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SEROLOGICAL, PARASITOLOGICAL AND MOLECULAR ASSESSMENT OF *Babesia bovis* AND *Babesia bigemina* IN CATTLE FROM STATE OF MARANHÃO[1]

FRANCISCO BORGES COSTA[2] , SOLANGE ARAÚJO MELO[2] , FLÁBIO RIBEIRO ARAÚJO[3] , CARLOS ALBERTO DO NASCIMENTO RAMOS[4], ALCINA VIEIRA CARVALHO-NETA[2] , RITA DE MARIA SEABRA NOGUEIRA DE CANDANEDO GUERRA[2]*

ABSTRACT - The aim of the present study was to determine the prevalence of *Babesia bovis* and *Babesia bigemina* in dairy cattle from São Luis Island in the state of Maranhão, Brazil. A total of 281 blood samples were collected. In total, 275 (97.9%) animals were *B. bovis*-reactive and *B. bigemina*-reactive in the Enzyme-Linked Immunosorbent Assay (ELISA). The microscopy examination detected 22 (7.8%) animals that were positive for *Babesia* sp. and the Polymerase Chain Reaction (PCR) analysis showed that 91 animals (32.38%) and 23 animals (8.18%) were positive for *B. bovis* and *B. bigemina*, respectively, while 17 animals (6.04%) were co-infected. There is a high level of transmission of these protozoa in Maranhão, and the animals were naturally exposed. Therefore, it is possible to characterize the island as enzootic stability for babesiosis, indicating a risk of financial losses when susceptible animals are introduced from areas of enzootic instability or free regions of *B. bovis* and *B. bigemina*.

Keywords: Dairy cattle. Babesiosis. Diagnose.

AVALIAÇÃO SOROLÓGICA, PARASITOLÓGICA E MOLECAR DE *Babesia bovis* E *Babesia bigemina* NO GADO DO ESTADO DO MARANHÃO

RESUMO - O objetivo deste trabalho foi determinar a prevalência de *Babesia bovis* e de *Babesia bigemina* em bovinos leiteiros na ilha de São Luis, estado do Maranhão, Brasil. Foram coletadas 281 amostras de sangue e 275 (97,9%) estavam soropositivas para *B. bovis* e *B. bigemina* pelo ensaio imunoenzimático indireto (ELISA). O exame microscópico dos esfregaços sanguíneos detectou 22 (7,8%) de animais positivos para *Babesia* sp. e na Reação em Cadeia da Polimerase (PCR) detectou-se, 91 (32,38%) e 23 (8,18%) animais positivos para *B. bovis*, *B. bigemina*, respectivamente e 17 (6,04%) animais co-infectados. Verifica-se alta transmissão desses protozoários no estado do Maranhão e os animais foram expostos naturalmente; portanto, é possível caracterizar a ilha de São Luís como área de estabilidade enzoótica para a babesiose, indicando risco de perdas financeiras quando animais susceptíveis de regiões de instabilidade enzoótica ou livres de *B. bovis* e *B. bigemina* são introduzidos.

Palavras-chave: Gado de Leite. Babesiose. Diagnóstico.

*Autor para correspondência
[1]Recebido para publicação em 24/04/2014; aceito em 11/02/2015.
[2]Universidade Estadual do Maranhão, Cidade Universitária Paulo VI, Caixa Postal 9, São Luis-MA, 65055-301, Brasil, grita62@hotmail.com, franc.borges@yahoo.com.br, sol_vet80@hotmail.com, avcn@yahoo.com.br.
[3]Embrapa Gado de Corte, Av. Rádio Maia, 830, 79106-550, Campo Grande, MS, Brasil, flabio.araujo@embrapa.br.
[4]Universidade Federal de Mato Grosso do Sul, Faculdade de Medicina Veterinária e Zootecnia, Av. Senador Filinto Muller, 79070900, Campo Grande, MS, Brasil, carlos.nascimento@ufms.br.

INTRODUCTION

Babesia bovis and *Babesia bigemina* are tick-borne hemoparasites that cause babesiosis in cattle worldwide. Babesiosis is responsible for financial losses due to a reduction in the meat and milk yield, and the application of control measures. It also has an impact on the international cattle trade (BOCK et al., 2004).

Babesiosis occurs primarily in tropical and subtropical areas, where the ixodid tick vector, predominantly *Rhipicephalus* (*Boophilus*) *microplus*, is found. The clinical signs of the disease are fever, anemia, anorexia, lethargy, ataxia, tachypnea, hemoglobinuria (SILVA et al., 2007). In Brazil, *B. bovis* is the etiological agent of most outbreaks (ANTONIASSI et al., 2009; CÂMARA et al., 2009). Although *B. bovis* is considered to be more pathogenic, the effect of the disease caused by *B. bigemina* is severe if not treated (BROW et al., 2006).

Several serological studies have reported different prevalence levels around the world: 21% to 42.2% for *B. bovis* and 10.8% to 40% for *B. bigemina* in Morocco, Southern Italy, Mozambique and Syria (SAHIB et al., 1998; CRINGOLI et al. 2002; ALFREDO et al., 2005; TERKAWI et al., 2012). In Latin America countries, including Brazil, the prevalence has been reported to range from 12.8% to 99.2% for *B. bigemina* and 22.5% to 98.8% for *B. bovis* (JAMES et al., 1985; PAYNE & OSÓRIO, 1990; RAMOS et al., 1992; MAS et al., 2000; BARROS et al., 2005; TEGLAS et al., 2005; TRINDADE et al., 2010; BRITO et al., 2013; SOUZA et al., 2013).

Serological methods are essential tools for epidemiological studies since the microscopic examination of blood smears can lead to false negative results. On the other hand, even when it's present in low quantities, molecular methods as PCR enable the direct identification of the agent. According to Terkawi et al. (2011), a combination of molecular and serological detection of the infection leads to an accurate diagnosis, and epidemiological investigation.

According to Souza et al. (2013), studies of the epidemiological situation of babesiosis in the northeast of Brazil are extremely scarce, although *B. bovis* and *B. bigemina* have been studied in the neighboring states of Maranhão, Pará and Piauí, North and Northeast regions of Brazil respectively, showing different situations of enzootic stability (Souza et al 2013;. Silva et al 2014). Since dairy cattle play a significant social and economic role on São Luis Island, epidemiological studies are necessary.

No previous reports have been published regarding babesiosis infection in cattle in the state of Maranhão. Therefore, the aim of the present study was to investigate the prevalence of *B. bovis* and *B. bigemina* using blood smears, the Indirect Enzyme-linked Immunosorbent assay (ELISA) and the Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Characterization of the studied area

São Luís Island, in the State of Maranhão, is located near the Equator line (2°31' Lat. S; 44°18' Long. W). The temperature and humidity are practically stable throughout the year, although there are variations in precipitation in certain months. The island is composed of four municipalities (São Luís, Raposa, Paço do Lumiar, and São José de Ribamar). It has two distinct periods of weather: the rainy season (From January to May) and the dry season (From June to December). The mean monthly temperature is 28°C, with a mean relative humidity of 78% and a mean rainfall of 152mm (Geoprocessing Laboratory - UEMA/LABGEO).

Sampling

The size of the cattle population studied was determined through a mathematical model developed by the *Centro Pan Americano de Zoonosis* (1979) for the epidemiological study of chronic diseases. The formula is the following: $n=p\ (100-p)\ Z^2/(s.d/100)^2$, where n is the sample number, p is the expected prevalence, Z is the degree of confidence, and d is the error margin. The minimal sample size confers 95% confidence with a 3% error probability. The total number of animals in São Luís Island is 1.443. The expected prevalence used was 92.5% which was obtained in a study performed in the Brazilian state of Pará (GUEDES-JÚNIOR et al., 2008).

Blood samples were collected randomly, with five ml of blood collected from the caudal vein using vacuum tubes. Blood samples with anticoagulant (EDTA) were used for DNA extraction, and serum samples were obtained for the ELISA. The samples obtained were stored at -4°C until analysis. A total of 281 male and female crossbred dairy cattle were sampled using the following age groups: less than 12 months old; 12 to 24 months old and over 24 months old.

Detection of antibodies against B. bovis and B. bigemina

Serum samples were analyzed by two Indirect Enzyme-linked Immunosorbent Assays (ELISA), which have been reported to exhibit sensitivity of 98% and specificity of 99% for *B. bigemina* (MADRUGA et al., 2001), and sensitivity of 98% and specificity of 98.1% for *B. bovis* (MADRUGA et al., 2000).

The antigens were composed of purified lyses

merozoites from infected cattle blood. The antigen protein concentrations were determined by Folin's reagent (LOWRY et al., 1951), resulting in 10.74mg/mL and 5mg/mL for *B. bovis* and *B. bigemina*, respectively. The antigen concentrations per well/plate were 1.074mg and 0.5mg, for *B. bovis* and *B. bigemina*, respectively.

The antigen was diluted to 1:1.000 in carbonate/bicarbonate buffer with a pH of 9.0 (200 mM Na_2CO_3, 199 mM $NaHCO_3$), and 100 µL was then placed into each well of 96 well plates (Costar, flat bottom, high binding, 3590). The adsorption time of the antigen was four hours at 40°C. Subsequently, the plates were washed five times with phosphate buffer saline (PBS, containing 0.1% Tween 20 (PBST). One hundred microliters of bovine serum diluted to 1:1000 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, A-0705), which was diluted to 1:12.000 in PBST, was 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 p-nitrophenyl phosphate (1.0 mg/mL in substrate buffer: 0.1 M diethanolamine, 0.1 M MgCl2, 0.1 M NaCl) was added to each well. The reaction was stopped 20 minutes later by adding 100 µL of 0.2M NaOH to each well. The optical density levels were measured in a microplate reader with a 405 hm filter.

The ELISA cut-off was determined as the mean optical density (OD) of 10 cattle sera in duplicates from the tick-free area of beef cattle at Embrapa, Campo Grande, MS, Brazil, plus two standard deviations.

Microscopy exam

A total of 281 thin blood smears from ear capillaries were subjected to Giemsa staining and observed with the help of an oil immersion lens (1000X) to detect the presence of *Babesia* sp.

PCR analysis

DNA extraction was performed with 300 µL total blood using the Wizard Genomic DNA Purification kit (PROMEGA), following the manufacturer's recommendations. PCR was performed to amplify specific DNA from *B. bovis* and *B. bigemina.* As described by FIGUEROA et al. (1993), in which a fragment was amplified from *B. bovis* with 350 bp and from *B. bigemin*a with 278 bp. The primers used were BoF 5'CACGAGGAAGGAACTACCGATGTTGA3' and BoR 3'CCAAGGAGCTTCAACGTACGAGGTCA 5' for *B. bovis* and BA 5'CATCTAATTTCTCTCCATACCCCTCC3' and BB 3'CCTCGGCTTCAACTCTGATGCCAAAG5' for *B. bigemina*. The Immunoblot Laboratory kindly provided the positive and negative controls.

RESULTS

The ELISA method confirmed that 275 (97.9%) of the 281 samples screened were *B. bovis*-reactive and *B. bigemina*-reactive. No significant differences were found for the frequency of antibodies in relation to gender and age using the x^2 square test (Table 1).

Table 1 – Sero prevalence of dairy cattle for *Babesia bovis* and *Babesia bigemina* in São Luis Island, Maranhão - Brazil using the Enzyme-Linked Immunosorbent Assay (ELISA)

Categories		*Babesia bovis*		*Babesia bigemina*	
		RS (NT)	%	RS (NT)	%
Gender	Male	45(45) [a]	100	45(45) [a]	100
	Female	230(236) [a]	97.5	230(236) [a]	97.5
Age (months)	Age < 12	74(75) [a]	98.7	74(75) [a]	98.7
	12 ≤ Age ≤ 24	84(87) [a]	96.6	84(87) [a]	96.6
	Age > 24	117(119) [a]	98.3	117(119) [a]	98.3
Mean		275(281)	97.9	275(281)	97.9

RS (Reactive sera); NT (Number of samples); % (Percentage of positive samples)
Same letters between lines to each group means there was no statistical difference (P>0.05).

The frequency of antibodies nm. to both agents was 95.4% for São Luis and 100% for São José de Ribamar, Raposa, and Paço do Lumiar respectively. There was no significant difference between the four municipalities. The microscopic exam revealed that 22 samples (7.8%) were positive for *Babesia* sp.

The PCR assay analysis of the samples diagnosed 91 (32.38%) animals as positive for *B. bovis* and 23 (8.18%) animals as positive for *B. bigemina.*

Seventeen animals (6.04%) were co-infected (Table 2). No statistical difference (p>0.05) was found between positivity for *B. bigemina* and *B. bovis* when the gender of the animal was considered. Animals less than 12 months old exhibited a significant difference (p<0.05) for *B. bovis* infection when compared to those aged between 12 and 24 months or above. For *B. bigemina* infection, animals aged between 12 to 24 months exhibited a significant difference (p<0.05) when compared to animals less than 12 months old and over 24 months old. There was no significant difference (p>0.05) between co-infected animals for the variables gender and age (Table 2).

Table 2 - Prevalence of *Babesia bovis* and *Babesia bigemina* in dairy cattle from São Luis Island, Maranhão - Brazil using the Polymerase Chain Reaction (PCR)

Categories		*Babesia bovis*		*Babesia bigemina*		Co-infected	
		PS (NT)	%	PS (NT)	%	PS(NT)	%
Gender	Male	10 (45)[a]	22.22	2 (45)[a]	4.44	3 (45)[a]	6.67
	Female	81(236) [a]	34.32	21(236) [a]	8.90	14(236)[a]	5.93
Age	Age < 12	9(75)[a]	12.0	1(75)[a]	1.33	3(75)[a]	4.0
	12 ≤ Age ≤ 24	35(87)[b]	40.23	15(87)[b]	17.24	8(87)[a]	9.19
	Age > 24	47(119)[cb]	39.49	7(119)[ac]	5.88	6(119)[a]	5.04
Mean		91(281)	32.38	23(281)	8.18	17(281)	6.04

PS (Positive sample), NT (Number total samples), % (Percentage of positive animals). Same letters between lines to each group means there were no statistical differences (P>0.05).

DISCUSSION

High *B. bovis* and *B. bigemina* seroprevalence rates observed in this study, allow us to consider São Luís Island as an area of enzootic stability for babesiosis, based on epidemiological concepts proposed by Mahoney & Ross (1972) that describes its methods of evaluating the *Babesia* status of herds in enzootic areas and its application to control possible outbreaks.

The data presented herein are similar to those obtained in other geographical regions of Brazil (BARROS et al., 2005, GUEDES-JÚNIOR et al., 2008, TRINDADE et al., 2010, BRITO et al., 2013), although lower prevalence rates have been detected (MADRUGA et al., 1983, OSAKI et al., 2002, SOUZA et al., 2013). In areas where the climate is not suitable for the tick vector, such as in the South of Brazil, a high prevalence is not expected due to low temperatures. It is worth noting, that Brazil is a huge country with different climatic and environmental conditions in its five geographic regions. In other countries in Latin America, such as Mexico, Venezuela, Paraguay and Bolivia, the prevalence ranged from 12.8% to 79% (JAMES et al., 1985, PAYNE & OSÓRIO, 1990, RAMOS et al., 1992, MAS et al., 2000). Several factors influence *Babesia* sp. infection, including size and the tick population, the capacity of the vector tick to transmit the parasite and host susceptibility (BOCK et al., 1997; JONSSON et al., 2008). In addition, the environmental conditions of each geographical region must be taken into account (TEMBUE et al., 2011).

In the present study, no association was found between gender and parasitism. This result was also reported in other studies performed in the states of Rio de Janeiro and Tocantins (SOARES et al., 2000; SOUZA et al., 2000; TRINDADE et al., 2010) and in southern Mozambique (TEMBUE et al., 2011). No differences were found in the prevalence between age groups in the ELISA assay. However, both Souza et al. (2000) and Trindade et al. (2010) reported differences when age was considered. TEMBUE et al (2011) found that the frequency of cattle that were positive for *B. bovis* and *B. bigemina* increased among age groups, suggesting that infection and reinfection persisted even after the primary infection.

Tick specimens were randomly collected during the sampling period. Only infestation by *R.* (*B.*) *microplus* (larvae, nymphs, and adults) was identified throughout the year. Therefore, we can say that the island exhibits environmental conditions that enable this tick species to complete its biological cycle. However, at the end of the rainy season and the beginning of the dry season, there was an increase in the tick population. Generally, in areas infested by ticks during most months of the year, babesias coexist with cattle without causing disease. In cases where the equilibrium between the tick vector, the agent, and the host is broken, the disease appears. Therefore, bovine susceptibility can be affected by age, breed, and environmental stress.

São Luis Island has high temperatures all year round, although humidity can vary in the rainy season. Thus, the climatic conditions are suitable for the life cycle of the tick vector, and the same can be observed in the State of Maranhão, where the tick population is controlled by the use of acaricides. *R.* (*B.*)

microplus has previously been identified on São Luis Island in domestic animals (GUERRA & BRITO, 2004).

Blood smears do not efficiently reveal the epidemiological situation of a region. Furthermore, a positive result in the test depends on the degree of parasitemia and the immunological response of the animal, as demonstrated in previous studies (ALMERIA et al., 2001; IÇA et al., 2007). The microscopic examination of blood smears detected in the present study was similar to a study by Kalkan et al. (2010). However, Chaudhry et al. (2010) detected more positive animals through morphometric analysis to differentiate *B. bovis* from *B. bigemina* and concluded that, it is difficult to differentiate the two species by microscopic examinations.

The frequency of infection with *B. bovis* in cattle determined in this study by PCR assay was similar to frequency reported previously in cattle in the dairy basin of Parnaíba – PI (Souza et al 2013), however higher frequencies were observed in the samples from Rondônia and Acre (Brito et al 2013). Infection with *B. bigemina* in São Luís Island was lower, than the one found in the dairy basin of Parnaíba.

Researchers have pointed out that serologic assays detect a higher number of infected *Babesia* sp. cattle than nested-PCR (SILVA et al., 2009). These differences are consistent with previous reports that *B. bovis* and *B. bigemina* parasites are difficult to detect using PCR techniques due to the small number of parasites in the peripheral blood during chronic infections (PIPANO et al., 2002). Another possible reason could be that the antibodies remain in circulation for a long period of time after acute infection. In this case, it is possible that the parasites are cleared from circulation, or that the parasite concentration in the blood decreases to the limit of detection of the nested-PCR (PIPANO et al., 2002; TJORNEHOJ et al., 1996).

Although the facts mentioned above should be taken into consideration, molecular techniques such as PCR have proven to be sensitive in detecting *B. bovis* and *B. bigemina* in carrier cattle (CALDER et al., 1996), as well as in the early phase of the infection (CHAUDHRY et al., 2010). Therefore, this kind of technique is useful in identifying the state of the carrier, which serves as a reservoir for infection in a herd. Animals which are not clinically ill may continue to infect the tick vector, so it can be used as a tool for epidemiological investigation. Moreover, an accurate early diagnosis of babesiosis in carrier cattle is essential to overcome economic losses (CHAUDHRY et al., 2010).

Molecular diagnosis of *Babesia* is a sensitive technique that can characterize the species accurately, enabling the identification of new species. ELISA, as a serological technique, provides knowledge of the status of the herd and the possibility of potential outbreaks. In summary, PCR assays can detect and differentiate Babesia species and are particularly useful in carriers. A positive result in a microscopic examination depends on the level of parasitemia. Parasites can be easily found in the blood during the acute infection.

The advantage of combining PCR and ELISA involves an increase in sensitivity while diagnosing these hemoparasites. Therefore, it is possible to distinguish recent infections and established infections, from which the animal has recovered. Furthermore, in cases of *B. bovis* infection, the animals are thought to remain as carries for life after infection. In contrast, *B. bigemina* may clear infection and antibody levels can decline below the negative threshold within months of the infection. Inconclusive results may occur in relation to negative threshold values, and this phenomenon can provide a diagnostic challenge in animals whose titers are declining, if the animal clears infection (GOFF et al., 2008).

Based on the results obtained in the present study, sanitary prophylaxis should be assigned. The eradication of ticks is not desirable, so their population must be controlled by the use of acaricides. A single infection with *B. bovis* or *B. bigemina* can lead to long-lasting immunity. Consequently, vaccinated native animals are not required in the studied area. However, endemic environments should be monitored because the introduction of new species or strains of the parasite, or interruptions in the exposure of ticks due to changes in climate, host factors, or management, could lead to infection.

Efforts should also be made to discover new tick antigens and chemical acaricides which, in combination with a vaccine, could be used to develop an efficient program to control tick infestation and protozoa transmission (BASTOS et al., 2010).

The present study provides the first epidemiological data of *Babesia bovis* and *Babesia bigemina* in the State of Maranhão (Brazil), using microscopic, serological and molecular methods with samples collected from dairy cattle that were naturally infected on São Luis Island.

CONCLUSION

There is a high level of transmission of these protozoa, and the animals were naturally exposed to both *B. bovis* and *B. bigemina*. Therefore, it is possible to characterize the island with enzootic stability for babesiosis, indicating a risk of financial losses when susceptible animals are introduced from areas of enzootic instability or parasite-free regions.

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