

## Evaluation of an enzyme-linked immunosorbent assay to detect antibodies against *Anaplasma marginale*<sup>1</sup>

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**ABSTRACT.**— Madruga C.R., Marques A.P.C., Leal C.R.B., Carvalho C.M.E., Araújo F.R. & Kessler R.H. 2000. Evaluation of an enzyme-linked immunosorbent assay to detect antibodies against *Anaplasma marginale*. *Pesquisa Veterinária Brasileira* 20(3):109-112. Embrapa Gado de Corte, Rodovia BR 262 Km 4, Cx. Postal 154, Campo Grande, MS 79002-970, Brazil.

A rapid indirect enzyme-linked immunosorbent assay (ELISA) was developed for measuring antibodies against *Anaplasma marginale* using a partially soluble antigen prepared from semi-purified initial bodies from erythrocytes with 80.0% of rickettsiaemia. This technique utilized alkaline phosphatase and p-nitrophenyl phosphate as reaction indicators. The high sensitivity (100.0%) was confirmed with sera from 100 calves experimentally-infected with *A. marginale*. All of these animals showed seroconversion before or at the same time of the first rickettsiaemia or even when it was not detected. Also the elevated specificity (94.0%) was confirmed by the low percentage of cross-reactions with sera from animals experimentally-infected with *Babesia bigemina* and *Babesia bovis* (1.4 and 6.6%, respectively). Performances of ELISA and indirect fluorescent antibody test (IFAT) with 324 sera from enzootically stable area did not show statistical difference ( $P > 0.05$ ), since the former showed 96.9% and the latter 97.2% of positive reactions. The advantage of this ELISA is a shorter execution time than others developed until now, allowing more samples to be analyzed.

INDEX TERMS: *Anaplasma marginale*, ELISA, serological tests, cattle.

**RESUMO.**— [Avaliação de uma prova de imunoabsorção enzimática para detecção de anticorpos contra *Anaplasma marginale*.] Um ensaio de imunoabsorção enzimática (ELISA) indireto, foi desenvolvido para detecção de anticorpos contra *Anaplasma marginale*, utilizando um antígeno parcialmente solúvel preparado a partir de corpúsculos iniciais semipurificados de eritrócitos com 80,0% de parasitemia. Esta técnica utilizou a fosfatase alcalina e o p-nitrofenil fosfato como indicadores de reação. A alta sensibilidade (100,0%) foi comprovada com soros de 100 bezerros experimentalmente infectados com *A. marginale*. Todos estes animais soroconverteram antes ou ao mesmo tempo que a primeira parasitemia foi detectada, ou mesmo quando não se obteve parasitemia. A elevada especificidade (94,0%) foi confirmada pela baixa percentagem de reações cruzadas com soros de animais

infectados experimentalmente com *Babesia bigemina* e *Babesia bovis* (1,4 e 6,6%, respectivamente). Os desempenhos do ELISA e da imunofluorescência indireta (IFI) com 324 soros de uma área de estabilidade enzoótica não diferiram estatisticamente ( $P > 0,05$ ), uma vez que o primeiro teste detectou 96,9% de reações positivas, enquanto o segundo, 97,2%. A vantagem deste ELISA foi um menor tempo de execução do que outros desenvolvidos até o momento, permitindo a análise de um maior número de soros.

TERMOS DE INDEXAÇÃO: *Anaplasma marginale*, ELISA, testes sorológicos, bovino.

### INTRODUCTION

Anaplasmosis is an infectious and transmissible disease of cattle and some wild ruminants, caused by the intraerythrocytic rickettsia *Anaplasma marginale*. This microorganism is widespread in tropical and subtropical areas of the world causing important economic losses, such as weight loss, reduced milk production, mortality and costs with treatment and prevention (Kessler et al. 1992).

In Brazil, as well as other Latin American countries, the economic impact is unknown but it is estimated to be high.

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In the United States the losses due to anaplasmosis were figured out around US\$ 300 millions per year (Palmer 1989).

Prevention may therefore be required in many circumstances and the best tool to make this decision is the prevalence data given by serological tests. Additionally, the serological tests are also important for evaluation of preventive measures employed to control anaplasmosis.

Many serologic techniques have been developed in the last decades, however the enzyme-linked immunosorbent assay (ELISA) that detect antibodies against various infectious diseases is the most advantageous, mainly because it is automated. ELISAs with higher sensitivity than the indirect fluorescent antibody technique (IFAT) (Goff & Winward 1983), card agglutination (Rose et al. 1974) and complement fixation (Mahoney 1975) have been developed recently to detect antibodies against *A. marginale* (Barry et al. 1986, Nakamura et al. 1988, De Echaide et al. 1998, Reyna-Bello et al. 1998, Saliki et al. 1998, Molloy et al. 1999). However, these ELISAs are more time-consuming than the IFAT.

The present paper describes the development and standardization of an indirect ELISA, based on semi-purified antigen, for the detection of antibodies against *A. marginale*.

## MATERIALS AND METHODS

The antigen source was calf blood with 80.0% rickettsaemia of Mato Grosso do Sul *A. marginale* isolate (Kessler et al. 1987). The infected erythrocytes were washed three times with phosphate buffer solution (10 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 6.4 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 99 mM NaCl), pH 7.2 (PBS), and stored at -72°C. During the antigen preparation, after thawing, the lysed erythrocytes were washed three times by centrifugation at 10,000 x g during 30 minutes at 4°C with PBS. After the last centrifugation, the intermediate layer was collected and lysed with a buffer solution containing 100 mM Tris, 10 mM EDTA, 0.2 mM N-a-p-tosyl-L-lysyl chloromethyl ketone, 2.0 mM phenylmethyl sulfonyl fluoride and 2.0% Tergitol NP-40 (v/v), and by ultrasonic disruption (100 W) for 10 minutes. The material was centrifuged for 60 minutes at 14,000 x g at 4°C and the supernatant was harvested and used as antigen. The total protein concentration of the antigen, measured by bicinchoninic acid method was 16.9 mg/ml.

The following procedure was the choice after a series of experiments based on the largest optical density difference between positive and negative control sera. The antigen was diluted 1:5,000 in carbonate/bicarbonate buffer, pH 9.0 (200 mM Na<sub>2</sub>CO<sub>3</sub>, 199 mM NaHCO<sub>3</sub>) and 100 µl/well were added to 96 well plates (Costar). 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 µl of rabbit anti-bovine IgG alkaline phosphatase conjugate (Sigma), diluted 1:24,000 in PBST, were added to each well and the plates were incubated for 30 minutes. After washing the plates 10 times with PBST, 50 µl of the substrate 1.0 mM p-nitrophenyl phosphate were added to each well. The reaction was stopped 15 minutes later by adding 100 µl of 0.2 M NaOH to each well and results were obtained in a microplate reader with a 405 nm wavelength.

A plate with negative and positive sera was setup as a standard and it was used for adjustment of the test plate each time the ELISA was performed. The same three positive sera and three negative

sera were used as controls in the test plates and were used for adjustment of the optical density (OD) using the following formula:  $F = (P_0 - N_0) / (P_t - N_t)$ , where F is the adjustment factor, N<sub>0</sub> is the mean OD for the negative sera and P<sub>0</sub> is the mean OD for the positive sera in the standard plate. N<sub>t</sub> and P<sub>t</sub> are the mean ODs for the same negative and positive sera in the test plate. The adjusted OD (AOD) for the test serum was obtained by  $AOD = F(P_t - N_t) + N_0$ , where St is the mean OD for the serum tested.

The cut-off was determined as the OD mean of 66 cattle sera from tick-free area plus two standard deviations. The validity of the test (sensitivity, specificity, positive and negative predictive values and precision) was determined according to Coggon et al. (1993).

The ELISA sensitivity was determined with 100 sera from cattle infected with *A. marginale*. The ELISA specificity was determined with 100 sera kept in a bank of the Beef Cattle National Research Center (Campo Grande, MS, Brasil), obtained from *Anaplasma*-free calves, held in an isolation area of this Center. Blood smears from these animals were analyzed for *A. marginale*. Also serum samples were analyzed by IFAT for *A. marginale* antibodies. Only calves negative in both tests were included in this study.

The *A. marginale* ELISA was also evaluated with 140 sera from 10 intact 6-8 months-old Nelore calves free from *Babesia* sp. and *Anaplasma* sp. infection and then experimentally-inoculated with 10<sup>10</sup> *B. bigemina* infected erythrocytes. Also, 30 sera from six calves experimentally-inoculated four times with 10<sup>7</sup> *B. bovis* infected erythrocytes were tested.

Sera of 324 cattle from an enzootically stable region (Bahia State, Brazil) were analyzed by ELISA and IFAT for detection of antibodies against *A. marginale*. The IFAT was performed according to Goff & Winward (1983). The comparison between the prevalences of positive sera detected by both tests were carried out by qui-square test, with a 95.0% interval of confidence.

## RESULTS AND DISCUSSION

The cut-off of the 66 cattle sera from tick-free area was 0.085. The results showed that all sera from *A. marginale*-infected cattle analyzed were positive for antibodies against this rickettsia, corresponding to a sensitivity of 100.0%. Six out of the 100 sera from *Anaplasma*-free calves showed false-positive reactions, corresponding to a specificity of 94.0%. Positive and negative predictive values were, respectively, 94.3% and 100.0% and precision of the test was 97.0% (Table 1).

With regards to the cross-reaction analyzes, from the 30

Table 1. Results of the ELISA with sera from calves experimentally-inoculated with *Anaplasma marginale* or free from infection with this rickettsia and analysis of validity of the test<sup>a</sup>

ELISA	Infection status of the calves		Total
	Positive	Negative	
Positive	100 (a)	6 (b)	106 (a+b)
Negative	0 (c)	94 (d)	94 (c+d)
Total	100 (a+c)	100 (b+d)	200 (a+b+c+d)

<sup>a</sup>According to Coggon et al. (1993):

Sensitivity:  $a / (a+c) \times 100 = 100/100 \times 100 = 100.0\%$

Specificity:  $d / (b+d) \times 100 = 94/100 \times 100 = 94.0\%$

Positive predictive value:  $a / (a+b) \times 100 = 100/106 \times 100 = 94.3\%$

Negative predictive value:  $d / (c+d) \times 100 = 94/94 \times 100 = 100.0\%$

Precision  $(a+d) / (a+b+c+d) \times 100 = 194/200 \times 100 = 97.0\%$

anti-sera to *B. bovis* tested against *A. marginale* antigen, 2 (6.7%) showed positive reactions. Cross-reactions with *B. bigemina* were found in 2 out of the 140 anti-sera tested on *Anaplasma* ELISA (Table 2). In both cases, the cross-reactions occurred in the acute phase of the infection (data not shown).

Table 2. Cross-reactions analyzes with sera from calves experimentally-infected with *Babesia* spp. on *Anaplasma* ELISA

Anti-sera to	N° of sera tested	N° of cross-reactions (%)
<i>Babesia bovis</i>	30	2 (6.7)
<i>Babesia bigemina</i>	140	2 (1.4)

With regards to the comparative performance of the ELISA and IFAT (Table 3), the differences between the prevalences detected by both tests (96.9% and 97.2%, respectively) were not statistically significant ( $P > 0.05$ ). From 324 sera analyzed, 308 were ELISA+/IFAT+ and 3 were ELISA-/IFAT-, corresponding to an agreement of 96.0% between the results shown by both tests.

Table 3. Comparison of the results obtained with ELISA and IFAT in detecting antibodies against *Anaplasma marginale* with sera of calves from an enzootically-stable area

IFAT	ELISA		Total
	Positive	Negative	
Positive	308	7	315
Negative	6	3	9
Total	314	10	324

The data showed that the ELISA evaluated showed a high sensitivity, comparable to other ELISAs described (Montenegro-James et al. 1990, Nielsen et al. 1996, Molloy et al. 1999). Nevertheless, the present ELISA had the advantage to be performed in shorter period of time than others developed until the present time (Duzgun et al. 1988, Shkap et al. 1990, Knowles et al. 1995, Nielsen et al. 1996, Reyna-Bello et al. 1998, Saliki et al. 1998, Molloy et al. 1999).

The specificity of this ELISA was also elevated and was comparable with other ELISAs based on crude antigens (Duzgun et al. 1988, Shkap et al. 1990) and not much lower than the ELISAs with recombinant protein (De Echaide et al. 1998, Molloy et al. 1999).

The reduced number of cross-reactions with animals experimentally-inoculated with *B. bigemina* and *B. bovis* endorses the high specificity of this serological test. Although this ELISA antigen contains erythrocyte stroma contamination, probably it was not a relevant factor of false-positive reactions.

The comparison between ELISA and IFAT showed that the performances of both tests in detecting antibodies against *A. marginale* in an enzootically stable area did not differ statistically ( $P > 0.05$ ) and showed a concordance degree very high. Likely, the antigen prepared from blood with high rickettsaemia, without antibodies against *Anaplasma*, and before accentuated packed cell volume decrease had been occurred, were important factors for the antigen quality. Furthermore, the utilization of Tergitol NP-40 in the antigen

preparation not only may have contributed for the high sensitivity, but may have increased the specificity; what is in agreement with Nakamura et al. (1988).

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

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