Ticks and Tick-borne Diseases xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Babesia bovis and *Babesia bigemina* infection levels estimated by qPCR in Angus cattle from an endemic area of São Paulo state, Brazil

R. Giglioti[a,*], H.N. Oliveira[a], C.H. Santana[a], A.M.G. Ibelli[b], T.A. Néo[c], T.B. Bilhassi[a], M.D. Rabelo[d], R.Z. Machado[a], L.G. Brito[e], M.C.S. Oliveira[d]

[a] *Universidade Estadual Júlio de Mesquita Filho, Jaboticabal, SP, Brazil*
[b] *Embrapa Suínos e Aves, Concórdia, SC, Brazil*
[c] *Universidade Federal de São Carlos, SP, Brazil*
[d] *Embrapa Pecuária Sudeste, São Carlos, SP, Brazil*
[e] *Embrapa Rondônia, Porto Velho, RO, Brazil*

ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form
16 December 2015
Accepted 10 February 2016
Available online xxx

Keywords:
Babesiosis
R. microplus
qPCR
Resistance

ABSTRACT

The levels of infection by *Babesia bovis* and *Babesia bigemina* were estimated by absolute quantification through the quantitative PCR technique (qPCR). Fifty-one contemporaneous Angus cattle were evaluated on two occasions. The number of standard female *Rhipicephalus microplus* ticks present on the left side of the body was counted and blood samples were drawn from the tail vein into tubes containing the anticoagulant EDTA. The blood samples were submitted to DNA extraction and used to quantify the number of copies (NC) of DNA from *B. bovis* and *B. bigemina* by qPCR. The data on tick count and number of DNA copies were transformed for normalization and analyzed by a mixed model method. A multivariate model with repeated measures of the same animal, including the effects of collection, parasite species and their interaction, was used. The repeatability values were obtained from the matrix of (co)variances and were expressed for each species. The correlations between the counts of different species on the same animal, in the same collection or different collections, were also estimated. The results showed the qPCR could distinguish the two between infection by the two *Babesia* species. Infection levels by *B. bovis* and *B. bigemina* were detected in 100% and 98% of the animals, respectively. Significant differences were found ($P < 0.05$) between the NC of the two *Babesia* species, *B. bovis* 1.49 ± 0.07 vs. *B. bigemina* 0.82 ± 0.06. Low repeatabilities were found for the counts of *R. microplus* and NC of *B. bovis* and *B. bigemina*: 0.05, 0.10 and 0.02, respectively. The correlations between *R. microplus* count and NC of *B. bovis* and *B. bigemina* were both very near zero. However, an association was observed between the NC of the two species, with a correlation coefficient of 0.30 for measures from the same collection. The absence of associations between the quantity of DNA from *B. bovis* and *B. bigemina* and the tick counts suggests that the variation of parasitemia by the hemoparasites did not depend on the tick infestation levels at the moment of each collection. The repeatability values estimated indicate that under the study conditions, the variations in the tick infestation levels and of parasitemia by *B. bovis* and *B. bigemina* depend more on factors related to each collection than on intrinsic factors of the animal.

1. Introduction

Babesiosis causes morbidity and mortality in cattle herds throughout the world (McCosker, 1981), although it occurs with greater frequency in tropical and temperate regions (Figueroa et al., 1998). In Brazil, *Babesia bovis* and *Babesia bigemina* are the agents that cause bovine babesiosis, and the cattle tick *Rhipicephalus microplus* is considered to be the only vector (Guglielmone, 1995). Both young and adult animals are susceptible to the disease when exposed for the first time to ticks. However, young animals have stronger innate resistance. This phenomenon, unusual in the case of infectious diseases, is known as "inverse age resistance". Therefore, the symptoms of the disease can be attenuated if the first infection occurs when the animals are very young. After recovery, low parasitemia levels persist for long periods without causing apparent harm to the animals (Zintl et al., 2005).

* Corresponding author at: Embrapa Pecuária Sudeste, Rodovia Washington Luiz, Km 234, CP 339, 13560-970 São Carlos, São Paulo, Brazil.
E-mail address: gigliotirodrigo@gmail.com (R. Giglioti).

http://dx.doi.org/10.1016/j.ttbdis.2016.02.011
1877-959X/

In regions where the vector *R. microplus* is present throughout the year, animals are typically infected while young, after which constant reinfection maintains parasitemia at subclinical levels, guaranteeing lifetime immunity (Mahoney and Ross, 1972). This situation, called endemic stability, occurs virtually throughout Brazil. Nevertheless, clinical cases can appear in calves with ages between 30 and 120 days, a period that coincides with a significant fall in the levels of colostral antibodies (Madruga et al., 1984), although humoral immunity appears to be independent of the resistance level of calves (Guglielmone et al., 1997). Farms located in regions of endemic stability with inadequate tick control are also subject to babesiosis outbreaks (Sserugga et al., 2003).

Several studies have shown that genetic factors are associated with resistance to tick infestations and babesiosis, with *Bos taurus indicus* animals being more resistant compared to *B. t. taurus* (Guglielmone, 1995; Bock et al., 1997; Jonsson et al., 2008; Piper et al., 2010; Bilhassi et al., 2014). This finding led Brazilian stockbreeders mainly to use zebu animals, so that at present about 80% of the nation's herd is composed of this breed and its crosses (Tizioto et al., 2012).

Techniques based on PCR are increasingly used to diagnose diseases, principally because of their high sensitivity and specificity (Figueroa et al., 1992; Smeenk et al., 2000; Oliveira et al., 2005; Oliveira-Sequeira et al., 2005; Guerrero et al., 2007; Buling et al., 2007). However, conventional PCR tests only allow qualitative detection of infection. In contrast, the quantitative real-time PCR technique (qPCR) overcomes these limits, and besides offering a simpler and more efficient diagnostic option, enables quantifying the number of copies of *Babesia* DNA fragments in samples extracted from cattle blood. Using this technique, Bilhassi et al. (2014) found significant differences in the levels of infection by *B. bovis* in animals of different ages and genetic groups (*B. t. taurus* and *B. t. indicus*). To our knowledge, no similar studies have been reported in the literature carried out with animals of a specific breed, living under conditions of endemic stability for babesiosis. There also is no information that permits verifying whether the infection level can be attributed to intrinsic animal factors (genetic and non-genetic) and the influence of the level of infestation by *R. microplus* on the levels of infection by *Babesia* species. Thus, the aims of this experiment were: (i) to find indicators of resistance/susceptibility to babesiosis by applying qPCR to quantify the DNA from *B. bovis* and *B. bigemina* in blood samples of Angus cattle; (ii) to estimate the repeatability of these measurements; and (iii) to investigate the occurrence of associations of the levels of infection by the two *Babesia* species, between each other and with the *R. microplus* counts.

2. Materials and methods

2.1. Animals and sample collection

We used 51 Angus males, with age about 24 months, from a herd kept in the municipality of José Bonifácio, São Paulo state, Brazil (21°03′10″ S and 49°41′18″ W), where infestations by the tick *R. microplus* occur throughout the year. The animals were evaluated on two occasions, in May 2012 (autumn) and July 2012 (winter), during which the numbers of *R. microplus* ticks longer than 4.5 mm were counted on the left side of each animal (Utech et al., 1978; Hermans et al., 1994). At the same time, blood samples for DNA extraction were obtained from the tail vein using Vacuteiners® (Becton Dickinson) containing the anticoagulant EDTA.

The animals of the herd studied were submitted to strategic treatments with a commercial acaricide based on pyrethroids and organophosphates (Colosso®, Ourofino Saúde Animal). The treatments were suspended in the 30 days before the tick counts and blood sample collections.

This experiment is in agreement with ethical principles of animal experimentation of Embrapa Southeast Livestock ethics committee for animal experiments (CEUA-EMBRAPA).

2.2. DNA extraction

The genomic DNA for quantification of DNA from *B. bovis* and *B. bigemina* was extracted from 300 μL of each blood sample, using the illustra blood genomicPrep Mini Spin® kit (GE Healthcare), following the vendor's recommendations, according to the protocol for extraction of genomic DNA from between 50 and 300 μL of blood.

2.3. qPCR reactions

The quantitative polymerase chain reactions were carried out with a CFX™ Real-Time PCR Detection System from BioRad, with the primers cbosg 1 (forward) 5′-TGTTCCTGGAAGCGTTGATTC-3′ and cbosg 2 (reverse) 5′-AGCGTGAAAATAACGCATTGC-3′ and cbisg 1 (forward) 5′-TGTTCCAGGAGATGTTGATTC-3′ and cbisg 2 (reverse) 5′-AGCATGGAAATAACGAAGTGC-3′ (Buling et al., 2007), which flank the gene fragment of cytochrome B of *B. bovis* and *B. bigemina*, respectively. Each produces amplicons with 88 base pairs (Buling et al., 2007; Salem et al., 1999). Genomic DNA extracted from isolates of *B. bovis* and *B. bigemina* (kindly provided by Professor Rosângela Zacarias Machado of Unesp de Jaboticabal, São Paulo) were used to construct the calibration curve and as positive control.

The qPCR was conducted in a volume of 12 μL, with 5 μL of SsoFast™ EvaGreen® Supermix (BioRad), 0.22 μM of each primer (cbosg 1 and 2; cbisg 1 and 2), 4.4 μL of ultrapure water (Invitrogen, USA) and 2.0 μL of DNA sample (genomic DNA extracted from 300 μL of blood). The thermocycle conditions were one step of 2 min at 95 °C, followed by 45 cycles at 95 °C for 5 s and 57 °C (annealing/extension) for 30 s. To prevent contamination, pipettes with barrier tips were used. The samples were analyzed in duplicate, as were the positive and negative controls (for the negative control, 2 μL of water was added in the reaction instead of the DNA solution).

To assess the specificity of the primers for detection of *B. bovis* and *B. bigemina* in the qPCR, a test with two reactions was used. The first reaction employed the primers cbosg 1 and 2 (*B. bovis*) in samples from animals and isolates of *B. bovis* and *B. bigemina*. The second reaction contained the same samples and *B. bovis* and *B. bigemina* isolates, but the primers were cbisg 1 and 2 (*B. bigemina*). The specificity was determined based on the results of the amplification and the melting curve values.

2.4. Construction of calibration curve and quantification of the number of copies

To construct the calibration curve, DNA samples from the isolates of the two species were amplified using the specific primers for *B. bovis* and *B. bigemina* (cbosg and cbisg). The PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen™), cloned using the pGEM®-T Easy Vector System (Promega, Madison, USA), and then the recombinant clones were transformed in *Escherichia coli* DH5α cells. The white colonies were selected, amplified to confirm the vector insert and then grown in SOB medium at 37 ± 1 °C overnight under shaking. The DNA was extracted using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen™) and sequenced with an Applied Biosystems ABI Prism 3130 Avant® genetic analyzer and submitted to BLAST analysis to confirm the sequences obtained (Altschul et al., 1990).

Please cite this article in press as: Giglioti, R., et al., *Babesia bovis* and *Babesia bigemina* infection levels estimated by qPCR in Angus cattle from an endemic area of São Paulo state, Brazil. Ticks Tick-borne Dis. (2016), http://dx.doi.org/10.1016/j.ttbdis.2016.02.011