Detection of antibodies against *Babesia bovis* and *Babesia bigemina* in calves from the region of Araguaína, State of Tocantins, Brazil

Detecção de anticorpos anti-*Babesia bovis* e anti-*Babesia bigemina* em bezerros na região de Araguaína, Estado do Tocantins, Brasil

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Abstract

The aim of this study was to determine the seroprevalence of antibodies against *B. bovis* and *B. bigemina* in calves from the region of Araguaína, State of Tocantins, Brazil. In this research we used sera obtained from 506 calves, from both genders and of 8 to 24 months old, to detect antibodies by indirect Enzyme-Linked Immunosorbent Assay (ELISA-test). Statistical analysis of the data was performed using the Chi-square (χ^2) test with Yates correction. The seroprevalence obtained was 90.5 and 91.7% for *B. bigemina* and *B. bovis*, respectively, characterizing the region as an area of enzootic stability for the species analyzed. The seroprevalence to *B. bovis* showed higher positivity among calves 19-24 months old.

Keywords: Bovine babesiosis, calves, sorology, Tocantins, Brazil.

Resumo

O objetivo desse estudo foi determinar a soroprevalência de anticorpos anti-*B. bovis* e anti-*B. bigemina* em bezerros da região de Araguaína, Estado do Tocantins, Brasil. Nesta pesquisa foram coletadas 506 amostras de soros de bezerros com faixa etária entre 8 e 24 meses, fêmeas e machos, as quais foram processadas pelo ensaio imunoenzimático indireto (ELISA-teste). Como análise estatística utilizou-se o Qui-Quadrado (χ^2) com correção de Yates. As prevalências obtidas em bezerros foram de 90,5 e 91,7% para *B. bigemina* e *B. bovis*, respectivamente, caracterizando esta região como de estabilidade enzoótica para as espécies analisadas. A soroprevalência para *B. bovis* apresentou maior positividade entre os bezerros com faixa etária de 19-24 meses.

Palavras-chave: Babesiose bovina, bezerros, sorologia, Tocantins, Brasil.

Introduction

Bovine babesiosis is a hemoparasitoses caused by the protozoa *Babesia bovis* and *Babesia bigemina* that has the tick *Rhipicephalus* (*Boophilus*) *microplus* as its biological vector in the Americas. The clinical signs of the disease are fever, anemia, anorexia, lethargy, ataxia, tachypnea, hemoglobinuria, and muscle tremors (GUGLIELMONE, 1995; SOARES et al., 2000; SILVA et al., 2007; SINGH et al., 2009). The economic importance of this disease is due to the high animal morbidity and mortality especially among calves, and to its effects on weight gain and milk production, leading to the use of costly disease control and prevention measures (MARTINS et al., 1996; GRISI et al., 2002; BARROS et al., 2005).

Babesiosis has worldwide distribution, with greater economic importance in tropical and subtropical regions (MUNOZ et al., 2008). The epidemiological status of the disease varies among free, instable, and stable areas. The free areas are those where

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climatic conditions do not support tick vector reproduction (BERTO et al., 2008). In Brazil, these regions are considered restricted (KESSLER et al., 1987). The areas of enzootic instability occur in climate conditions and/or in livestock management situations that affect tick vector reproduction (KESSLER et al., 1983; BARROS et al., 2005; FOLLY et al., 2009). Animals that had no contact with the pathogen in the first months of life are more susceptible to the disease, especially when transported to areas considered to be of enzootic stability for babesiosis (SANTOS et al., 2001).

In areas considered to be of enzootic stability, calves are protected by maternal antibodies obtained through colostrum, and when infected during the first months of life they develop their own active immunity, resulting in fewer cases of clinical disease at later stages (MADRUGA et al., 1984; GONÇALVES, 2000; OSAKI et al., 2002).

In Brazil, some studies have revealed areas of instability in the region of Londrina (Paraná) with a prevalence of 69.30% for *B. bigemina* and 60.19% for *B. bovis* (VIDOTTO et al., 1997). For areas of enzootic stability, we highlight the municipalities of Goiânia (Goiás) with a prevalence of 98.90% for *B. bovis* and 94.40% for *B. bigemina* (SANTOS et al., 2001); of Paudalho (Pernambuco) with 76.59% for *B. bovis* and 97.61% for *B. bigemina* (BERTO et al., 2008); of Pindamonhangaba (Vale do Paraíba, São Paulo), showing a prevalence of 88% for *B. bovis* and 94% for *B. bigemina* (BARCI et al., 1994) and of Campos dos Goyatacazes (Rio de Janeiro) with a prevalence of 90.50% for *B. bovis* and 78.70% for *B. bigemina* (FOLLY et al., 2009).

Knowing the prevalence of these hemoparasites is of great importance for determining the epidemiological status of a region, indicating whether there is a situation of enzootic stability or instability. This information serves as a support for the employment (or not) of prophylactic measures to minimize the effects of this disease (MADRUGA et al., 2000).

There are several serological methods to detect antibodies against *Babesia* spp.; the most used are the indirect immunofluorescence assay (IIFA) and the indirect immunoenzimatic ELISA assay (IIEA), as they are sensitive and specific tests and therefore considered appropriate for epidemiological studies of this disease (SANTOS et al., 2001; OSAKI et al., 2002; JULIANO et al., 2007). But a comparative study between IIFA and ELISA demonstrated that the latter was more sensitive and specific, allowing the analysis of a larger number of samples (MARTINS et al., 1996; ARAÚJO et al., 1998).

Considering the scarcity of data on the epidemiology of babesiosis in the region of Araguaina (Tocantins), this study aimed to investigate the seroprevalence of anti-*B. bovis* and anti-*B. bigemina* in calves in this region.

Material and Methods

1. Study area

This work was carried out in the region of Araguaína, Northern part of Tocantins State, which occupies an area of 4000.40 km², at latitude 07° 11' and longitude 48° 12', with average altitude of 227 m, and featuring a wet tropical climate (SECRETARIA DE PLANEJAMENTO E MEIO AMBIENTE DO ESTADO DO TOCANTINS, 2009). The region is considered to be part of the Eastern Amazon (KAMPEL et al., 2001).

2. Sera sampling

Sample size calculation was performed using Epi Info 6.04 by considering the population of cattle aged 0-24 months, in 98,715 animals (AGÊNCIA DE DEFESA ANIMAL DO ESTADO DO TOCANTINS, 2008), the possibility of detecting the disease by 50% (corresponding to disease occurrence unknown in a given population), confidence interval of 95% and odds ratio 10%. This calculation resulted in a sample (N) of 384 animals, to which 10% was added to cover for potential losses. The resulting N was 423 animals (CENTRO PAN-AMERICANO DE ZOONOSIS, 1979). Sera were collected from 506 Nelore and Tabapuá animals reared extensively, of both genders and aged 8-24 months. Twenty beef cattle farms registered in the Agência de Defesa Agropecuária do Tocantins (ADAPEC) of Araguaina were chosen so that 1% of the animals could be randomly selected for the study.

Blood collection was performed throughout March-June 2009 by puncturing the coccygeal vein. The collected blood sample (10 mL) was centrifuged at 2,000 g for 10 minutes, and the resulting serum split into 2 mL microtubes and stored at –20 °C, until the appropriate time for routine HIV testing. During blood collections animals were checked for tick infestation and, when ticks were found, control measures were carried out.

3. Indirect immunoenzimatic ELISA assay

The method used to detect serum anti-*Babesia* sp. was the ELISA assay described by Machado et al. (1997), with some modifications. The collected material was analyzed at the Imunoparasitology Laboratory at the Faculty of Agricultural Sciences and Veterinary Medicine of UNESP (Jaboticabal).

In each well of the ELISA microplate (NunclonTMSurface, Nunc, Denmark), we added 100 µL of crude soluble antigen diluted in carbonate-bicarbonate 0.05 M pH 9.6 buffer. The protein concentration for both *B. bovis* and to *B. bigemina* was standardized at 10 µg.mL⁻¹. Plates were incubated overnight at 4 °C and the excess of antigen was removed by three washes with phosphate buffer solution (PBS) plus 0.05% Tween 20 (PBST). Later, 200 µL of PBST 20 containing 5% skimmed milk was added for 90 minutes at 37 °C and washed off three times with PBST 20. Then serum samples previously diluted (1:100) in PBST 20 with 5% normal rabbit serum were added in duplicates (negative control, positive control, serum test). Plates were then incubated for 90 minutes at 37 °C and afterwards washed again as described before. Further, we added 100 µL total bovine anti-IgG conjugated with alkaline phosphatase (Sigma Chemical Company, St. Louis, USA) diluted to 1:30,000 and incubated for 90 minutes at 37 °C. Plates were washed again as before in PBST 20. Finally, 100 µL of the substrate p-nitrophenylphosphate (1 mg.mL⁻¹) diluted in a diethanolamine buffer pH 9.8 was added to each well and the plates were incubated at room temperature for 30 minutes.

Reaction was read using a microplate reader (Dynex Technology) and 405 ηm wavelength. Sera absorbance values were grouped in ELISA levels (EL), which vary between zero and nine, as recommended by Machado et al. (1997). Absorbance breakdown (cut-off value) was determined to be two and a half times the average value of absorption obtained for the negative control serum. Readings above this value were considered positive.

4. Statistical analysis

We used the statistical test Chi-Square (χ^2) with Yates correction for the statistical analysis of the variables *B. bovis*, *B. bigemina*, gender and age. Yates correction was not applied for analysis regarding the seropositive prevalence distribution according to age. *Odds Ratio* (OR) was calculated to check whether gender was a risk or a protection factor. All statistical analyses were performed using the statistical program Graphpad Prism. 5 - Windows (2009), with a confidence interval (CI) of 95%.

Results and Discussion

The search for antibodies showed positive reaction rates of 91.7% for *B. bovis* and 90.5% for *B. bigemina*, with no significant difference regarding the species prevalence in the region (p > 0.05) (Table 1). Frequency of antibodies against *B. bovis* and *B. bigemina* in calves coming from Araguaína, Tocantins, demonstrated a high prevalence of seropositive animals.

These data are similar to results found for cattle in the Northeast of Pará, where a prevalence of 98.8% for *B. bovis* and 99.2% for *B. bigemina* was observed (GUEDES JUNIOR et al., 2008). The data is also considerably higher than the values reported for the states of Rondonia and Acre, where prevalence of 7.1 and 9.0% respectively were found for *B. bovis*, and prevalence of 3.0 and 8.0% respectively were found for *B. bigemina* (BRITO et al., 2007). These data demonstrate that the disease has a varied distribution within the region.

The differences found in the same region may be due to variation in the rate of infection by ticks and, subsequently, a variation in the rate of cattle inoculation by the infected ticks (SALCEDO et al., 1987). A study of natural infection in bovines of the Nellore breed from the region of Umuarama (Paraná) showed a variation of 36.7 to 92% among animals seropositive for *B. bovis* (OSAKI et al., 2002). Thus, in the same locality, there are significant differences among herds. These differences may result from seasonal increase in the tick population, or in a lack of control of such increase or from the introduction of susceptible animals derived from areas free of ticks.

An enzootic stability area is one in which herds have antibody frequency above 75%, while an area of instability is one herds have antibody frequency lower than 75% (MAHONEY; ROSS, 1972; D'ANDREA et al., 2006). The seroprevalence in this study was higher than 75%, characterizing the region of Araguaína as an area of enzootic stability for bovine babesiosis. In a situation of enzootic stability, calves are infected in the early months of life and are protected by colostral antibodies (passive immunity), thus enabling the development of active immunity without presenting clinical disease (KESSLER et al., 1983). However, a study of calves of 1 to 50 days of age concluded that under conditions of high tick infestation high morbidity and mortality can occur even in the period of protection by colostral antibodies (SILVA et al., 2007).

Other regions of the country were considered areas of stability, such as the city of Goiania (SANTOS et al., 2001) in Goiás, the Curraleiros Experimental Station for Bovine Studies (CESBS) also in Goiás (JULIANO et al., 2007), the semiarid region of Bahia (BARROS et al., 2005), and the Forest Zone in Minas Gerais (SALCEDO et al., 1987). Other locations were considered areas of instability, such as the States of Rondonia and Acre in northern Brazil (BRITO et al., 2007) and Londrina in the State of Parana (VIDOTTO et al., 1997). The Greater Metropolitan area of Rio de Janeiro was considered an area of stability for *B. bovis* (SILVA et al., 2007) and of instability for *B. bigemina* (SOUZA et al., 2000).

During blood collection, presence of ticks was observed in all farms, despite the frequent use of tick control drugs containing avermectins, as well as a high prevalence of babesiosis. Host and environment are factors that affect the prevalence of babesiosis in a particular region (MAHONEY; ROSS, 1972). The Amazon region is considered an endemic area for babesiosis due to the existence of ticks throughout the year (LIMA et al., 1999).

In this study, no effect of sex on parasitism (p > 0.05) was found; that is, in the case of *B. bovis* prevalence in males was 94.0% and females 90.2%, while in the case of *B. bigemina* prevalence in males was 87.5% and in females 92.5% (Table 2). These results are similar to those found by Soares et al. (2000) and Souza et al. (2000), in studies regarding seroprevalence of *Babesia* spp. in cattle from the Northern region of the State of Rio de Janeiro.

Analysis for *B. bovis* revealed a prevalence of 90.9, 83.5 and 98.9% for ages 8-12, 13-18 and 19-24 months, respectively, showing a higher positivity among the age groups 19-24 months as compared to the other two age groups (p < 0.0001) (Table 3). For *B. bigemina* seroprevalence was 90.2, 90.8 and 90.5%, and no significant difference was found among the age groups (p > 0.05) (Table 3). Similar data were reported by Souza et al. (2000).

Table 1. ELISA assay results indicating the prevalence of positive and negative calves for antibodies against *Babesia bovis* and anti-*Babesia bigemina* in the region of Araguaína, Tocantins State, Brazil.

Parasite	Positive		Total nº		χ^2	P value	OR (CI 95%)
	Nº	%	Nº	%			
B. bovis[ns]	464	91.7	506	100	0.3049	0.5808	1.158 (0.7503 a 1.787)
B. bigemina[ns]	458	90.5	506	100			

[ns]not significant by Chi-square (χ^2) test with Yates at 5% probability; OR = *Odds Ratio*; CI = Confidence Interval.

Table 2. Prevalence of antibodies against *Babesia bovis* and *Babesia bigemina* in calves according to gender. Region of Araguaína, Tocantins State, Brazil.

	Gender	Positive		Total nº		χ^2	P value	OR (IC 95%)
		Nº	%	Nº	%			
Babesia bovis	Male[ns]	188	94.0	200	100	1.827	0.1765	1.703 (0.8500 a 3.911)
	Female[ns]	276	90.2	306	100			
Babesia bigemina	Male[ns]	175	87.5	200	100	2.942	0.0863	0.5689 (0.3132 a 1.033)
	Female[ns]	283	92.5	306	100			

[ns]not significant by Chi-square (χ^2) test with Yates at 5% probability; OR = *Odds Ratio*; CI = Confidence Interval.

Table 3. Prevalence of antibodies against *Babesia bovis* and anti-*Babesia bigemina* in calves according to age. Region of Araguaína, Tocantins State, Brazil.

	Age (months)	Positive		Total nº		χ^2	P value
		Nº	%	Nº	%		
Babesia bovis	8-12[b]	149	90.9	164	100		
	13-18[b]	127	83.5	152	100	26.52	0.0001
	19-24[a]	188	98.9	190	100		
Babesia bigemina	8-12[ns]	148	90.2	164	100		
	13-18[ns]	138	90.8	152	100	0.0274	0.9864
	19-24[ns]	172	90.5	190	100		

Variables followed by the same letter do not differ statistically among themselves by Chi-square (χ^2) test with Yates at 5% probability.

In summary, the Araguaína region was considered an area of enzootic stability for *B. bovis* and *B. bigemina*. This region offers risk for babesiosis to susceptible animals coming from areas of enzootic instability. Therefore, use of appropriate preventive measures is needed, especially for the control of ticks and/or vaccination.

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