Seasonal fluctuations in Babesia bigemina and Rhipicephalus microplus in Brangus and Nellore cattle reared in the Cerrado biome, Brazil

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Abstract

Background: *Rhipicephalus microplus* (Ixodida: Ixodidae, Canestrini, 1888) is a tick that causes great economic and health losses related to the cattle industry and is the main vector of *Babesia bigemina* (Piroplasmidae: Babesidae, Smith & Kilborne, 1893). *B. bigemina* is responsible for a tick-borne disease known as babesiosis that can cause hemolytic anemia, fever, and death. This study investigated the relationship between the number of ticks per animal and the number of *B. bigemina cbisg* gene copies in the blood of Brangus and Nellore cattle reared without acaricidal treatment in the Brazilian Cerrado over a one-year period.

Methods: The ticks on 19 animals (9 Brangus and 10 Nellore cattle) were counted every 18 days, and blood was collected every 36 days for 12 months. Serological samples were analyzed with iELISA. Genomic DNA (gDNA) was analyzed by PCR and qPCR. The PCR products were sequenced by the Sanger method.

Results: These two breeds showed similar weight development and no clinical signs of babesiosis. Statistically significant differences ($p< 0.05$) were observed in the number of ticks and the number of *B. bigemina cbisg* gene copies between the breeds.

Conclusion: No babesiosis clinical signs were observed, and no correlation between the number of ticks and the number of circulating copies of *cbisg* was observed.

Introduction

Babesia bigemina is a bovine hemoparasite mainly present in tropical and subtropical regions that can cause clinical signs such as hemolytic anemia, fever, occasional hemoglobinuria and death [1]. Babesiosis is transmitted by ticks [2]. *Rhipicephalus microplus* is responsible for its transmission in the Brazilian Cerrado, presenting a strong preference for cattle and causing large economic losses related to the cattle industry [3].

Bos indicus cattle present fewer engorged ticks than other types of cattle and are known as a breed resistant to tick infestation [4, 5]. They also exhibit lower *Babesia* spp. parasitemia [6] than other taurine breeds and their crosses; however, the degree of parasitism may vary with animal age and breeding system [7]. Crossbreeding with taurine cattle is used to increase the genetic propensity for weight gain and precocity in beef cattle, and Brazil's Cerrado region has increased its use of the Brangus crossbreed at the cost of lower resistance to tick infestation [8].

Moreover, the absence of a well-defined, effective and well-deployed vaccine against babesiosis [9], tick resistance to chemical control [10], the capacity for transovarial *Babesia* spp. transmission in ticks [11] and the endemism of babesiosis in Brazil render monitoring and control difficult.

This study sought to investigate the number of ticks and assess the *B. bigemina* parasitary intensityin naturally infested Brangus and Nellore cattle reared in the growth phase without any acaricide intervention in the Brazilian Cerrado.

Material And Methods

The study was conducted in the municipality of Água Clara, Mato Grosso do Sul State, Brazil (20° 46'24"S 52° 32'24"W). Nineteen animals (9 Brangus and 10 Nellore cattle) in the growth phase aged eight to 10 months and with a mean weight of 219.5 kg were studied. These animals were naturally infested and kept together at a density of 0.6 animals per hectare. Acaricides were not used, and prophylactic control of tick-borne diseases (TBDs) was not performed.

Data collection was performed from June 2016 to June 2017, with intervals of 18 days between tick counts according to the method of Wharton and Utech [12]. Ticks with lengths greater than 4.5-8 mm on both sides of each animal were counted. Tick taxonomic classification was performed following the system of Pereira et al. [13]. Additionally, every 36 days, the animals were weighed on a digital scale (Coimma®, Dracena - SP) to assess animal welfare [14], and blood was harvested from the caudal vein using Vacutainer® tubes. In total, 228 samples were collected; these samples were kept at 4°C and transported to the laboratory for serum and gDNA extraction.

Genomic DNA (gDNA) was extracted from whole blood as described by Rodrigues et al. [15] using the phenol/chloroform method. The quantity and purity of each sample were estimated by spectrophotometry with a NanoDrop™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the absorbance readings at 260 nm and the 260/280 nm absorbance ratios, respectively. Then, the 228 samples corresponding to each animal and collection time point were stored at -80°C until further use.

These gDNA samples were analyzed by PCR in duplicate following the methodology of Guerrero et al. [16] using the primers KB-18 (5'-GAT-GTA-CAA-CCT-CAC-CAG-AGT-ACC-3' forward) and KB-19 (5'-CAA-CAA-AAT-AGA-ACC-AAG-GTC-CTA-C-3' reverse), which produce a PCR product of 262 bp. Two negative controls (a blood sample extracted from a bovine healthy donor and water) and one positive control (a sample obtained from a bovine blood smear positive for *B. bigemina*) were tested.

The final product was visualized on a 1.5% agarose gel stained with ethidium bromide (EtBr). Twelve samples yielding the expected PCR product size for *B. bigemina* were purified using a PureLink quick gel extraction kit (Invitrogen, Carlsbad, CA, USA). These DNA samples were sequenced at the Human Genome and Stem-Cell Research Center (Universidade de São Paulo – USP, Brazil) in an automatic sequencer (ABI 3730 DNA Analyzer, Applied Biosystems, USA) with a 48-capillary DNA analysis system. The sequenced PCR products were analyzed with MEGA X software [17], and the consensus sequences of the analyzed samples were deposited in GenBank under accession number MZ542450.1.

gDNA samples were diluted to a concentration of 100 ng/µl and subjected to qPCR analysis to quantify the circulating copies of the *cbisg* gene. Absolute quantification was performed using primers (PrimeTime® Std qPCR Assay-IDT-Integrated DNA Technologies®) based on the *B. bigemina* LK054939.1

sequence in GenBank and designed using the PrimerQuest Tool (IDT Technologies, Coralville, Iowa, USA), which generated an 88-bp product of the *cbisg* gene (forward primer 5'CGAAGTGCCCAACCATATTA-3', probe 5'-/56-FAmQCGAGTGTGT/Zen/TATCAGAGTATTAACTGAGGT/3IABkFQ/-3', and reverse primer 5'TGTTCCAGGAGATGTTGATTCTT-3').

Primer-dimer formation was tested with the OligoAnalyzer tool (https://www.idtdna.com/pages/tools/oligoanalyzer).

Specificity *in silico* was tested using the NCBI BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The organisms searched were limited to "bovine", "human" and "babesia".

The efficiency and reproducibility (Additional Files 1, 2 and 3) of the reaction were calculated as stated by Okino et al. [18]. Serial dilutions (1:10) from 10^1 to 10^{10} were used to construct a standard curve with different concentrations of synthetic DNA gBlocks® Gene Fragments (IDT, Coralville, IA, USA) containing the sequence of *B. bigemina* (5'-
TGACCTTTTATTATGTTCCAGGAGATGTTGATTCTTTCGAGTGTGTTATCAGAGTATTAACTGAGGTTAATATGGGTTGGGCACTTCGTTATTTCCATGCTCAATGTGTTT
TTTTTCTTTATGATGTTACATATGTTAAAAGGTTTATG-3 ' - also constructed based on the sequence under accession number LK054939.1). Positive controls and duplicate negative template and negative control samples were added to each qPCR run.

The reaction volume was 10 µL per well and consisted of 5 µL of Taqman™ Universal PCR Master Mix (Thermo Fisher Scientific), 0.5 µL of each primer (10 µM), 3 µL of Milli-Q H_2O and 1 µL of 100 ng/µL gDNA. The reactions were run in duplicate. Ultrapure water was used instead of gDNA as a negative control.

A five-point standard curve (concentrations of 10^5 to 10^{10} gBlocks®) was used in triplicate as an internal control in each 98-well plate. The samples were analyzed using a StepOnePlus™ Real-Time PCR System using a *hydrolase probe* activation cycle of 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 45 s and annealing/extension at 60°C for 1 min.

The reaction signal was recorded during the extension step, and the data were analyzed using StepOne v2.3. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed [19].

Using the qPCR results, the number of target DNA molecules in each reaction (copy number – CN) was calculated according to the method of Ke et al. [20], as follows: CN (L) = [(6.022 × 10^{23} (copies/mol) × concentration (g/mol))/molecular mass (g/L)], where 6.022 × 10^{23} is Avogadro's number and the molecular mass is the average molecular weight of double-stranded DNA (330 × 2) multiplied by the size of the cloned fragment.

For antigen detection of anti-*B. bigemina* immunoglobulin G (IgG), the indirect ELISA (iELISA) technique was used following a protocol based on that of Machado et al. [21].

Total antigen from *B. bigemina* (produced by the Immunoparasitology Laboratory of the Faculty of Agricultural and Veterinary Sciences [FCAV]/São Paulo State University [UNESP] Jaboticabal) was diluted to an optimal concentration of 10 µg/mL in 0.5 M carbonate/bicarbonate buffer (pH 9.6). After 12 h of incubation at 4°C, blocking was performed with PBS Tween 20 (pH 7.2) and 6% powdered skim milk (Molico®, Nestlé, Brazil). The ninety-six-well plates (Maxisorp®; Nunc, Thermo Scientific, Brazil) were incubated for 90 min at 37°C within a moist chamber.

After three washes with PBS Tween 20 buffer, the positive, negative, and reference sera were added (all diluted 1:400 in PBS Tween + 5% rabbit normal serum). The plates were then incubated at 37°C for 90 min in a moist chamber. After three washes with PBS Tween 20, alkaline phosphatase-conjugated bovine anti-IgG (Sigma®, St. Louis, USA) diluted 1:30000 in PBS Tween + normal 5% rabbit serum was added, and the plates were washed again.

Then, the alkaline phosphatase substrate p-nitrophenyl phosphate (Sigma®, St. Louis, MO, USA) was diluted in 1 mg/mL diethanolamine buffer (pH 9.8). The plates were sealed in aluminum foil and incubated for 30 min at room temperature. The plates were then read at a wavelength of 405 nm on a micro-ELISA reader (B.T.-100; Embrabio, São Paulo, Brazil).

The R Project for Statistical Computing (R version 3.6.3) [22] and RStudio (8.15 build 180091) [23] were used for statistical analysis.

The Kolmogorov–Smirnov test was performed to check data normality, and then the Mann–Whitney U test was used to compare the weight, number of ticks and *B. bigemina cbisg* gene CN between the breeds because the data did not present a normal distribution.

The CNs and numbers of ticks were $\log_{10}(n + 1)$-transformed and then analyzed by Spearman's rho statistic to estimate a rank-based measure of association among the weight, $\log_{10}$(CN) and $\log_{10}$(ticks).

Results

During the sampling period, the mean environmental temperature was 24.95 ± 2.77°C, the humidity was 66.86 ± 4.53%, and the rainfall was 33.41 ± 18.60 mm (Fig. 1). The Brangus cattle had a mean monthly weight gain of 13.69 ± 8.1 kg; for Nellore cattle, the weight gain was 16.46 ± 8.7 kg (Fig. 1). No significant difference was observed comparing the weight mean at the start (227.22 ± 17.26 for Brangus and 209.8 ± 24.36 for Nellore) and at the end of the experiment (378.88 ± 31.59 for Brangus and 395 ± 40 for Nellore) ($p > 0.05$).

Brangus cattle had a higher tick count (45.5 ± 20.9) than did Nellore cattle (10.08 ± 2) ($p < 0.01$) (Fig. 2B). All PCR and qPCR samples detected the presence of *B. bigemina*, none were positive for *B. bovis*. When the circulating *B. bigemina cbisg* gene CNs were analyzed (Fig. 2A) using qPCR, the Nellore breed presented a higher mean CN (3.25 ± 0.18) than the Brangus breed (2.5 ± 0.15) ($p = 0.005$) (Table 1).

Table 1
qPCR results for the detection of the *B. bigemina cbisg* gene.

					Cq		Quantitative (Log10 DNA copies)	
	E	R^2	Slope	y-Intercept	Nellore	Brangus	Nellore	Brangus
Max	103.18	1	3.349	41.89	38.10	37.27	4.91×10^{5}	1.04×10^{3}
Mean	99.0	0.990	3.345	38.14	32.72	34.10	2.23×10^{2}	7.45×10^{2}
Min	96.14	0.987	3.291	33.28	28.65	24.7	9.01×10^{-1}	0×10^{0}

E: efficiency of amplification; R^2: determination coefficient; Cq: quantification cycle;

Serological samples from each time point were analyzed by iELISA with a cutoff of 0.277. Brangus animals had 13% (15/108) positive samples, while Nellore animals had 15% (18/120) positive samples.

The Brangus breed presented a Spearman correlation of r_s= -0.23 between weight and CN (p = 0.01), a Spearman correlation of r_s= -0.47 between weight and tick count (*p = 0.00)*, and a Spearman correlation of r_s= 0.25 between tick count and CN (p = 0.01). The Nellore breed presented a Spearman correlation of r_s= -0.11 between weight and CN (p = 0.22), a Spearman correlation of r_s= -0.42 between weight and tick count (*p = 0.00)*, and a Spearman correlation of r_s= 0.12 between tick count and CN (p = 0.16).

Discussion

Brazil is an endemic region for cattle tick fever. Beef cattle breeders in the Cerrado region have introduced other taurine breeds into the genetic lines of their herds to increase the production of beef cattle per hectare and to meet the demand from the consumer market for higher-quality meat [24]; however, this strategy has increased the sensitivity of the animals to ticks and increased the risk for TBD outbreaks.

The Água Clara region is characterized by 3–4 tick cycles per year, three of which occur in the rainy season from October to April, when most infestations occur [25]. Our data suggest that the high tick infestation rates in Brangus cattle in the initial month of the experiment may have been related to the stress generated at the end of the weaning period and the beginning of the growth phase combined with the lower pasture quality at this time of the year in the Cerrado [8] and subsequent nutritional stress, which suppressed the immune system of the cattle [26].

No significant difference in weight was detected between the two breeds during the experimental period, but a weak negative correlation was observed between the number of ticks and body weight. As reviewed by Jonsson [27], this effect of ticks on cattle weight gain is expected and may cause economic loss over time [28].

During the experiment, larvae and nymphs were observed at the same infestation proportions in both breeds but were not quantified. Nellore animals showed few engorged tick females, which led to the end of the parasitic phase. The resistance in cattle like Nellore cattle could be associated with increased numbers of mast cells, eosinophils, and basophils in the skin, while recruitment of neutrophils is potentially associated with tick susceptibility [4]. The increased numbers of mast cells, eosinophils, and basophils cause the release of histamines from these cells, inhibiting tick attachment and leading to itching, increased grooming and tick removal [4].

Poor correlations between *Babesia* spp. CNs and tick counts have also been observed in recent studies, suggesting that there is no correlation between these factors at the time of data collection [29, 30]. However, Giglioti et al. [31] have suggested that a high positive correlation coefficient in bovine parasitemia may be dependent on or determine the parasitemia burden for ticks.

Our results contradict previous data reported by Bilhassi et al. [32] for *Babesia* spp. indicating that pure Zebu animals should be expected to have a low number of ticks, resulting in relatively low levels of parasitemia. However, one might question the methodology proposed by Wharton and Utech [12], as only the number of engorged parasites is taken into account; nymphs also have the ability to transmit *Babesia* spp. [33] but are not counted in the methodology of Wharton and Utech [12].

Babesia bigemina can establish long-lasting chronic infections that are often accompanied by *Anaplasma marginale* and *B. bovis*, causing the TBD complex. Even if a bovine host is able to establish an immune response that controls the disease, the parasite continues to proliferate in the bloodstream at levels that may be below detection by microscopy [34]. Although no clinical signs of babesiosis were observed during the study, subclinical cases cannot be discarded.

Água Clara is a region known to be endemic for the TBD complex, and the farm on which the experiment was carried out employs an extensive production system with nursing, weaning and rearing phases.

Some studies have observed serological prevalence rates of 23% for *B. bigemina* in the State of Mato Grosso do Sul [35], 87.7–98.9% in the Pantanal region [36] and 97% in Pará State [37]. In this study, we observed low iELISA responses (13% for Brangus and 15% for Nellore cattle) that did not reflect the PCR and qPCR results. This finding could be attributed to a state of equilibrium between *B. bigemina* and the host immune system (the cattle in this study were considered to be in good nutritional condition based on the weight gain curves) due to the ability to vary the antigens expressed on the surfaces of infected red cells, thus making the host a chronic carrier [38].

Enzootic stability of babesiosis in a herd occurs when the inoculation rate from ticks is sufficient to infect most calves before innate resistance to clinical disease disappears somewhere between six and nine months of age, ensuring that most cattle are infected and immune before they reach an age at which they are susceptible to clinical disease [39]. However, Jonsson et al. [40] criticized the concept of enzootic stability and did not recommend its application to *Bos taurus indicus* or diseases that have inverse immunity because the Mahoney and Ross [39] experiments did not test these breeds and ticks, nor did they perform serological assays. Thus, the degree of suppression of the host immune response in the field could not be evaluated as a function of infestation in the present study.

Conclusions

This work analyzed field data from growth-phase animals in a extensive breeding system without acaricide treatment in an endemic environment, the Cerrado region. The system might not have been the ideal system for these observations; however, the information gained contributes to comprehension of the seasonal dynamics of *B. bigemina* and may help lead to future identification and classification of strains that are less pathogenic to herds throughout this period.

In conclusion, Nellore cattle presented lower tick counts and higher CNs of the *B. bigemina cbisg* gene than Brangus cattle. Despite the low iELISA results, the pathogen was observed to be present throughout the entire period by molecular methods. On the other hand, the two breeds showed similar weight development and no symptoms of babesiosis throughout the study period. This was the first study performed on two cattle breeds in the rearing phase in the Cerrado region to quantify tick counts and circulating *B. bigemina* CNs.

Abbreviations

Bp
base pair
gDNA
genomic deoxyribonucleic acid
cbisg
Babesia bigemina cytochrome b gene
PCR
polymerase chain reaction
qPCR
real-time quantitative polymerase chain reaction
iELISA
indirect enzyme-linked immunosorbent assay.

Declarations

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Availability of data and materials

The majority of the data are included in the present manuscript. The nucleotide sequences are available under accession nos. LK054939.1 and MZ542450.1 in GenBank. All other relevant data are included in the manuscript and the references or are available upon request by the corresponding author.

Authors' contributions

KRM, MVG, JCB and RA designed the study; MVG, PB, POD, LOSH, NPZ, JCB and RA performed all sample collections; KRM and POD performed all molecular analyses; KRM, NPZ, LOSH and JCB performed statistical analyses; KRM, MVG, JCB and RA drafted the manuscript; and all authors reviewed the manuscript.

Ethics approval and consent to participate

The procedures with the animals were performed according to the guidelines of the Ethics Committee for Animal Experimentation (Comitê de Etica para Experimentação Animal - CEUA) (Embrapa Gado de Corte, Protocol 01/2016).

Consent for publication

Not applicable.

Declaration of competing interests

The authors declare that there are no conflicts of interest.

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Figures

Figure 1

Mean rainfall (gray background); mean temperature (gray column); Nellore cattle mean weight (gray line and gray circle); Brangus cattle mean weight (black line and black diamond).

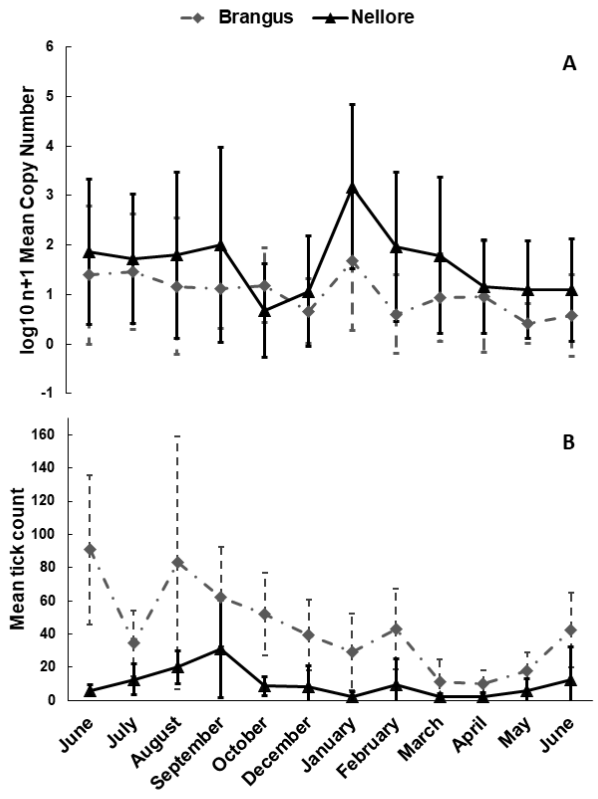

Figure 2

Mean *Babesia bigemina cbigs* gene copy number (data transformed to Log 10+1) for Brangus (dashed gray line and gray diamond) and Nellore (black line and black triangle) cattle (A); Variation across 12 months for mean *Rhipicephalus microplus* tick count for Brangus (dashed gray line and gray diamond) and Nellore (black line and black triangle) (B).

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