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Bovine babesiosis and anaplasmosis complex: diagnosis and evaluation of the risk factors from Bahia, Brazil

Complexo tristeza parasitária bovina: diagnóstico e avaliação dos fatores de risco na Bahia, Brasil

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Abstract

Direct diagnoses were made by using - blood smears and nested PCR (nPCR) tests on 309 blood samples from crossbred dairy cattle in the municipality of Ibicaraí, Bahia. From diagnostic blood smear slides, the observed parasitic frequencies were 31.1% for *Anaplasma marginale* and 20.4% for *Babesia* sp. From nPCR diagnoses, they were 63% for *A. marginale*, 34% for *Babesia bigemina* and 20.4% for *Babesia bovis*. There were significant differences ($P < 0.01$) between the two diagnostic methods (nPCR and blood smear slides). The compliance obtained from the kappa test was 0.41 and 0.48 for *A. marginale* and *Babesia* sp., respectively. The tick samples from the six farms analyzed using nPCR were only positive for *A. marginale*. Evaluation of the risk factors relating to the presence of ticks and the age of the animals showed that there was a significant association ($P < 0.01$) with the frequency of animals infected with both pathogens. Therefore, under the conditions studied, nPCR proved to be a good tool for diagnosing the agents of the bovine babesiosis and anaplasmosis complex because of its sensitivity and specificity in comparison with blood smears. The municipality of Ibicaraí is an area with endemic prevalence of bovine babesiosis and anaplasmosis confirmed by nPCR and *A. marginale* is the main agent of the disease.

Keywords: Bovine Babesiosis and Anaplasmosis Complex, blood smear, nPCR, risk factors.

Resumo

Realizou-se o diagnóstico direto por esfregaço sanguíneo e nested PCR (nPCR) em 309 amostras de sangue de bovinos mestiços leiteiros provenientes do município de Ibicaraí, Bahia. A frequência observada no diagnóstico por lâminas de esfregaço sanguíneo foi 31,1% para *Anaplasma marginale* e 20,4% para *Babesia* sp. Enquanto que no diagnóstico por nPCR foi 63% para *A. marginale*, 34% para *Babesia bigemina* e 20,4% *Babesia bovis*. Verificaram-se diferenças significativas ($P<0,01$) na comparação entre os dois métodos de diagnósticos (nPCR e esfregaço sanguíneo). A concordância ao teste KAPPA obtida foi de 0,41 e 0,48 para *A. marginale* e *Babesia* sp., respectivamente. As amostras de carrapatos das seis propriedades analisadas por nPCR foram positivas apenas para *A. marginale*. Na avaliação dos fatores de risco verificou-se que a presença de carrapato e idade dos animais apresentaram associação significativa ($P<0,01$) com a frequência de animais infectados por ambos os patógenos analisados por nPCR. Portanto, nas condições estudadas, a nPCR revelou-se uma boa ferramenta para diagnóstico dos agentes do complexo tristeza parasitária bovina (TPB) devido a sensibilidade e especificidade, quando comparado ao esfregaço sanguíneo. O município de Ibicaraí apresenta-se como uma área endêmica para TPB com prevalência comprovada através de nPCR e, *A. marginale* o principal agente encontrado.

Palavras-chave: Complexo TPB, esfregaço sanguíneo, nPCR, fatores de risco.

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Introduction

In Brazil, the main etiological agents of the bovine babesiosis and anaplasmosis (BBA) complex are *Anaplasma marginale* Theiler, 1910, *Babesia bovis* Babes, 1888, and *B. bigemina* Smith; Kilborne, 1893. The first is a bacterium belonging to the order Rickettsiales, of the Anaplasmataceae family (DUMLER et al., 2001), and the other two are protozoa belonging to the Babesiidae family. The main vectors of *Babesia* are *Rhipicephalus* (*Boophilus*) ticks (BOCK et al., 2004). *Anaplasma marginale* is transmitted mechanically via blood-contaminated fomites or mouthparts of biting flies (SHIMADA et al., 2004). Biological transmission of *A. marginale* is effected by ticks. In general, the tick vectors of *A. marginale* include *Rhipicephalus* spp., *Dermacentor* spp. (*D. albipictus*, *D. andersoni*, *D. hunteri*, *D. occidentalis* and *D. variabilis*) and *Ixodes ricinus*, while *Amblyomma* spp. do not appear to transmit *A. marginale* (KOCAN et al., 2004). Although transovarial transmission has been reported for some ticks, it has not been demonstrated for *R. microplus* (RIBEIRO et al., 1996; KESSLER, 2001).

Agents for the BBA complex are distributed across almost the entire Brazilian territory and their occurrence is directly related to the distribution of the vectors, which find ideal conditions for development in countries with tropical and subtropical climates. This complex of diseases causes economic losses and is a limiting factor for the growth of cattle, due to high rates of morbidity and mortality in these animals (PEREIRA, 2006).

The epidemiological profile of the BBA complex of a region can be determined by evaluating several factors, such as breed, age, climate, stress and pasture management. Regions can be defined as free, unstable or stable. Areas that are free from BBA are those where weather conditions are not favorable for development of the vector (BERTO et al., 2008). Unstable areas are those where the climatic conditions and/or livestock management affect occurrences of BBA at certain periods of the year (KESSLER et al., 1983, BARROS et al., 2005). In stable areas, the disease is present throughout the year, and therefore the animals have high enough levels of antibodies to promote protection that can be passed to calves with the colostrum (MADRUGA et al., 1984; GONÇALVES, 2000; OSAKI et al., 2002). It is necessary to know the epidemiological situation of a region in order to measure occurrences of outbreaks and the need to implement preventive measures (GUIMARÃES et al., 2011).

In most regions of Brazil, BBA presence is epidemiologically stable, with a few areas of instability (ARTILES et al., 1995; GUIMARÃES et al., 2011). A similar situation is observed in the state of Bahia, where the areas of instability are strictly due to semi-arid climatic conditions that are unfavorable for development of the vector (ARAÚJO et al., 1998).

Worldwide, many techniques have been standardized to indirect and direct diagnose the BBA complex. The diagnosis consists of indirect detection of antibodies against the agents of the BBA complex. Several serological tests are used for detection of bovine babesiosis and anaplasmosis, and the following stand out among them: the rapid conglutination test (RCT), latex agglutination, hemagglutination, complement fixation (CF) (MARANA et al., 2006), test card (TC), indirect immunofluorescence assay (IFA) (SOUZA et al., 2001; MARANA et al., 2009), indirect enzyme-linked immunosorbent assay (iELISA) and competitive ELISA (cELISA) (MARANA et al., 2009).

The BBA complex can be directly diagnosed based on viewing parasites within red blood cells in thin blood smears stained with Giemsa, or by means of parasite DNA amplification using the polymerase chain reaction (PCR) (VIDOTTO; MARANA, 2001). The parasitological examination, performed by means of blood smears, is the oldest method and is routinely used for diagnosing hemoparasites. In the acute phase of the disease, when parasitemia is high, parasites are easily detected in bovine erythrocytes (FARIAS, 1995). However, when the animals recover and become carriers with low parasitemia, viewing the parasites using this method becomes extremely difficult and may fail, since the parasite can easily be mistaken for an artifact. Thus, the sensitivity of blood smears is low and false-negative results are common (BOSE et al., 1995). However, this event is common when the technician is not trained to reach a diagnosis.

Advances in the field of molecular biology have made it possible to use parasite DNA amplification techniques as diagnostic methods. Molecular diagnosis of hemoparasites identifies the agent directly, even when the parasites are circulating in small quantities, and this method has high sensitivity and specificity in comparison with other diagnostic techniques, such as blood smears. Among the molecular diagnosis techniques, the PCR technique has been used to demonstrate the presence/absence of *Babesia* spp. and *Anaplasma* spp. in asymptomatic carrier cattle (BRITO et al., 2006).

The municipality of Ibicaraí, located in southern Bahia, stands out as a milk production region that provides raw material for the dairy industry in this state. Given the importance of the BBA complex, together with the lack of information on the epidemiology of these diseases in southern Bahia, the aims of the present study were to compare the diagnostic methods for direct blood smears and nPCR, determine the frequency of infection by agents for the BBA complex and evaluate the risk factors associated with such infection in crossbred dairy cattle in the municipality of Ibicaraí, Bahia.

Materials and Methods

Study area

This study was conducted in the municipality of Ibicaraí, which is located in the southern region of Bahia, covering a total area of 217,914 km^2, at a latitude of 14°51' south and longitude of 39°35' west, with an average altitude of 162 m. The vegetation consists of seasonal and dense rain forest, with rainfall between 1200 and 1400 mm/year and an average annual temperature of 27 °C. The municipality has a herd of approximately 17,900 cattle, of which 4,500 are dairy cattle with an average production of 2.6 million liters/year (IBGE, 2010).

The sample size was calculated using Epi Info 6.0, taking into consideration the total population of the herd, an 80% possibility of detecting the disease with a 95% confidence interval and a

statistical error of 5%. This calculation resulted in a sample (N) of 243 animals. However, blood samples were collected from 309, with 169 samples of adult females and 140 samples from calves of both sexes aged between one and 270 days.

Samples were collected on six farms with extensive rearing on pasture. In some cases, the cattle were given protein supplementation. The sample collection period was between November 2010 and February 2011. Blood samples (5 mL volume) were collected by coccygeal vein puncture with needle and Vacutainer tubes containing EDTA anticoagulant. The samples were packed in insulated containers with ice packs, and were transported to the laboratory for processing, on the same day.

To obtain data on the risk factors relating to the BBA complex, a questionnaire was applied, which was filled out at the time of sample collection. This contained objective discursive questions, which had the purpose of collecting information on: ticks (yes or no); flies (yes or no); animal movements (yes or no); examinations for hemoparasites (yes or no); use of larval packet test (yes or no); type of calf management (individual or collective); breed of animals (crossbred or Girolando); pasture areas; breeding systems (intensive or extensive); age (adult or calf); and sex of animals (male or female).

Laboratory processing

The blood samples collected were used to blood smears preparation, which were fixed using methanol and stained by Giemsa. To direct diagnosis, the slides were observed under an optical microscope (Olympus BX 51®).

Extraction of genomic DNA

A volume of 300 µL of each blood sample was aliquoted into 2 mL microcentrifuge tubes, to which 500 µL of distilled water was added. This mixture was homogenized by vortexing for 30 s to break the cells, and was then centrifuged for 6 min at 12,000 g. The supernatant was discarded and the pellet was eluted in T.E. (10 mM Tris-HCl, pH 8.0; 200 mM EDTA, pH 8.0; proteinase K 120 mg/mL; and 1% Triton X-100). Each sample was gently stirred on a vortex and was incubated in a water bath at 50 °C for about 30 min. Then SDS (0.5%) was added, homogenized by vortexing for 30 s and incubated in a water bath for 30 min. Genomic DNA was extracted using a mixture of phenol, chloroform and isoamyl alcohol, and was precipitated with 100% ethanol and ammonium acetate at a ratio of 1/10. The pellet was washed with 85% ethanol, resuspended in 10 mM Tris-HCl (pH 8.0) and quantified by means of spectrophotometric absorbance at 260 nm and 280 nm. The DNA samples were stored at –20 °C in a microtube in order to perform- PCR diagnostic, later on.

To check the feasibility of total DNA extraction from the blood of crossbred cattle, reactions with primers specific to the promoter region of the DGAT1 gene (which is responsible for production of milk fat) were performed: forward - 5'-TCA GGATCCAGAGGTACCAG-3' and reverse - 5'-GGGGTCCAAGGTTGATACAG-3' (KUHN et al., 2004).

DNA extraction from ticks

Around 10 teleogine ticks were collected from each animal on each of the six farms. These were fixed in ethanol and stored at –20 °C. All the ticks collected on a single farm were mixed to form a representative sample. From each of these six samples, five subsamples were withdrawn, weighing approximately 300 mg (around 3 to 5 ticks per sample), thus totaling 30 subsamples, in order to increase the likelihood of identifying the DNA of parasites in ticks. Each subsample was macerated with liquid nitrogen, followed by genomic DNA extraction using the same protocol as used for the blood samples.

Polymerase chain reaction (PCR)

For the molecular diagnosis of *A. marginale*, *B. bovis* and *B. bigemina*, nPCR reactions were performed, using the primers described in Table 1. The reactions were prepared in a volume of 25 µL containing 200 mM dNTP, 5.0 mM $MgCl_2$, 2 U Taq DNA polymerase (Promega®), 3x amplification buffer (supplied by the manufacturer), 11 pmol of every primer (Ana Ana R and F for *A. marginale*, BoF and Bor for *B. bovis* and Bilhah and Bilb for *B. bigemina*) and 60 ηg of genomic DNA. The amplification protocol was as follows: initial denaturation at 95 °C for 5 min

Table 1. Primers used for the detection of *A. marginale* (TORIONI DE ECHAIDE et al., 1998), *B. bovis* and *B. bigemina* (FIGUEROA et al., 1993).

Pathogen	Primer	Sequence	Bp
B. bovis	BoF	CACGAGGAAGGAACTACCGATGTTGA	350
	BoR	CCAAGGAGCTTCAACGTACGAGGTCA	
	BoFN	TCA ACA AGGTACTCTATATGGCTACC	290
	BoRN	CTACCGAGCAGA ACCTTCTTCACCAT	
B. bigemina	BiIA	CATCTAATTTCTCTCCATACCCCTCC	278
	BiIB	CCTCGGCTTCAACTCTGATGCCAAAG	
	BiIAN	CGCAAGCCCAGCACGCCCCGGTGC	170
	BiIBN	CCGACCTGGATAGGCTGTGTGATG	
A. marginale	Ana F	GCATAGCCTCCCCCTCTTTC	458
	Ana R	TCCTCGCCTTGCCCCTCAGA	
	AnaFint	TACACGTGCCCTACCGACTTA	345

followed by 35 cycles of 94 °C for 1 min, annealing at 65 °C for 1 min for *A. marginale*, 55 °C for 1 min for *B. bovis* and 64 °C for 1 min for *B. bigemina*, and extension at 72 °C for 40 s, with a final step at 72 °C for 5 min in the MJ96G thermocycler (Biocycler®). The amplification products were separated on 1.5% agarose gel stained with ethidium bromide and photographed.

In the second reaction, 25 µL of DNA from the product of the first reaction was used as the template. nPCR was only done with samples that did not amplify (i.e. were negative) in the first reaction. In the reaction with 25 µL, the primers F int Ana and Ana R were used for *A. marginale*, BoFN and Born for *B. bovis* and B. Bilan and BilBN for *B. bigemina*, with an amplification scheme similar to that used in the first reaction, except for changing the annealing temperatures, which were 65 °C for *A. marginale* and *B. bovis*, and 69 °C for *B. bigemina*. The amplification products were separated on 1.5% agarose gel stained with ethidium bromide and photographed.

In all reactions, positive and negative controls were used. DNA samples from Embrapa Beef Cattle in Campo Grande, Mato Grosso do Sul, were used as the positive control. The negative control, ultrapure water was used in the PCRs.

Statistical analysis

For data analysis, logistic regression was performed to construct multivariate models that could explain the dependent variables (presence of DNA de *Babesia* sp. and *A. marginale*) as a function of the 11 independent variables, dichotomously (Table 2). The independent variables were subjected to univariate analysis and those with a statistical association (here considered to be $P < 0.20$, using the chi-square test, for this first analysis) were tested in the multivariate model by means of the stepwise forward method (COSTA et al., 2013). The variables were included in

Table 2. Risk factors for the etiologic agents of bovine babesiosis complex in crossbred dairy cattle in the municipality of Ibicarai, BA, Brazil.

Risk factors	*Babesia* sp.				*Anaplasma marginale*			
	Samples +/Total (%)	X^2	P	OR (CI 95%)	Samples +/Total (%)	X^2	P	OR (CI 95%)
Presence of tick								
Yes	119/174 (68.4)	98.44	0.00	17.3 (9.2-32.3)	132/174 (75.9)	29.34	0.00	4.32 (2.6-7.0)
No	15/135 (11.1)				60/134 (44.8)			
Transit of animals								
Yes	61/134 (45.5)	0.30	0.57	1.02 (0.6-1.6)	83/134 (61.9)	0.00	0.95	1.02 (0.6-1.6)
No	73/175 (41.7)				109/175 (62.3)			
Test to hemoparasites								
Yes	21/61 (34.4)	2.04	0.15	0.62 (0.3-1.1)	37/61 (60.7)	0.01	0.90	0.87 (0.4-1.5)
No	113/248 (45.6)				155/248 (62.5)			
*Adult Immersion Test								
Yes	21/61 (34.4)	2.04	0.15	0.62 (0.3-1.1)	37/61 (60.7)	0.07	0.90	0.87 (0.4-1.5)
No	113/248 (45.6)				155/248 (62.5)			
Type hutch								
Aggregate	109/256 (42.6)	0.21	0.64	0.83 (0.4-1.5)	160/256 (62.5)	0.01	0.89	1.15 (0.6-2.1)
Single	25/53 (47.2)				32/53 (60.4)			
Age of animals								
Calves	78/140 (55.7)	14.98	0.00	2.53 (1.5-4.0)	108/140 (77.1)	23.35	0.00	3.62 (2.1-5.9)
Cows	56/169 (33.1)				84/169 (49.7)			
Breed								
Blended	109/256 (42.6)	0.21	0.64	0.83 (0.4-1.5)	160/256 (62.5)	0.01	0.89	1.15 (0.6-2.1)
Girolando	25/53 (47.2)				32/53 (60,4)			
Cattle run								
> 50 ha	73/175 (41.7)	0.30	0.57	0.85 (0.5-1.3)	109/175 (62.3)	0.00	0.95	0.97 (0.6-1.5)
< 50 ha	61/134 (45.5)				83/134 (61.9)			
System breeding								
Extensive pasture	88/195 (45.1)	0.48	0.48	0.82 (0.5-1.3)	123/195 (63.1)	0.10	0.74	0.83 (0.5-1.3)
Extensive /supplementation	46/114 (40.4)				69/144 (60.5)			
Sex								
Female	93/243 (38.3)	11.06	0.10	1.58 (0.8-2.8)	146/243 (60.1)	1.65	0.19	1.58 (0.8-2.8)
Male	41/66 (62.1)				46/66 (69.7)			
Presence of flies								
Yes	58/146 (39.7)	1.22	0.26	0.75 (0.4-1.1)	95/146 (65.1)	0.31	0.57	1.17 (0.7-1.8)
No	87/163 (53.4)				63/163 (55.3)			

*Bioassay of acaricide susceptibility by Drummond et al. (1973).

the multivariate model if they displayed statistical significance of $P < 0.05$, considering also the significance level of the final model. All analyses were performed using the Epi-Info software, version 3.5.1. Principal component analysis (PCA) also was made using SAS (2003) statistical program, where the factors associated with significant effect by chi-square test were used as variables in terms of positive and negative diagnoses for *A. marginale* and *Babesia* sp. The PCA is a multivariate analysis technique which consists in transforming an original set of variables into a set of transformed variables, the principal components, that retains as much of the original information in terms of total variation (CRUZ et al., 2011). The kappa test was used to measure the agreement between the tests under real conditions, in accordance with Thrusfield (1986).

Results

Amplification of total DNA extracted from the blood of crossbred cattle with primers specific to the promoter region of the gene DGAT1 resulted in fragments of 145 bp, thus proving the feasibility of analyzing DNA for use in molecular diagnosis of BBA.

The first PCR amplification resulted in fragments of 458 bp for *A. marginale*, 350 bp for *B. bovis* and 278 bp for *B. bigemina*. The second amplification reaction resulted in fragments of 345 bp for *A. marginale*, 290 bp for *B. bovis* and 170 bp for *B. bigemina*. The animals were considered to be positive for one of the three agents of the BBA complex when the amplifications resulted in the fragment patterns described above. The analysis on 309 blood samples from cattle, that were subjected to nPCR, showed that 195 (63.1%) were positive for *A. marginale*, 105 (34%) for *B. bigemina* and 63 (20.4%) for *B. bovis*. From the blood smear slides, 96 (31.1%) were positive for *A. marginale* and 60 (19.41%) for *Babesia* sp.

In this study, 73.13% (226/309) of the animals that were analyzed by nPCR were found to be infected; 24.27% (75/309) were infected by two of the three pathogens evaluated and 10.03% (31/309) by all three (Figure 1). A total of 24 subsamples (80%) of ticks from the six farms that were analyzed by nPCR were positive for *A. marginale*. All the tick samples analyzed by nPCR were negative for *Babesia* spp.

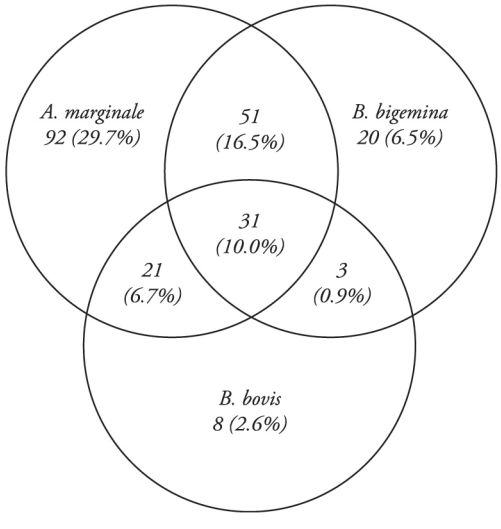

Figure 1. Venn diagram PCR results for BPS complex.

There were significant differences ($P < 0.01$) between the two diagnostic methods (nPCR and blood smear slides). Out of the 195 blood samples that were positive for *A. marginale* from nPCR, 96 (49.2%) showed positive on blood smears (Table 3). The agreement obtained in the kappa test was 0.41. Out of the 134 blood samples (43.4%) that were positive for *Babesia* sp. from nPCR, 63 (47%) showed positive on blood smears (Table 3). The agreement obtained in the kappa test was 0.48.

In assessing the risk factors for the etiological agents of the BBA complex, it was found through the logistic regression, which the presence of ticks and the age of the animals showed a significant association ($P < 0.01$) with the frequency of animals infected by both pathogens, in the analysis by nPCR (Table 2 and 4).

The first two principal components were sufficient to explain 81.60% (CP1 and CP2 = 51.70% = 29.90%) of the variation between the positive and negative individuals for *A. marginale*. The first principal component was given by CP1 = - 0.632 + 0.659 * SEX * + 0.406 * PCARR FET, and it was observed that the variables age (FET) and presence of ticks (PCARR) were associated factors that characterize most animals with positive diagnosis. Sex has negative weighing value and was slightly associated with positive animals for this disease

For *Babesia* sp. was observed that the first three principal components are needed to explain 86.60% (=39.7% CP1, CP2 and CP3 = 25.6% = 21.3%) variance in the original data set. The first principal component was given by CP1 = - 0.584 + 0.648 * SEX * + 0.415 * FET PCARR - 0.254 * TESTCARR. In this component will also be highlighted the variables age (FET) and presence of ticks (PCARR), and these were the associated factors related to the majority animals with positive diagnosis. SEX and the TESTCARR (Test to hemoparasites) variables showed negative values.

Discussion

In Brazil, diagnostic techniques to investigate the molecular epidemiology of the BBA complex are little used, with only a small number of published studies. In Bahia, this is the first study in which the nPCR technique was used to detect the presence of agents for the BBA complex in crossbred cattle and ticks. The amplification patterns found in this study for *B. bovis*, *B. bigemina* and *A. marginale* were similar to those described by Figueroa et al. (1993), Torioni de Echaide et al. (1998), Moura et al. (2002) and Brito et al. (2007), who used the same sets of primers.

The southern region of Bahia is characterized as an area of enzootic stability (ARAÚJO et al., 1998), according to the concepts of Mahoney and Ross (1972). This region has annually climatic conditions that favor the development of *R. microplus*, the main vector of the agents for the BBA complex.

On the six farms analyzed, it was found that 63.1% of the animals were positive for *A. marginale*, using the nPCR diagnostic test. This was similar to what was found by Torioni de Echaide et al.

Table 3. Comparison of PCR and blood smears as diagnostic methods of *Anaplasma marginale* and *Babesia* sp. in crossbred dairy cattle herds from the municipality of Ibicarai, BA, Brazil.

Anaplasma marginale				
		PCR		**Total**
		Positive	**Negative**	
Blood smear	Positive	96 (49.2%)	0 (0%)	96 (31.1%)
	Negative	99 (50.8)	114 (100%)	213 (68.9%)
	Total	195 (63.1%)	114 (36.9%)	309 (100%)
***Babesia* sp.**				
		PCR		**Total**
		Positive	**Negative**	
Blood smear	Positive	63 (47%)	0 (0%)	63 (20.4%)
	Negative	71 (53%)	175 (100%)	246 (79.6%)
	Total	134 (43.4%)	175(56.6%)	309 (100%)

Test χ^2 (P<0.01).

Table 4. Multivariate analysis of risk factors associated with the presence of *Anaplasma marginale* and *Babesia* sp. in crossbred dairy cattle in the municipality of Ibicarai, BA, Brazil.

	***Babesia* sp.**			***Anaplasma marginale***		
Variable	**OR**	**CI 95%**	***P* Value**	**OR**	**CI 95%**	***P* Value**
Age of Animals						
Calves	2.18	1.24-3.81	0.006	3.21	1.89-5.45	0.0000
Cows	1.00			1.00		
Presençe of Ticks						
Yes	16.43	8.74-30.90	0.0000	3.91	2.35-6.51	0.0000
No	1.00					
	2*Final Log-Likelihood: 303.7129			2*Final Log-Likelihood: 350.7693		

(1998) in Oregon, USA, with 64% of positive animals, and lower than what was observed by Brito et al. (2010) in Rondônia and Acre, Brazil, who found that 98.6% and 92.4% of the animals were positive, respectively, among animals aged between 4 and 12 months, using PCR in both studies. These higher results obtained by Brito et al. (2010) may have been influenced by several factors, including age, since their study was conducted exclusively using calves. In the results from the present study, the calves also showed higher values (P < 0.01). The frequency of positive samples for *B. bigemina* was 34%, similar to what was found by Smeenk et al. (2000), who observed in Zimbabwe that 35% of the animals were positive. These findings were higher than what was found by Brito et al. (2007) in Rondônia, with a frequency of 3.09%, also using molecular diagnostic PCR. On the other hand, 20.4% of the animals were found to be positive for *B. bovis*, a frequency similar to what was observed by Martins et al. (2010) in Mozambique, where 27% of the animals were positive. Furthermore, mixed infections with the hemoparasites *B. bigemina* and *B. bovis* were identified and were correlated with the ticks responsible for transmitting these diseases to cattle (MARTINS et al., 2010).

As expected, the frequencies of *Babesia* sp. and *A. marginale* detected by DNA amplification (43.4 and 63.1%, respectively) were significantly higher than those obtained through microscopic examination of blood smears (20.4 and 31.1% respectively) (P < 0.01). Similar results were observed by Cassini et al. (2011) from comparing these same techniques for detecting *Babesia* sp. in Italy, who found that 21.6% of the animals were positive by PCR and 6.5% by blood smears. The method of microscopic examination of blood smears has low sensitivity and does not detect positive animals in the early or chronic stages of infection, when the number of circulating parasites is very low (OLIVEIRA-SEQUEIRA et al., 2005), although this procedure is used by veterinarians to monitoring clinical cases (SANTANA et al., 2008).

The multivariate logistic regression analysis results and principal components showed the influence of the risk factors presence of ticks and age for of *A. marginale* and *Babesia* sp infection. It was found that the calves were 3.62 and 2.53 times more susceptible when compared to adult cows for *A. marginale* and *Babesia* sp., respectively. Similar results were observed for *A. marginale* by Ashuma et al. (2013). The results of this paper disagree with Terkawi et al. (2012) that found greater positivity of *Babesia* in adult cattle.

The presence of ticks positive for *A. marginale* was in agreement with the findings of Shimada et al. (2004), who confirmed the presence of *A. marginale* DNA in *R. microplus* larvae by PCR. *Rhipicephalus microplus* is a monoxenic tick, but adult stages can migrate between animals by physical contact (MASON; NOVAL, 1981). Intrastadial transmission by male ticks may be important in maintaining the organism in enzootic areas. Transstadial

transmission has been reported in relation to many ticks including *R. microplus* (CONNELL, 1974; STICH et al., 1989).

The fact that the ticks analyzed were negative for *Babesia* sp. does not rule out their importance as *Babesia* sp. vectors for the studied animals. The presence of ticks was considered to be an important risk factor (OR = 16.4) for *Babesia* positive animals. These negative results can be explained by low positivity for *Babesia* sp. in the ticks or by molecular detection failure due to low amounts of the pathogen's DNA in the analyzed samples. In fact, transmission of *B. bigemina* and *B. bovis* by *R. microplus* has been confirmed in some reports (SPARAGANO et al., 1999; OLIVEIRA-SEQUEIRA et al., 2005).

Determination of the stability or instability profile of a region depends on assessment of several factors, such as those inherent to the animal (breed and age), along with climate change, stress management, pasture type and presence of vectors (GUIMARÃES et al., 2011). In areas of enzootic stability, immunity is acquired early enough in the life of most animals, and this is the reason that clinical disease only occurs rarely. However, management and climatic conditions can affect the immune status and tick population, making the animals susceptible. Certain risk factors may affect the presence of hemoparasites in animals, such as the presence of ticks and the age of the animals, as seen in this study, in which these two risk factors were significantly associated with the presence of *A. marginale* and *Babesia* sp. (Table 2). Persistent presence of ticks throughout the year allows continuous inoculation of the pathogens into animals from the time of their birth onwards, which allows them to develop specific immunity that is sufficient to make them resistant (FOLLY et al., 2009). Thus, calves have higher prevalence of agents for the BBA complex, as observed by Madruga et al. (2000) in Mato Grosso do Sul, even without evidence of clinical signs (ZAUGG et al., 1986).

Endemic (or enzootic) stability is a widely used term in the epidemiology of ticks and tick-borne diseases. The main features of endemic stability in this context are a high level of challenge with hemoparasite-infected ticks and a concurrent low incidence of clinical disease. However, Jonsson et al. (2012) disagreed with the definition of endemic stability and reliance on seroprevalence as an indicator of exposure that could be generally accepted and extrapolated to other host-tick-pathogen systems. These authors described the importance of including data generated to correlate tick infection rates by pathogens, tick infestation rates among cattle, serial seroprevalence in cattle and disease incidence among animals of diverse genotypes. Further studies are needed to confirm these claims. They concluded that the concept of endemic stability was not applicable to *B. t. indicus* cattle or to tick-borne diseases in which inverse age immunity (as seen in *B. bovis, B. bigemina* and *A. marginale* infections) is not a feature.

In short, within the conditions studied, nPCR proved to be a good tool for diagnosing BBA agents because of its sensitivity and specificity in comparison to blood smears. The municipallity of Ibicaraí presents all three parasites, and *Anaplasma marginale* is the main agent for BBA. Moreover, in the municipality of Ibicaraí, the factors associated with BBA that presented greatest significance were the presence of ticks and the age of the animals, in comparison with the observed frequency of BBA agents.

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