

Development of an Enzyme-linked Immunosorbent Assay for Detection of IgM Antibodies to *Babesia bigemina* in Cattle

Patrícia M Gonçalves Ruiz, Lygia MF Passos/⁺, Rosângela Z Machado*, José D Lima**, Múcio FB Ribeiro**

Escola de Veterinária **Departamento de Parasitologia, Universidade Federal de Minas Gerais, Caixa Postal 567, 30123-970 Belo Horizonte, MG, Brasil *Faculdade de Ciências Agrárias e Veterinárias, Unesp, Jaboticabal, SP, Brasil

A crude antigenic preparation of Babesia bigemina was used to develop an ELISA for the detection of IgM antibodies. Optimal dilutions of the antigen, using positive and negative reference sera, were determined by checkerboard titrations. Negative sera from cattle imported from tick-free areas, serum samples collected from infected B. bigemina cattle were used to validate the test. The specificity was 94% and sensitivity of the ELISA 87.5%. Sera from 385 cattle deriving from areas free from tick-borne diseases, which were submitted to a preimmunization process, were screened by this technique. The ELISA detected seroconversion on the 14th day post-inoculation in animals either infested with Boophilus microplus ticks (infected with B. bigemina), or inoculated with B. bigemina infected blood. Antibody titers decreased after day 33; however, all animals remained positive until the end of the experiment (124 days). The ELISA described may prove to be an appropriate serological test for the detection of IgM antibodies against B. bigemina.

Key words: *Babesia bigemina* - ELISA - IgM

Bovine babesiosis is an economically important tick-transmitted protozoan parasite of cattle in tropical and subtropical areas (McCosker 1981). In Brazil, the two species present are *Babesia bigemina* and *B. bovis*. The acute infections with *Babesia* spp. are characterized by a systemic inflammatory response as evidenced by hyperthermia, anemia with concomitant hemoglobinemia, apathy and jaundice, many times leading to death (Wright & Goodger 1988).

Accurate diagnosis of *B. bigemina* infection is essential for disease control measures and for epidemiological studies. During the acute phase of babesiosis the direct identification of the causative agent is possible by examination of stained blood smears, however, during early stages of infection detection of *Babesia* is difficult, due to very low parasitemia levels.

Several serodiagnostic techniques have been developed and applied for the diagnosis of bovine babesiosis (O'Donoghue et al. 1985, Waltisbuhl

et al. 1987, Böse et al. 1990, Machado et al. 1997), but these are designed to identify chronic infections since they detect IgG antibodies.

Detection of IgM antibodies may help to identify infection of different species of *Babesia* and therefore it will be useful for epidemiological studies as well as to evaluate the immune response after immunizations.

The aim of this study was to develop an ELISA system for detection of IgM antibodies against *B. bigemina* in cattle.

MATERIALS AND METHODS

ELISA standartization

Source of B. bigemina - The *B. bigemina* isolate used in this study was maintained cryopreserved in 10% dimethyl sulphoxide, in liquid nitrogen (Jaboticabal stock, Machado et al. 1994).

B. bigemina ELISA antigen - Five to six month old calves, free from hemoparasites, were splenectomized and inoculated intravenously with cryopreserved *B. bigemina* and monitored daily for the presence of parasites by microscopic examination of Giemsa-stained blood smears. Infected blood was collected in an equal volume of Alsever's solution during the peak of parasitemia (40 to 60%) that occurred between the 8th and the 10th days after inoculation. The blood was diluted 1:4 in normal saline and the infected erythrocytes

Partial financial support: Fapemig and Capes

⁺Corresponding author. Fax: +55-31-3499.2080. E-mail: lygia@dedalus.lcc.ufmg.br

Received 28 May 1999

Accepted 14 November 2000

subjected to lyses with ammonium chloride (Martin et al. 1971). A 0.8%-ammonium chloride solution was prepared using pyrogen-free deionised water (Boyle 1968). To each 90 ml of this solution 10 ml of 0.17M Tris buffer (pH 7.65) were added. The final pH of the buffered ammonium chloride solution was adjusted to 7.4 at room temperature and then it was warmed to 37°C. Nine volumes of this solution were mixed with one volume of the suspension of infected erythrocytes. The mixture was incubated at 37°C for 3 min during which time hemolysis became evident. The mixture was centrifuged at 1,500 g for 10 min and the erythrocyte-free sediment was washed three times in sterile normal saline. The pellet was resuspended in 5 vols of PBS containing protease inhibitor (1 mM PMSF, 2 mM TPCK and 0.1 mM TLCK). *B. bigemina* free merozoites were disrupted by freezing/thawing cycles using liquid nitrogen. The supernatant obtained after centrifugation at 12,000 x g for 60 min at 4°C was stored at -20°C until required for ELISA (Machado 1991).

Serum bank - To check the specificity of *B. bigemina*, serum samples from 50 Holstein cattle living in tick-free areas were collected before they were imported to Brazil. These sera were considered negative controls. A positive reference group produced by experimental infections consisting of 24 serum samples anti-*B. bigemina* provided by Embrapa, CNPGC, Campo Grande, MS, Brazil, four anti-*Babesia bovis* and four anti-*Anaplasma marginale* serum samples (serum bank - DMVP, UFMG, MG, Brazil) was used.

ELISA procedure - The method used was essentially as described by Wilchek and Bayer (1984). The reactions were carried out on polystyrene plates (Costar, USA). One hundred ml of antigen diluted in sodium bicarbonate-carbonated 0.05M buffer pH 9.6, were added to each well. After incubation for 18 h at 4°C, the excess of antigen was removed by three washes with PBS Tween 80 at 0.05%. The blocking step was carried out using 100 µl of PBS casein 5%, pH 7.6, during 1 h at 37°C. Following washing, the plate was incubated at 37°C for 60 min, with 100 µl of each serum sample diluted in PBS Tween (1:800). Unbound antibodies were removed by washing the plates as described above. One hundred µl of monoclonal anti-bovine IgM Biotin Conjugate (Sigma B 0774), diluted in PBS-Tween, were added to each well and the plates incubated for 60 min and washed. One hundred µl of Avidin-Peroxidase Conjugate (Sigma A 7419) diluted in PBS-Tween were added to each well of the plate. The anti-bovine IgM biotin conjugate (Sigma) was used at a dilution of 1:30,000 and avidin-peroxidase (Sigma) at 1:100,000, according to the manufacturer's rec-

ommendation. The plate was washed after incubation for 1 h at 37°C, and 100 µl of orthophenylenediamine (OPD) substrate solution were added. The reaction was interrupted using sulphuric acid 4N (32 µl per well). Absorbances were read using a microplate reader (Sigma) with a filter of 492 nm.

Standardization of assay - The antigen was attached to the solid-phase on microtitre plates at a concentration of 5 ml ml⁻¹ in carbonate buffer, pH 9.6 and then reacted against bovine immune and negative reference control sera. Optimal dilutions of antigen and positive and negative sera were determined by checkerboard titrations (Woodford et al. 1990). The antigen was tested against the panel of sera of known origin, including 50 "known negative samples" and 24 samples collected in infected *B. bigemina* cattle. The discriminant absorbance value (cut-off) was determined by the mean absorbance values of the negative group plus two standard deviations (Richardson et al. 1983).

Specificity was defined as the proportion of known negative serum samples detected as negative, and the sensitivity was defined as the proportion of known positive serum samples detected as positive.

***B. bigemina* serological profile of infected cattle** - Sera collected from 385 animals deriving from areas free from tick-borne diseases, which were submitted to a preimmunization process (Lima 1991), were tested in ELISA. Since it is known that differences regarding infectivity, pathogenicity and virulence may occur if strain of *B. bigemina* are originated from ticks or from blood, both inocula were used to compare the dynamics antibody production in cattle. Animals were divided into two groups - group A: five animals infested with approximately 2,000 larvae of *Boophilus microplus* infected with *B. bigemina* and *B. bovis* and group B: six animals inoculated with blood containing *B. bigemina* and *B. bovis* (10⁶ infected erythrocytes). All animals were challenged with infected blood (*B. bigemina* and *B. bovis* - 10⁶ infected erythrocytes) 30 days after the first inoculation/infestation. During the first 19 days, tail blood smears were examined daily for parasitemia determination (at least 100 fields were examined under the microscope). After this period, parasitemia was determined every week. Serum samples were collected daily during 19 days after inoculation and thereafter weekly during 15 weeks. Statistical analysis of serological data (absorbance values) was carried out using the Mann Whitney Wilcoxon test (Stell & Torrier 1960).

RESULTS

Standardization of assay - Checkerboard titrations showed a dilution of 1:2,000 (5 µg antigen

ml⁻¹ in carbonate buffer, pH 9.6) as optimum for the antigen. Serum samples were diluted at 1:800. The mean absorbance value of negative sera was 0.048 ± 0.038 , resulting in a calculated cut-off value of 0.124. Absorbance values observed with anti-*A. marginale* and anti-*B. bovis* sera were lower (≤ 0.071) than the calculated cut-off value. The mean absorbance value of the anti-*B. bigemina* serum group was approximately 7.2 times greater than that obtained with pre-infection sera, clearly discriminating between the mean absorbances of the positive and negative reference sera. This data gave a specificity of 94%, with only three among 50 "known negative samples" being detected as positive. Three among 24 anti-*B. bigemina* sera collected after experimental inoculation with infected *B. bigemina* blood were detected as negative, giving the assay a sensitivity of 87.5%. The medians of the absorbance values⁻¹ of serum samples from the animals submitted to the preimmunization process are shown in the Figure.

Serologic profile of B. bigemina - Parasitemia in blood smears was detected two to four weeks after either *B. microplus* larva infestation (group A) or inoculation of blood (group B).

The detection of IgM anti-*B. bigemina* antibodies occurred 14 days p.i. in the animals infected with *B. microplus* larvae and 19 days p.i. in animals inoculated with infected blood (Figure). A progressive increase in titers was observed up to 26 days p.i., and all the animals proved positive. The challenge given on day 30 p.i. evoked a rapid IgM response up to the 33rd day, when there was a progressive decline in antibody levels. All the ani-

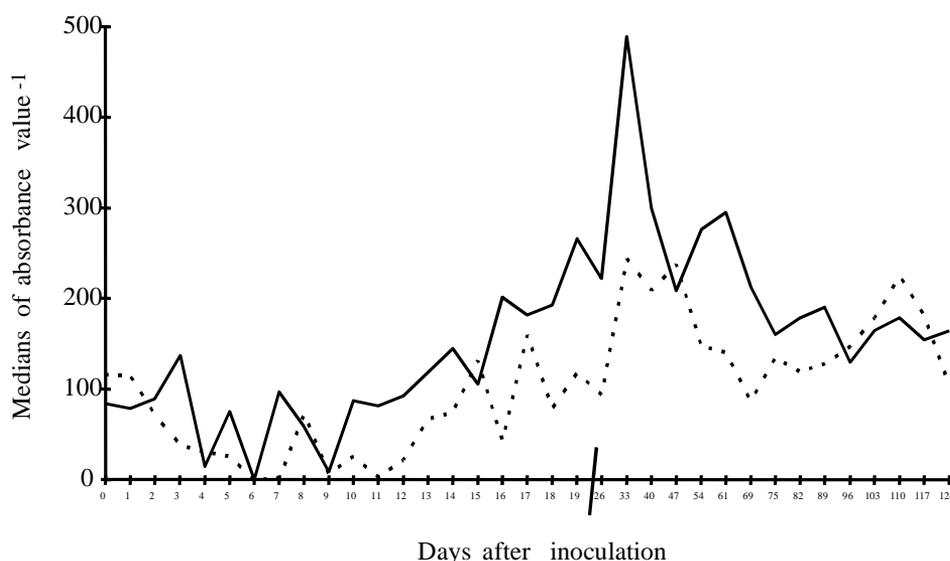
mals remained positive, until the end of the experiment at 124 days (Figure). The highest absorbance values for *B. bigemina* were recorded between days 26 and 33 p.i. Significant differences were recorded for the antibody production of the two types of inoculum ($p > 0.05$) from the 19th to the 68th days p.i. No significant differences were found in the other post-inoculation periods.

DISCUSSION

The developed ELISA system proved to have sensitivity, allowing the detection of specific IgM antibodies against *B. bigemina* in cattle experimentally infected with *B. bigemina*. Such test has a potential use as diagnostic tool in detecting early seroconversion of infected animals, as described for *B. bovis* (Gonçalves et al. 1999).

The kinetics of IgM antibodies in *B. bigemina* infections reported in the present study differs from that described by O'Donoghue et al. (1985), who found the first antibodies seven days after experimental infection with a cryopreserved stabilate. These authors report a maximum production of antibodies occurring between 12 and 22 days p.i. with a decrease to low levels around 28 days p.i. The differences between their results and the ones reported here are probably related to the fact that their study was based on a single animal, while in the present study a large number of animals was evaluated. The pattern of antibody production differs among the animals. The overall seroconversion occurred around day 14 p.i.

The diagnosis of bovine babesiosis by blood smear examination during the acute phase is lim-



Medians of Elisa absorbance values⁻¹ of bovine sera collected during a preimmunization process and tested against *Babesia bigemina* antigen. — *B. bigemina* and *B. bovis* infected *Boophilus microplus* larvae; · · · *Babesia bigemina* and *B. bovis*

ited not only due to the low parasitemias, generally observed at the beginning of infections, but also because a reduced number of samples can be examined in a day. An IgM-ELISA system makes possible the diagnosis at an early stage, which coincides with the beginning of parasite multiplication, contributing to the success of treatment.

Good crude antigenic preparations of intracellular parasites are difficult to produce, particularly due to the presence of host contaminant components, such as red blood cell fragments. These contaminants make the standardization of immunological assays a crucial step (Mahoney & Goodger 1981) since they increase the occurrence of non-specific reactions (Böse et al. 1990). El-Ghaysh et al. (1996) suggest that the use of ELISA for *B. bigemina* is inappropriate unless purified antigens or specific monoclonal antibodies are used. The use of semi-defined antigens has allowed the standardization of the ELISA for detection of anti-*B. bigemina* antibodies with good repeatability of results (O'Donoghue et al. 1985). In the present paper the antigenic preparation was produced using a very simple methodology, which requires little material manipulation at a very low cost. The infected red blood cells were lysed with ammonium chlorite, according to the method described by Martin et al. (1971), resulting in *B. bigemina* merozoites free from red blood cell membranes. In addition the merozoites kept their structural integrity, following the same results obtained by Machado et al. (1994).

In conclusion, the developed ELISA for detection of anti-*B. bigemina* IgM antibodies shows to be an appropriate test for epidemiological studies in endemic areas as well as in evaluating the antibody response of cattle after immunizations.

REFERENCES

- Böse R, Jacobson RH, Gale KR, Waltisbuhl DJ, Wright IG 1990. An improved ELISA for the detection of antibodies against *Babesia bovis* using either a native or a recombinant *B. bovis* antigen. *Parasitol Res* 76: 648-652.
- Boyle W 1968. An extension of the 51 Cr-release assay for the estimation of mouse cytotoxins. *Transplantation* 6: 761-764.
- El-Ghaysh A, Sundquist B, Christensson DA, Hilali M, Nassar AM 1996. Observations on the use of ELISA for detection of *Babesia bigemina* specific antibodies. *Vet Parasitol* 62: 51-61.
- Gonçalves PM, Passos LMF, Ribeiro MFB 1999. Detection of IgM antibodies against *Babesia bovis* in cattle. *Vet Parasitol* 82: 11-17.
- Lima JD 1991. Premunicação: uma alternativa para o controle da Tristeza Parasitária. Seminário Brasileiro de Parasitologia Veterinária, São Paulo, p. 39-43.
- Machado RZ 1991. *Estudo de Imunidade Celular e Humoral na Babesiose Bovina*, PhD Thesis, UNESP, Jaboticabal, 150 pp.
- Machado RZ, Montassier HJ, Pinto AA, Lemos EG, Machado MR, Valadão IF, Barci LG, Malheiros EB 1997. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Babesia bovis* in cattle. *Vet Parasitol* 71: 17-26.
- Machado RZ, Valadão CAA, Melo WR, Alessi AC 1994. Isolation of *Babesia bigemina* and *Babesia bovis* merozoites by ammonium chloride of infected erythrocytes. *Brazilian J Med Biol Res* 27: 2591-2598.
- Mahoney DF, Goodger BV 1981. The isolation of *Babesia* parasites and their products from the blood. In M Ristic, JP Kreier (eds), *Babesiosis*, Academic Press, New York, p. 323-332.
- Martin WJ, Finerty J, Rosenthal A 1971. Isolation of *Plasmodium berghei* (malaria) parasites of ammonium chloride lysis of infected erythrocytes. *Nature New Biol* 67: 260-261.
- McCosker PJ 1981. The global importance of babesiosis. In M Ristic, JP Krier (eds), *Babesiosis*, Academic Press, New York, p. 1-24.
- O'Donoghue KT, Friedhoff OG, Vizcaino OG, Weyreter H 1985. The detection of IgM and IgG antibodies against *Babesia bigemina* in bovine sera using semi-defined antigens in enzyme immunoassays. *Vet Parasitol* 18: 1-12.
- Richardson MD, Turner A, Warnock DW, Llewellyn PA 1983. Computer-assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. *J Immunol Meth* 9: 57-562.
- Stell RGD, Torrier JH 1960. *Principles and Procedures of Statistics*, McGraw-Hill Book Company, New York, 481 pp.
- Waltisbuhl DJ, Goodger BV, Wright IG, Commins MA, Mahoney DF 1987. An enzyme-linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle. *Parasitol Res* 73: 126-131.
- Wilchek M, Bayer EA 1984. The avidin-biotin complex in immunology. *Immunol Today* 5: 39-43.
- Woodford JD, Jones TW, Rae PF, Boid R, Bell-Sakyi L 1990. Seroepidemiological studies of bovine babesiosis on Pemba Island, Tanzania. *Vet Parasitol* 37: 175-184.
- Wright IG, Goodger, BV 1988. Pathogenesis of babesiosis. In M Ristic, *Babesiosis of Domestic Animals and Man*, Academic Press, New York, p. 99-118.