). An infection nowadays is usually detected via determination of circulating antibodies against *Borrelia*-specific antigens in body fluids.

Epidemiologically, reservoir hosts in which the pathogen can be amplified, and non-competent hosts not developing a systemic infection, are of importance (3). Ecological studies on Lyme borreliosis have shown that certain genospecies are associated with different host species. The prevalence of bovine borreliosis ranges from 30.0% to 90.0% in the state of Rio de Janeiro, Brazil (4).

For detection of antibodies against *Borrelia*, antigens purified from in vitro cultures have been used in immunoassays, such as ELISA or immunoblot, for more than two decades. However, the use of native preparations is hampered by their complicated cultivation in complex and costly culture media as well as the marked variations in growth rates and gene expression patterns (5). Thus, the aim of this study was to detect circulating antibodies against *B. burgdorferi* by enzyme-linked immunosorbent assay (ELISA) in cattle.

MATERIALS AND METHODS

Study area

The field activities were conducted in 2008 and 2010, in the dairy cow section at the Seropédica Experimental Station, which belongs to the Agricultural and Livestock Research Company of the State of Rio de Janeiro, located in the Rio de Janeiro Metropolitan microregion (latitude 22°45'S, longitude 43°41'W and altitude 33 meters). This average annual temperature of the area is approximately 22,7°C with an average annual precipitation of 1291,7 mm. This region is characterized by two well-defined seasons. The dry season (March to September) has lower temperatures and rainfall, which leads to a reduction of vector populations; the rainy season (October to February) has higher temperatures and rainfall, resulting in an increased number of vectors.

Study design and sample size methodology

In the herd used in this study, the serological prevalence of *B. burgdorferi* was 70% and no clinical cases of this disease had been observed over the preceding 3 years (4). A longitudinal study was conducted. Twenty calves were selected from day of birth to three years of age. The calves used were specific pathogen free calves. The animals were bled monthly for three years to obtain a total of 240 serum samples per year. The animals were kept on pasture consisting of *Brachiaria decumbens* and *Panicum maximum* at a density of three animals per hectare. In both areas, the animals received water and mineral salt *ad libitum*.

Obtaining the antigen for *Borrelia burgdorferi*

The antigen used for the serological tests came from a cultivar of the G39/40 strain of *B. burgdorferi stricto*

sensu from North America. Spirochetes were grown in Barbour-Stoener-Kelly (BSK-H) medium and, at maximum bacterial growth, antigens were produced as described by Ishikawa *et al.* (6). The *B. burgdorferi* G39/40 strain was kindly provided by Professor Dr. Natalino Hajime Yoshinari, of the Medical Investigation Laboratory for Rheumatology, Hospital das Clínicas, School of Medicine of the University of São Paulo (LIM-17/HCFMUSP).

Production of positive and negative sera for *Borrelia burgdorferi*

The positive control for the serologic test was produced according to Ishikawa *et al.* (6). Sera were collected from specific pathogen free Holstein calves (n = 2) experimentally infected with *B. Burgdorferi* from day 5 to day 125 postinfection (PI). Negative sera (n = 15) were collected from calves prior to infection and before they suckled colostrum.

Serological testing

The ELISA method used was essentially that described in detail by Machado *et al.* (7) for *Babesia bovis* with minor modifications. Briefly, 100 µl of antigen diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, was added to each well of a micro-ELISA plate (Costar 3590, Corning Co.) and the protein concentration was adjusted to 5 µg/ml⁻¹, 10 µg/ml⁻¹, 15 µg/ml⁻¹, and 20 µg/ml⁻¹. The plates were sealed and incubated overnight at 4°C. Plates were blocked for 1 h at 37°C in a humid chamber with 3% ovalbumin in carbonate/bicarbonate buffer. After five washes with buffer (phosphate buffered saline, pH 7.2, and 0.05% Tween 20, PBS-Tween), 100 µl of diluted bovine sera (1:400 to 1:800) in PBS-Tween plus 5% normal rabbit serum were added in duplicate to the ELISA plate. The plates were incubated at 37°C in a humid chamber for 90 min and then washed five times with PBS-Tween. A 100 µl aliquot of a 1:5000 dilution of alkaline phosphatase conjugated anti-bovine IgG (Sigma Chemical Co.) was added to each well and the plates were incubated at 37°C under the same conditions for 90 min. The plates were washed five times with PBS-Tween. The appropriate substrate p-nitrophenyl phosphate (PNPP; Sigma Chemical Co.) was added and the plates were sealed and incubated for 30 min at room temperature. At the end of the incubation, the plates were read at 405 nm wavelength on a micro-ELISA reader (Labsystems iEMS Reader MF).

The immunological activity of each serum was calculated by determining the sample to positive serum ratio (S/P) at each dilution, considering positive and negative sera as reference, using the following equation:

$$S/P = \frac{((\chi \text{ sample absorbance}) - (\chi \text{ absorbance of negative serum reference}))}{((\chi \text{ absorbance of positive reference serum}) - (\chi \text{ absorbance of reference serum}))}$$

S/P values were grouped into ELISA levels (EL), which ranged from 0 (lowest level) to 9 (highest level). The subsequent levels were determined by increment of 35% as described by Wilson *et al.* (8) for the Newcastle system.

Statistical analyses

To investigate the influence of the variable year as a risk factor for occurrences of *B. burgdorferi* in dairy cows, the average antibody title observed at birth, first, second and third year were subjected to the qui-square or Fischer's exact tests to ascertain whether any association existed between them. A qui-square or Fischer's exact tests were used to determine significant differences in percentages of positive results. Values of $p < 0.05$ were considered to be statistically significant. The operational procedures were done using the R Foundation statistical computing software, version 2.12.2 (2011).

RESULTS

Selected conditions were as follows: (1) 15 μg antigen ml^{-1} in carbonate buffer, pH 9.4; (2) negative and positive sera diluted 1:400 in TBS, giving average absorbance of 1.013 ± 0.0084 for positive sera ($n = 15$) and 0.170 ± 0.0015 for negative sera ($n = 15$). The EL was determined as shown in Table 1.

As shown in Figure, sera from twenty calves naturally infected with *Borrelia* sp. were screened by ELISA from day of birth to three years of age. Seven samples (7 animals) showed a higher average antibody title (EL= 6) than the low average antibody title showed (EL= 0) by 103 samples during the three years of the study.

For antibodies response to *Borrelia* sp., the iELISA test showed 93.33% sensitivity and 86.66% specificity. The prevalence of antibodies against *Borrelia* sp. were 15%, 23.8%, 48.6% and 65.8% at birth, first, second and third year, respectively (Table 2).

DISCUSSION

The use of highly specific recombinant antigens in automated enzyme-linked immunosorbent assays has improved the sensitivity and specificity of detecting antibodies to *B. burgdorferi* in humans, dogs, and horses (9, 10). Whole-cell *B. burgdorferi* antigens have been used in indirect fluorescent antibody (IFA) staining, immunoblotting, and ELISA methods in studies of cattle and buffaloes (4, 11, 12), but there is little information available on the performance and suitability of ELISA with recombinant antigens for these animals.

Although a comparison of immunoblot and ELISA results revealed discrepancies between banding patterns and serum reactivities to *B. burgdorferi*

TABLE 1. S/P values for EL 0-9./ *Valores de S/P para EL 0-9.*

EL	A/P	% sample for levels Elisa (EL)		
		< 1 year	1 - 2 year	2 - 3 year
0	0 - 0.173	14.7%	0.0%	0.0%
1	0.174 - 0.235	22.9%	0.0%	0.0%
2	0.236 - 0.318	21.0%	11.4%	5.7%
3	0.319 - 0.430	18.1%	40.0%	28.6%
4	0.431 - 0.582	17.1%	34.3%	51.4%
5	0.583 - 0.787	6.7%	14.3%	11.4%
6	0.788 - 1.0.63	0.0%	0.0%	2.9%
7	1.064 - 1.436	0.0%	0.0%	0.0%
8	1.437 - 1.940	0.0%	0.0%	0.0%
9	> 1.941	0.0%	0.0%	0.0%

EL = Elisa levels; S/P = Absorbance sample

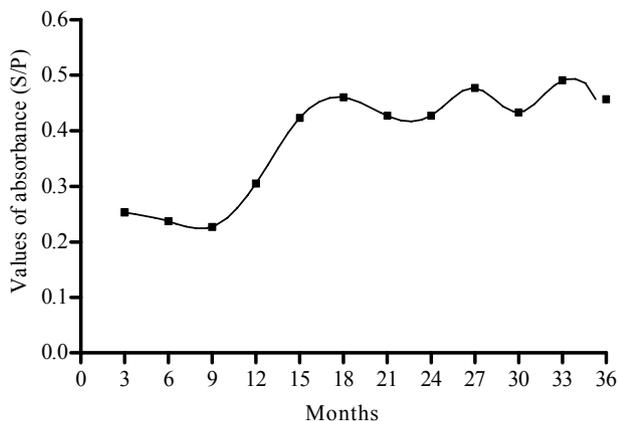


FIGURE. ELISA absorbance of sera from cattle naturally infected with *Borrelia burgdorferi*./ *Absorbancia del ELISA de sueros de bovinos infectados con Borrelia burgdorferi*.

recombinant antigens (13), each method of antibody detection had advantages and disadvantages regarding sensitivity, specificity, ease of standardization, and expense. Therefore, different antibody-detection assays should be used to help determine whether hosts were exposed to *B. burgdorferi*.

The occurrence of the Lyme disease-like syndrome referred by Mantovani *et al.* (14) in Brazil, justify the seroepidemiological study in cattle, because of the

cross-reacting antibodies between *Borrelia theileri* and other species of *Borrelia* genus (15). The rate of cattle that showed homologues antibodies to *B. burgdorferi* indicated the presence of the tickborne spirochaetal agent in the herds evaluated. The rate of seropositive cattle indicated the presence of some agent of tickborne spirochetosis in the herd assessed, which showed 54.9% seropositivity to *B. burgdorferi* in dairy cows in the state of Pará, Brazil (16).

Regarding age, a higher seroprevalence was observed in cows than in calves. Similarly, Stefanciková *et al.* (17) found a higher seroprevalence in older cows. Lyme disease has frequently been found in first-calf heifers when going into full milk production, often presenting a herd problem (18).

CONCLUSION

The rate of animal that showed homologues antibodies to *B. burgdorferi* indicated the presence of the tickborne spirochaetal agent in the cattle population in the studied region. Therefore, an ELISA could be used to confirm the exposure of cattle to *B. burgdorferi*.

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TABLE 2. Seroprevalence in calves, heifers and cows for *Borrelia burgdorferi*./ *Seroprevalencia en terneros, novillas y vacas para Borrelia burgdorferi*.

Risk factors	N	P(%)	χ^2	RP	P-valor	IC95%
Age						
(1) < 1 year*	240	22.85 %	-	-	-	-
(2) 1 – 2 year	240	48.75 %	16.8	1.90	0.03	[0.47 - 0.93]
(3) 2 – 3 year	240	65.85 %	9.4	1.76	0.04	[0.74 - 0.87]

* Reference value, N= number of sample evaluated, P(%)=Prevalence of seropositivity for *B.burgdorferi*, χ^2 = chi-square, OR= odds ratio, IC 95%= confidence interval

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