


qPCR estimates of *Babesia bovis* and *Babesia bigemina* infection levels in beef cattle and *Rhipicephalus microplus* larvae

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Abstract *Babesia* spp. are tick-transmitted intraerythrocytic apicomplexan parasites that infect wild and domestic animals. *Babesia bovis* and *B. bigemina* are endemic and responsible for enormous economic losses to the livestock industry in most of the Brazilian territory, wherein the tick *Rhipicephalus microplus* is the unique vector. Better understanding of epidemiology and parasite–host interactions may improve the tools for disease control and genetic management for selection of resistant animals. This study aimed to detect, quantify and measure the correlation between *B. bigemina* and *B. bovis* infection levels in bovine blood and into tick, by absolute quantification of hemoparasite DNA using qPCR. Blood bovine samples and larvae pools from 10 engorged *R. microplus* females were collected from each Canchim heifers (5/8 Charolais + 3/8 zebu, n = 36). All evaluated samples were positive for both *Babesia* species tested. Correlations of *B. bovis* and *B. bigemina* levels between cattle and tick host were 0.58 and 0.66, respectively. These high positive correlation coefficients indicate that parasitemia load in the bovine may be dependent on or may determine the parasitemia load in the ticks.

Keywords Correlation · Infection · Babesiosis · Cattle · Ticks

Introduction

Bovine babesiosis are tick-borne diseases that cause fever, anemia, hemoglobinuria and ataxia, leading to significant losses for the livestock industry in tropical and subtropical regions of the world (Uilenberg 1995; Brown and Palmer 1999; Gohil et al. 2013). Cattle that recover from acute infection remains persistently infected and can be continuing

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infecting ticks (Calder et al. 1996; Brown and Palmer 1999; Bock et al. 2004; Oliveira et al. 2005).

Endemic stability for *B. bovis* and *B. bigemina* is common in most of regions from Brazil (Kessler et al. 1983; Vidotto et al. 1997), wherein qPCR assays have been successfully applied for detection and quantification of these pathogens (Ramos et al. 2011; Bilhassi et al. 2014; Giglioti et al. 2016, 2017, 2018). Recent studies have demonstrated no association between *Babesia* DNA levels in naturally infected cattle and tick counts (Giglioti et al. 2016, 2017, 2018). However, studies regarding association between babesiosis infection levels in ticks and cattle host blood remain still scarce.

In this context, differential transmissibility rates of *B. bovis* and *B. bigemina* to the ticks was previously associated to bovine host's age (Oliveira-Sequeira et al. 2005). In this study, carried in endemic area for these hemoparasites, presenting similar infection rates for both *Babesia* species in adult and young cattle, it was verified that engorged female ticks collected from calves produced significantly higher frequency of eggs infected with *B. bigemina* compared to those infected with *B. bovis*, while no differences were observed for cows.

Similar *B. bovis* transmissibility rates from persistently or acutely infected calves to larval progeny through engorged tick females were observed in experimentally *B. bovis* challenged animals, leading authors to conclude that risk associated with introduction of both animals to non-endemic area for this pathogen is equal (Howell et al. 2007a). In another study, positive correlations were observed between *B. bovis* levels in host animal and percentage of engorged females containing high levels of kinetes in hemolymph and also between parasite levels in larval progeny and kinete levels in hemolymph of adult females, while no correlations were observed between kinete levels in hemolymph with percentage of infection rates of larval progeny (Howell et al. 2007b).

This study was carried aiming to better elucidate cattle host-babesia-tick triad interactions, and to evaluate correlations between the *B. bovis* and *B. bigemina* infection levels in naturally infected cattle and respective ticks.

Materials and methods

Experimental area, animals and sample collections

The experiment was conducted at the research farm from Embrapa Livestock Southeast (Embrapa Pecuária Sudeste), located at São Carlos, São Paulo state (22°01'S and 47°53'W). This region is considered endemic for the occurrence of *R. microplus* and the hemoparasites transmitted by these ticks (Oliveira et al. 2008; Giglioti et al. 2018). Thirty six Canchim heifers (5/8 Charolais + 3/8 Zebu) with 10–13 months of age, naturally infested with ticks, were submitted to blood sampling (using EDTA vacutainer tubes) and collection of engorged female ticks (body lengths greater > 5 mm) during spring season (October 2013). Acaricide treatments of animals were avoided during 50 days before samplings. For each animal, groups of 10 engorged female ticks presenting similar weight were washed, dried, placed in sterile Petri dishes and incubated in a BOD (biochemical oxygen demand) chamber at 27 ± 1 °C under relative humidity > 90%. At the end of laying period (15th day), eggs from all 10 engorged females per animal were pooled in transparent plastic syringes to verify the larval hatching rate. All procedures have been approved by Embrapa Southeast Livestock Ethical Committee for Animal Experimentation (CEUA-EMBRAPA),

in accordance with ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation (Process Number 03/2014).

DNA extraction

Blood samples were processed for total DNA extraction using Easy-DNA™ kit (Invitrogen USA, catalog number K180001) according to the manufacturer's protocol (Protocol #2-30 Minute DNA Extraction from Blood Samples). DNA extractions from tick larval pools (approximately 100 larvae) were performed in two steps. In the first, the larval pool was transferred and macerated into 1.5 mL microtubes using plastic pestil and liquid nitrogen during 15 s. The macerated was supplemented with 100 µL of DNA isolation buffer (1667 µL of 3 M KCl; 600 µL of 1 M Tris–Cl, pH 8.5; 400 µL of 1 M Tris–Cl, pH 8.0; 7333 µL of ultrapure water) and macerated with the frozen pestil for another 15 s. The homogenized material was incubated at boiling temperature during 5 min and processed with Axygen™ Axyprep™ Multisource Genomic DNA MiniPrep Kit (No. 14-223-003)—protocol “Purification of Genomic DNA from Animal Tissues”, as recommended by the manufacturer. All DNA samples were kept at –80 °C until further analysis.

qPCR

Absolute quantification of *B. bovis* and *B. bigemina* DNA levels were carried in a CFX™ Real-Time PCR Detection System (BioRad), using SsoFast™ EvaGreen® Supermix (BioRad) reagents and primers targeting mitochondrial cytochrome b gene (Buling et al. 2007). Standard curves and reaction conditions were performed as described (Giglioti et al. 2016, 2017, 2018). Samples and controls (positive and negative) were analyzed in duplicate. Negative and positive samples of *R. microplus* larval DNA for *Babesia* spp. obtained from the microbial collection of Embrapa Southeast Livestock, São Carlos, São Paulo state, were also included as negative and positive controls, respectively.

Statistical analysis

The analyses were carried out using the SAS statistical package (SAS 2002). DNA copy numbers (CN) data were transformed into $\log_{10}(n + 1)$ to approximate the normal distribution. After transformation, the data were analyzed using the PROC GLM and PROC CORR procedures. The first model was used to compare means of the *Babesia* spp. infection levels between each host. The CN Means values were compared by the Tukey test ($p \leq 0.05$). In the second analysis, the relationships of transformed CN data of *B. bovis* and *B. bigemina* between ticks and cattle were established using Pearson's correlation coefficient.

Results and discussion

High positive frequencies of both *Babesia* species were found, as all tested samples were positive, either from tick or bovines. The means of *B. bovis* and *B. bigemina* DNA copies followed by standard errors in cattle were 2.58 ± 0.45 and 2.85 ± 0.41 , respectively, while for ticks the values were 1.96 ± 0.84 and 1.96 ± 0.84 , respectively. There were no significant differences between DNA copy levels observed in cattle and ticks, for both *Babesia* species. Similar findings were already described for previous

experiments performed in this experimental region, classified as endemically stable for the occurrence of babesiosis (Oliveira et al. 2005, 2008; Giglioti et al. 2018). Despite existence of several studies evaluating babesiosis infection levels in ticks (Cafrune and Aguirre 1995; Oliveira et al. 2005; Oliveira-Sequeira et al. 2005; Ica et al. 2007; Oliveira et al. 2008), there are still few studies which have performed the comparative evaluation in the respective host. In this sense, there were no differences of *B. bovis* transovarial infection rates in the larval progeny from *R. microplus* females that fed on persistently infected calves or those females that fed on acutely infected calves (Howell et al., 2007a). In another study, Howell et al. (2007b) found a correlation between *B. bovis* infection levels in the blood of clinically affected cattle and kinete levels in the hemolymph of adult female *R. microplus* ticks. Although, in the same study, *B. bovis* infection levels in the engorged females hemolymph have not correlated to infection rates of larval progeny. Since we observed high correlations of *Babesia* spp. infection levels between the vertebrate and invertebrate hosts, our results diverge from these previous studies. Main reasons which may explain this difference are due to differential nature of infection (experimental versus natural field infection), and to our method for *Babesia* levels quantification.

According to Mahoney and Mirre (1971), there is a low transmissibility percentage of transovarial transmission of *Babesia* spp. even if they become infected from animals in the acute disease, characterized by markedly parasitemia. In this infection phase, the transmission rates ranges from 20 to 40 and 0.5 to 14.5%, for *B. bigemina* and *B. bovis*, respectively. Therefore, despite low transovarial transmissibility rates, our method comprised quantification of *Babesia* DNA levels from pools containing approximately 100 larvae (hatched from eggs laid by 10 engorged females), which may explain high positivity. Furthermore, we evaluated young animals (10–13 months of age) which may also have contributed to observed high infection levels. Oliveira et al. (2005) have observed higher frequency of *Babesia* spp. kinetes in ticks hemolymph collected from calves (77/260) compared to those collected from cows (7/236), by direct microscopy. Higher *B. bigemina* infection levels in calves compared to adults were also reported by Oliveira-Sequeira et al. (2005), wherein frequency of *B. bigemina* positive samples (156/258) was higher than frequency of *B. bovis* infection (23/258). In our study, the infection levels between *Babesia* species did not differ.

Although it is well established that the larval phase form of *R. microplus* does not transmit *B. bigemina* to the bovine host (Radostits et al. 2008), in the present study it was possible to quantify the DNA levels of this hemoparasite in a pool of approximately 100 larvae. According to Riek (1964), *B. bigemina* is only transmitted to cattle by infected nymphs and adults due to the presence of infective sporozoites of *B. bigemina* only 8–10 days after larval attachment. Regarding *B. bovis* infection, larvae are able to infect the host within 2–3 days after attachment, but the infectivity does not persist after the larval stage (CFSPH 2008).

High correlation coefficients ($p < 0.01$) of DNA levels of *B. bovis* and *B. bigemina* were found between cattle and ticks: 0.58 and 0.66, respectively (Fig. 1). Concerning correlation analysis, our study showed a high association of *Babesia* spp. between cattle and tick DNA samples. The DNA copy numbers of *B. bovis* and *B. bigemina* between the ticks and cattle indicates that parasitemia loads in the bovine is dependent or will determine the parasitemia loads in the ticks. Further studies must be performed to better understand this parasite-host interaction, including elucidation of which factors may be influencing on transovarial transmission fluctuations.

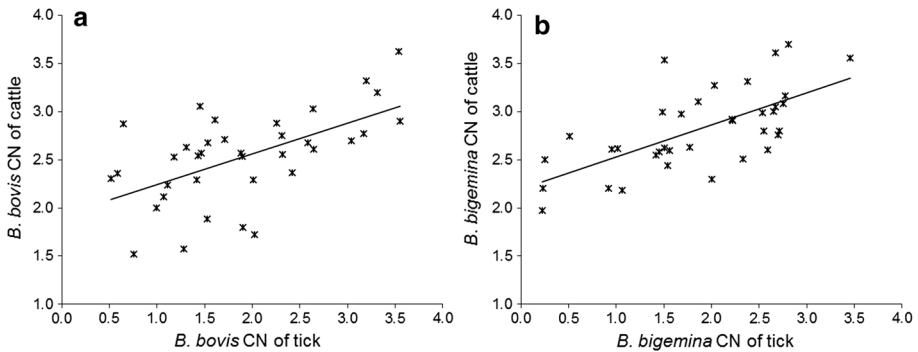


Fig. 1 Scatter plot correlation of copy number values of *Babesia bovis* (a) and *B. bigemina* (b) between DNA samples from cattle and ticks

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