Evaluation of an ELISA for detection of antibodies to *Babesia bigemina* in cattle and it's application in an epidemiological survey in Brazil[1]

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ABSTRACT.- Madruga C.R., Marques A.P.C., Araújo F.R., Miguita M., Carvalho C.M.E., Araújo F.S., Umaki A.C.S., Crocci A.J. & Queiróz R.A. 2001. **Evaluation of an ELISA for detection of antibodies to Babesia bigemina in cattle and it's application in an epidemiological survey in Brazil.** *Pesquisa Veterinária Brasileira 21(2):72-76.* Embrapa Gado de Corte, Rodovia BR 262 Km 4, Cx. Postal 154, Campo Grande, MS 79002-970, Brazil.

An indirect enzyme-linked immunosorbent assay (ELISA) using a crude antigen was evaluated for its performance to detect *Babesia bigemina* antibodies. The sensitivity and specificity were 98.0% and 99.0%, respectively. In agreement with the high specificity, no cross-reactions were verified with sera from calves inoculated three times with 10^7 *Babesia bovis* organisms. With regard to the comparison of ELISA and indirect fluorescent antibody test (IFAT) in detecting antibodies against *B. bigemina* in calves experimentally infected with five Brazilian geographical isolates of this hemoparasite, IFAT was able to detect antibodies one day earlier in most of the calves' sera. There was a good agreement between results shown by ELISA and IFAT with sera from an enzootically stable area (k=0.61). However, there was no agreement between these serological tests with sera from an enzootically unstable area (k=0.33). The ELISA was employed in an epidemiological survey using with 1,367 sera from four counties in the Pantanal of Mato Grosso do Sul and characterized this region as an enzootically stable area, since the prevalence ranged from 87.7 to 98.9%. Therefore, this ELISA with high sensitivity, specificity and performance similar to IFAT can be employed in serological diagnosis of *B. bigemina*.

INDEX TERMS: *Babesia bigemina*, ELISA, serological tests, cattle, Pantanal.

RESUMO.- [**Avaliação de um ELISA para detecção de anticorpos contra Babesia bigemina em bovinos e sua aplicação em um inquérito sorológico no Brasil.**] Um ensaio de imunoadsorção enzimática (ELISA) baseado em antígeno bruto foi avaliado na detecção de anticorpos contra *Babesia bigemina*. A sensibilidade e a especificidade do teste foram de 98,0% e 99,0%, respectivamente. Concordan-

do com a alta especificidade do teste, não foram verificadas reações cruzadas com soros de bezerros inoculados três vezes com 10^7 merozoítos de *Babesia bovis*. Com relação à comparação do ELISA com a imunofluorescência indireta (IFAT) na detecção de anticorpos contra *B. bigemina* em bezerros experimentalmente infectados com cinco isolados brasileiros geograficamente distintos deste hemoparasito, o IFAT foi capaz de detectar anticorpos um dia antes do ELISA na maioria dos soros dos animais. Houve uma boa concordância entre os resultados encontrados no ELISA e no IFAT com soros de bovinos de região de estabilidade enzoótica (k=0.61). No entanto, não houve concordância entre os testes sorológicos com soros de animais de área de instabilidade enzoótica (k=0.33). O ELISA foi empregado em um inquérito epidemiológico com 1.367 soros de quatro municípios do Pantanal de Mato Grosso do Sul e caracterizou esta região como uma área de estabilidade enzoótica, uma vez que as

prevalências variaram de 87,7 a 98,9%. Dessa forma, este ELISA, que apresentou alta sensibilidade, especificidade e desempenho similar ao IFAT, pode ser utilizado no diagnóstico sorológico de *B. bigemina*.

TERMOS DE INDEXAÇÃO: *Babesia bigemina*, ELISA, testes sorológicos, bovinos, Pantanal.

INTRODUCTION

Bovine babesiosis is a tick-borne protozoan disease of considerable economic importance in cattle herds within tropical and subtropical regions (Buening & Figueroa 1992). In these areas, the disease is caused by *Babesia bovis* and *Babesia bigemina*, which are transmitted by *Boophilus* spp ticks (McCosker 1981).

A large number of serological tests have been used for detection of antibodies to *Babesia* (Reiter & Weiland 1989, Katsande et al. 1999), as the basis for epidemiological surveys (Passos et al. 1998, Sahibi et al. 1998) and for evaluation of preventive measures, such as vaccination (Guglielmone et al. 1997). Among these tests, the enzyme-linked immunosorbent assay (ELISA) is preferred since it is less subject to operator error (el-Ghaysh et al. 1996).

Problems in using ELISA with crude antigen for detection of antibodies to *B. bigemina* have been reported, mainly concerning it's lack of specificity (Waltisbuhl et al. 1987, Gray & Kaye, 1991, el-Ghaysh et al. 1996). Such antigens frequently contain host cell components, which may affect the performance of the test by increasing the non-specific background (Passos et al. 1998). These problems led some researchers to consider crude antigen ELISAs for *B. bigemina* unreliable (Böse et al. 1995, el-Ghaysh et al. 1996).

Recently, a highly specific competitive ELISA, based on monoclonal antibodies to a single epitope (p58), was developed for *B. bigemina* (Molloy et al. 1998). In spite of the high specificity of this test, the techniques employed for its development require well-equipped laboratories, often not available in developing countries. For these reasons, the search for alternatives to increase the performance of crude antigens for *B. bigemina* ELISAs still must be encouraged.

The Pantanal region of Mato Grosso do Sul (MS), Brazil, has a low density of cattle, predominantly of the Nelore (*Bos indicus*) breed. It is characterized by a rainy season with flooding of large areas followed by a dry season. All these factors are not favorable for *Boophilus microplus* tick development, the only vector of *Babesia* spp in the region. Therefore, some Pantanal areas could be enzootically unstable. This paper describes the performance of an ELISA for *B. bigemina* antibodies based on a crude antigen and it's use to characterize the epidemiology of *B. bigemina* in the Pantanal region of MS.

MATERIALS AND METHODS

The ELISA antigen source was blood with 25.0% parasitemia from a splenectomized calf experimentally inoculated with an isolate of *B. bigemina* from MS, Brazil (Kessler et al. 1987). To avoid false-positive reactions, the calf blood was collected before *B. bigemina* antibodies were produced.

The infected blood was washed three times with phosphate buffer saline (PBS) (83.3 mM KH_2PO_4, 66 mM Na_2HPO_4 14.5 mM NaCl), pH 7.2, by centrifugation at 12,100 x g, for 30 minutes, for buffy coat removal, and stored at -72°C. At the moment of antigen preparation, the blood was thawed at 37°C and the erythrocytes were washed three times with PBS, pH 7.2, by centrifugation at 12,100 x g, during 30 minutes, at 4°C. After the last centrifugation, the intermediate layer of the pellet was collected and lysed with a solution containing 100 mM tris, 10 mM EDTA, 0.2 mM N-a-p-tosyl-L-lysil chloromethyl ketone, 2 mM phenylmethyl sulfonylfluoride and 2% Nonidet P40 (v/v), and by ultrasonic disruption (100 W for 10 minutes). The material was centrifuged at 23,700 x g for 60 minutes at 4°C and the supernatant was harvested and used as antigen. The total protein concentration of the antigen, estimated by the Folin's reagent method (Lowry et al. 1951), was 5 mg/mL.

After a series of experiments based on the largest optical density difference between positive and negative control sera, the following procedure was chosen. The antigen was diluted 1:1,000 in carbonate/bicarbonate buffer, pH 9.0 (200 mM Na_2CO_3, 199 mM NaHCO_3) and 100 mL were placed into each well of 96 well plates (Costar, flat bottom, high binding, 3590). The adsorption time of the antigen was four hours at 4°C. Subsequently, the plates were washed five times with PBS containing 0.1% tween 20 (PBST). One hundred microliters of bovine serum, diluted 1:1,000 in PBST, were added to each well and incubated for 45 minutes at 37°C. After washing as described above, 50 mL of rabbit anti-bovine IgG alkaline phosphatase conjugate (Sigma, A-0705), diluted 1:12,000 in PBST, were added to each well and the plates were incubated for 30 minutes. After washing the plates 10 times with PBST, 50 mL of the substrate p-nitrophenyl phosphate (1.0 mg/mL in substrate buffer: 0.1 M diethanolamine, 0.1 M $MgCl_2$, 0.1 M NaCl) were added to each well. The reaction was stopped 20 minutes later by adding 100 mL of 0.2 M NaOH to each well and optical densities measured in a microplate reader with a 405 hm filter.

The preparation of the indirect fluorescet antibody test (IFAT) antigen, as well as the test procedure, were described elsewhere (Madruga et al. 1986). Briefly, blood from splenectomized calves with 5.0% *B. bigemina* parasitemia was collected and washed with PBS, pH 7.2, by centrifugation at 1,090 x g, during 15 minutes. After each centrifugation, the buffy coat was removed. Then, thin blood smears were prepared and stored at −70°C. At the moment of the analyses, the smears were dried for 10 minutes at 37°C and circles were drawn with finger nail polish. An amount of 10 mL of serum, diluted 1:160 in PBS, was added to each circle on the slide and incubated for 30 minutes at 37°C. Following three washes with PBS, the smears were incubated for 30 minutes with rabbit anti-bovine IgG fluorescein isothiocyanate conjugate (Sigma, F-7887) diluted 1:320 in PBS. Finally, the smears were washed three times, dried and examined by epifluorescent microscopy.

The ELISA cut-off was determined as the optical density (OD) mean of 100 cattle sera from tick-free area of Embrapa Gado de Corte, Campo Grande, MS, Brazil, plus two standard deviations. All sera were negative for antibodies against *B. bigemina* in the IFAT.

The ELISA sensitivity was determined with 50 sera from cattle experimentally infected with *B. bigemina* and positive for antibodies to this hemoparasite in IFAT. The ELISA specificity was determined with 100 calf sera kept in the sera bank, obtained from six to ten month-old calves raised in a tick-free area of Embrapa Gado de Corte. To ensure that these animals were not *B. bigemina* carriers, blood

smears stained by May-Grünwald-Giemsa and IFAT were performed periodically. Only negative animals in both tests were included in this study.

The performances of the ELISA and IFAT were compared with 160 cattle sera from an enzootically stable area to *B. bigemina* (Campo Grande, MS, Brazil), and with 80 cattle sera from an enzootically unstable area (Bagé, Rio Grande do Sul, Brazil). The analyses of the agreement between ELISA and IFAT was done by kappa test (Altman 1996).

The ELISA and IFAT were also compared with sera from five groups of five calves, each one of them infected with 10^9 organisms of one of five Brazilian geographical isolates of *B. bigemina* (North, Northeastern, Southeastern, Mid-West and South). Sera from these calves were obtained until 30 days post-infection and the parasitemias were determined by thin smears stained by May-Grünwald-Giemsa.

To evaluate cross reactions with *B. bovis* antibodies, six 6-10 month-old calves, free of antibodies against *Babesia* spp, determined by IFAT, were inoculated with 10^7 *B. bovis* organisms three times at intervals of 15 days. Sera from these animals were collected at 15 days intervals from inoculation, until 60 days post-inoculation. The sera of these animals were seropositive to *B. bovis*, at different end titers ranging from 160 to 10,240 in the IFAT.

After *B. bigemina* ELISA standardization, an epidemiological survey was conducted in the Pantanal region, MS, Brazil. This area is an 138,000 Km^2 tropical seasonal wet land located in the center of South America between 16° and 21°S and 55° and 58°W and contains approximately 1,100 extensive cattle ranches, varying from 10,000 to 200,000 hectares and three million cattle (Seidl et al. 1998, Dávila et al. 1999). Sera from 1,367 beef cattle (predominantly Nelore breed), of both sexes and varying ages, were randomly selected from the counties of Corumbá, Porto Murtinho, Rio Verde and Aquidauana.

The sample size was determined by the following mathematical model of the Centro Pan-Americano de Zoonoses (1979): n = (p(100-p)Z^2/(dp/100)^2, in which: n = number of sera, p = estimated prevalence, Z = confidence degree and d = error probability.

Initially, 50 sera were analyzed in each region by IFAT for *B. bigemina* antibodies. Based on an estimated prevalence of seropositive cattle of 85.0%, with a degree of confidence of 95.0% and an error probability of 10.0%, a sample of 71 sera was determined for each county. As there was a higher number of sera available, we analyzed 618 sera from Corumbá, 92 from Porto Murtinho, 308 from Rio Verde and 349 from Aquidauana.

The comparison of the prevalences of seropositive cattle between the four counties was done by Goodman's test (Goodman 1965).

RESULTS

The mean OD of the 100 negative sera was 0.033, with a SD of 0.027, corresponding to a cut-off of 0.087.

The results showed that 49 out of the 50 serum samples from experimentally infected cattle were positive in ELISA, corresponding to a sensitivity of 98.0% (Table 1). Only one out of the 100 sera samples from cattle kept in a tick-free area was positive in ELISA, corresponding to a specificity of 99.0%.

With regards to the comparison of ELISA and IFAT in detecting antibodies against *B. bigemina* in cattle from enzootically stable area, there were 141 ELISA+/IFAT+ sera and nine ELISA-/IFAT- sera, corresponding to an agreement between both serological tests of 93.75%. There were also seven (4.4%) ELISA+/IFAT- sera and three (1.9%) ELISA-/IFAT+

Table 1. Validation of ELISA with sera from calves experimentally inoculated with *Babesia bigemina* or free from infection with this hemoparasite[a]

ELISA	Infection status of the calves		
	Positive	Negative	Total
Positive	49 (a)	1 (b)	50 (a+b)
Negative	1 (c)	99 (d)	100 (c+d)
Total	50 (a+c)	100 (b+d)	150 (a+b+c+d)

[a]Acoording to Coggon et al. (1993):
Sensitivity: a / (a+c) x 100 = 49/50 x 100 = 98.0%;
Specificity: d / (b+d) x 100 = 99/100 x 100 = 99.0%;
Positive predictive value: a / (a+b) x 100 = 49/50 x 100 = 98.0%;
Negative predictive value: d / (c+d) x 100 = 99/100 x 100 = 99.0%;
Precision: (a+d) / (a+b+c+d) x 100 = 148/150 x 100 = 98.7%.

Table 2. Comparison between ELISA and IFAT in detecting antibodies against *Babesia bigemina* in 160 cattle sera from an enzootically stable region and in 80 cattle sera from an enzootically unstable region of Brazil

Epidemiological condition		Serological status		
		ELISA+	ELISA -	Total
Enzootic stabilityl	FAT +	141	3	144
	IFAT -	7	9	16
	Total	148	12	160
Enzootic instability	IFAT +	6	2	8
	IFAT -	14	58	72
	Total	20	60	80

Table 3. Prevalences of seropositive cattle to *Babesia bigemina* in four counties of Pantanal region, Mato Grosso do Sul, Brazil, by ELISA

County	Number of sera tested	Number of positive sera	(%)
Corumbá	618	583	(94.3)[b*]
Porto Murtinho	92	91	(98.9)[a]
Rio Verde	308	270	(87.7)[c]
Aquidauana	349	345	(98.9)[a]
Total	1367	1289	(94.3)

*Different letters mean statistical differences between lines (P<0.05).

sera (Table 2). The statistical analyses showed a good agreement between ELISA and IFAT, with k=0.61.

In the analyses of 80 sera of cattle from enzootically unstable area, there were six ELISA+/IFAT+ sera and 58 ELISA-/IFAT- sera, corresponding to an agreement between both serological tests of 80.0%. There were also 14 (17.5%) ELISA+/IFAT- sera and two (2.5%) ELISA-/IFAT+ sera (Table 2). The statistical analyses showed no agreement between ELISA and IFAT, with k=0.33.

With some exceptions, most of the calves experimentally inoculated with the five Brazilian isolates of *B. bigemina* showed detectable immunoglobulins against *B. bigemina* on the fifth or sixth day post-infection in the IFAT and/or ELISA
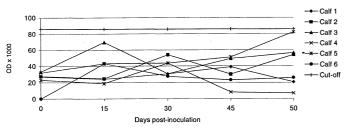
Fig. 1. Determination of cross-reactions in *Babesia bigemina* ELISA with sera from six calves experimentally inoculated with *Babesia bovis*

(data not shown). The IFAT showed positive reactions earlier than ELISA did in the sera of 11 calves, while the opposite occurred in the sera of nine calves. In the majority of cases, the intervals of first detection of antibodies between IFAT and ELISA were one day (14 out of 20 calves). The remaining five calves displayed positive reactions at the same time in both serological tests.

No cross-reactions with *B. bovis* antibodies were detected with the 30 sera from six calves experimentally infected with this hemoparasite (Fig. 1).

The results of the epidemiological survey conducted in the Pantanal region are shown in Table 3. The prevalences of seropositive cattle in the four counties ranged from 87.7% to 98.9%, what characterizes this region as enzootically stable.

DISCUSSION

Accurate diagnosis of *B. bigemina* infection is essential for the management of disease control and for epidemiological studies. Among the current methods of diagnosis, ELISA test has been increasingly used. Nevertheless, one of the problems in developing ELISAs for babesial infections is the contamination of antigen preparations with host proteins (Waltisbuhl et al. 1987, Gray & Kaye 1991, el-Ghaysh et al. 1996).

We have developed an ELISA with crude antigen, obtained from splenectomized calves experimentally infected with a Brazilian *B. bigemina* isolate, that has proven to be highly specific, as shown by the low number of false-positive reactions (1.0%) and the lack of cross-reactions with *B. bovis* antibodies. The test was also highly sensitive (98.0%) in detecting *B. bigemina* antibodies. Such results were comparable to those obtained by Molloy et al. (1998), using a competitive ELISA based on monoclonal antibodies directed against a 58 kDa *B. bigemina* merozoite antigen, which showed a sensitivity and specificity of 95.7% and 97.0%, respectively.

The performance of a crude antigen ELISA in detecting antibodies to *B. bigemina* in cattle sera from the enzootically stable area was very similar to the IFAT, with agreement between both tests. However, there was no agreement between the results shown by ELISA and IFAT with sera from the enzootically unstable area, according to the kappa test (Altman 1996). This could reflect the higher number of seropositive cattle detected by ELISA (20 out of 80) than IFAT (8 out of 80) and suggests that in such epidemiological

conditions, ELISA is more sensitive than IFAT. The possibility that these results could be false-positive reactions in ELISA seems minimal, since this test showed a high specificity (99.0%) with sera from calves raised in a tick-free area.

The analyses of the sera from cattle experimentally inoculated with different Brazilian isolates of *B. bigemina* showed that IFAT detected antibodies against this hemoparasite earlier than ELISA in the majority of the animals, except those inoculated with Northeastern isolate. One hypothesis to explain this tendency is that in IFAT, the antibodies predominantly detected are those against surface antigens, which are produced earlier in the course of the infection, while the ELISA detects predominantly antibodies against somatic antigens, that appear later in the course of the infection.

The similarity in the results obtained by the ELISA and IFAT with sera from calves inoculated with the five Brazilian isolates suggests that the possible antigenic variations between geographical isolates of *B. bigemina* did not influence the performance of both tests.

Although the IFAT is one of the most commonly used tests, ELISA has some advantages. The results can be obtained directly through an ELISA reader, making it possible to evaluate a larger number of serum samples and avoiding problems with doubtful interpretations, common in weak fluorescent reactions.

The prevalence of antibodies against *B. bigemina* in Pantanal region was similar to those found in other enzootically stable Brazilian regions, such as Bahia (95.0%) (Araújo et al. 1997). The characteristics of the Pantanal, such as low cattle density per area, zebu cattle breed, rainy season with flood lands followed by dry season did not create an enzootic unstable area. This probably occurred due to *B. microplus* efficiency in the transmission of *B. bigemina*. In Mato Grosso do Sul highlands, this tick exhibited a transmission rate of 0.0088 (Madruga et al. 1987), which was higher than that described in Australia (Mahoney 1975).

According to the results obtained in this study, the crude antigen ELISA developed is very sensitive and specific in the detection of antibodies against *B. bigemina*. Thus, it can be a useful tool in epidemiological studies.

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