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Molecular and serological detection of *Babesia Bovis* and *Babesia Bigemina* in cattle in the Rio de Janeiro, Brazil

Deteção molecular e sorológica de *Babesia Bovis* e *Babesia Bigemina* em bovinos no Rio de Janeiro, Brasil

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Abstract

Bovine babesiosis is an important disease of cattle where *Rhipicephalus microplus* acts as a vector for the two causal organisms *Babesia bovis* and *Babesia bigemina*. A total of 22 calves were randomly monitored during three years by semi-nested PCR assay and ELISA test to determine prevalence of *B. bovis* and *B. bigemina*. The overall prevalence of *B. bovis* and *B. bigemina* was 30% and 35% by nested PCR (nPCR), and 70% and 75% by ELISA, respectively. Statistical analysis of the characteristics of animals showed that age and tick infestations ($p < 0.05$) might play an important role in the spread of babesiosis as animal less than 6 months old. A high correlation (Kappa index of 0.70 for *B. bovis* and 0.65 for *B. bigemina*, respectively) between serological and molecular tests suggests that the combination of the utilized techniques in the present study is suitable for babesiosis diagnosis in an endemic unstable area.

Key words: Babesiosis, Brazil, diagnosis, ELISA, PCR

Resumo

A babesiose bovina é uma importante doença do gado, onde *Rhipicephalus microplus* atua como um vetor para os dois organismos causais *Babesia bovis* e *Babesia bigemina*. Um total de 22 animais foram aleatoriamente e monitorados durante três anos, por nested PCR e teste de ELISA para determinar a prevalência de *B. bovis* e *B. bigemina*. A prevalência global de *B. bovis* e *B. bigemina* foi de 30% e 35%, em nPCR, e 70% e 75% por ELISA, respectivamente. A análise estatística das características dos animais mostrou que a idade e carrapatos ($p < 0,05$) podem desempenhar um papel importante na propagação de babesiose em animais com menos de 6 meses de idade. Uma correlação elevada (índice Kappa de 0,70 para *B. bovis* e *B. bigemina* 0,65, respectivamente) entre os testes sorológicos e moleculares sugere que a combinação das técnicas utilizadas no presente estudo é adequado para o diagnóstico da babesiose numa área de instabilidade endêmica.

Palavras-chave: Babesiose, Brasil, diagnóstico, ELISA, PCR

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Introduction

Rhipicephalus microplus is considered the most important vector of pathogens that cause disease in cattle. *Rhipicephalus microplus*, are distributed in tropical and subtropical regions of the world, where they economically impact cattle industry by reducing weight gain and milk production, and by transmitting pathogens (PETER et al., 2005).

Bovine babesiosis is a tick-borne disease of cattle that is caused by one or more intraerythrocytic protozoa of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa and is generally characterized by significant morbidity and mortality worldwide (McCOSKER, 1981). Although outbreak reports and localized epidemiological studies have been published in the last decade, comprehensive and detailed descriptions of the epidemiology of bovine piroplasmoses are still lacking in Brazil.

The disease is clinically manifested by anemia, fever, hemoglobinuria, and marked splenomegaly and sometimes causes death. Of the species affecting cattle, *Babesia bovis* and *Babesia bigemina* are the most economically important species worldwide. The economic losses from the infection are incurred not only from mortality, loss of milk/meat production, and cost of control measures but also through its impact on the international cattle trade (BOCK et al., 2004).

Definitive diagnosis of babesial infection is generally made by microscopic identification of Giemsa or Wright stained blood smears. This technique is not reliable for detecting the infection in carrier cattle and for differentiating closely related infections (TERKAWI et al., 2011). Molecular detection based on PCR technique has been proven to provide reliable results with high sensitivity and specificity in detection of babesial DNA in blood, particularly when the parasitemia is very low or no detectable in microscopic examination (FIGUEROA et al., 1993). Serological tests, the enzyme-linked immunosorbent assays (ELISA) are capable of detecting the infection in carrier animals

and have been broadly used for surveillance and export certification (GOFF et al., 2006; SILVA et al., 2014).

Consequently, combination of molecular and serological test for the infection provides powerful tools for accurate diagnosis as well as for epidemiological investigations (TERKAWI et al., 2011). Thus, we investigated in a longitudinal study the detection of *B. bovis* and *B. bigemina* by ELISA test and PCR assay in cattle.

Materials and Methods

Background

Field activities were conducted from September 2008 to August 2011, at the Dairy Cattle Division of Seropédica Experimental Station, Agricultural Research Corporation of the State of Rio de Janeiro (Pesagro- Rio). The experimental area was located in the microregion of the Metropolitan Region of Rio de Janeiro (latitude 22° 45' S and longitude 43° 41' W and altitude 33 m).

Field blood samples

By means of proportional stratified sampling, 22 calves were randomly selected. Blood samples were collected every thirty days from birth until the three year of age. The herds are usually moved to pasture during the study. Cattle between 1 and 36 months old were divided into three groups based on their age; young (1 year), old (1-2 years) and older (2-3 years).

Blood was collected from the caudal or jugular vein of individual cattle with EDTA or without, incubated at room temperature for 2 h, and then centrifuged at 3000rpm for 10 min. The sera were collected and then stored at -20 °C until use. The genomic DNA samples were extracted from the whole blood using a commercial kit (QIAamp DNA Blood Mini-Kit, Madison, WI, USA) according to the manufacturer's instructions.

ELISA

The assay was then performed as earlier described (Machado et al., 1997). Briefly, 100 µl of antigen diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, was added to each well of a micro-ELISA plate (Immulon®; Dynatech Laboratories Inc.) and protein concentration was adjusted to 10 µg/ml. The plates were sealed and incubated overnight at 4 °C. Plates were blocked for 1 h at 37 °C in a humid chamber with 3% ovalbumin in carbonate/bicarbonate buffer. After five washes with buffer (phosphatebuffered saline, pH 7.2, and 0.05% Tween 20, PBS-Tween), 100 µl of diluted bovine sera (1:400) in PBS-Tween plus 5% normal rabbit serum were added in duplicate to the ELISA plate. Plates were incubated at 37 °C in a humid chamber for 90 min and then washed five times with PBS-Tween. A 100 µl aliquot of a 1:10000 dilution of alkaline phosphatase conjugated anti-bovine IgG (Sigma Chemical Co.) was added to each well and the plates were incubated at 37 °C under the same conditions for 90 min. Plates were washed five times with PBS-Tween. The appropriate substrate (p-nitrophenyl phosphate) was added and the plates were sealed and incubated for 40 min at room temperature. The plates were then read at 405 nm wavelength on a micro-ELISA reader (B.T.-100; Embrabio, São Paulo, Brazil). The cut-off values were calculated based on 10 non-*B. bovis* and *B. bigemina*-infected calves sera.

Nested-PCR

The reaction was conducted in a 25µl reaction mixture containing 5µl of extracted genomic DNA, 12.5µl of PCR master mix (Roche), and a 1.6 µM concentration of each primer set. Briefly, one universal forward primer and two unique reverse primers were designed to amplify the 18S rRNA gene. The primer sequences are as follows: *B. bovis* primers forward: 5'-AGTTGTTGGAGGAGGCTAAT-3' and reverse: 5'-TCCTTCTCGGCGTCCTTTTC-3' and *B. bigemina* primers forward 5'-

GAGTCTGCCAAATCCTTAC-3' and reverse 5'-TCCTCTACAGCTGCTTCG-3' (TERKAWI et al., 2011). PCR amplifications were performed at 94 °C for 3 min followed by 34 repetitive cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The *n*PCR conditions were the same as described above, 1 µl of the primary PCR product was used as template and amplified with 10 pmol of each of the primer (*B. bovis* forward 5'-GAAATCCCTGTTCCAGAG-3' and reverse 5'-TCGTTGATAACACTGCAA-3' and *B. bigemina* forward 5'-AGCTTGCTTTCACAACTCGCC-3' and reverse 5'-TTGGTGCTTTGACCGACGACAT-3'). PCR products (primary as well as nested) were checked for amplification by electrophoresis on a 2.0% agarose gel and visualized using gel documentation system (Syngene, UK).

Tick

To count the ticks, the animals were restrained individually, and all the fully or partially engorged females of *R. microplus* measuring between 4.5 and 8.0 mm that were found on the right side of each animal were counted as described by Wharton, Utech and Turner (1970). The result from each count was multiplied by 2 to obtain the monthly average for each animal. The chi-square test was used to evaluate significant differences ($P < 0.05$) of infection rate in animals by *B. bovis* and *B. bigemina*. The kappa coefficient was calculated to evaluate the agreement among the *n*PCR assay and ELISA test. The operational procedures were done using the R statistical software (R Foundation for Statistical Computing, version 2.12.2, 2011).

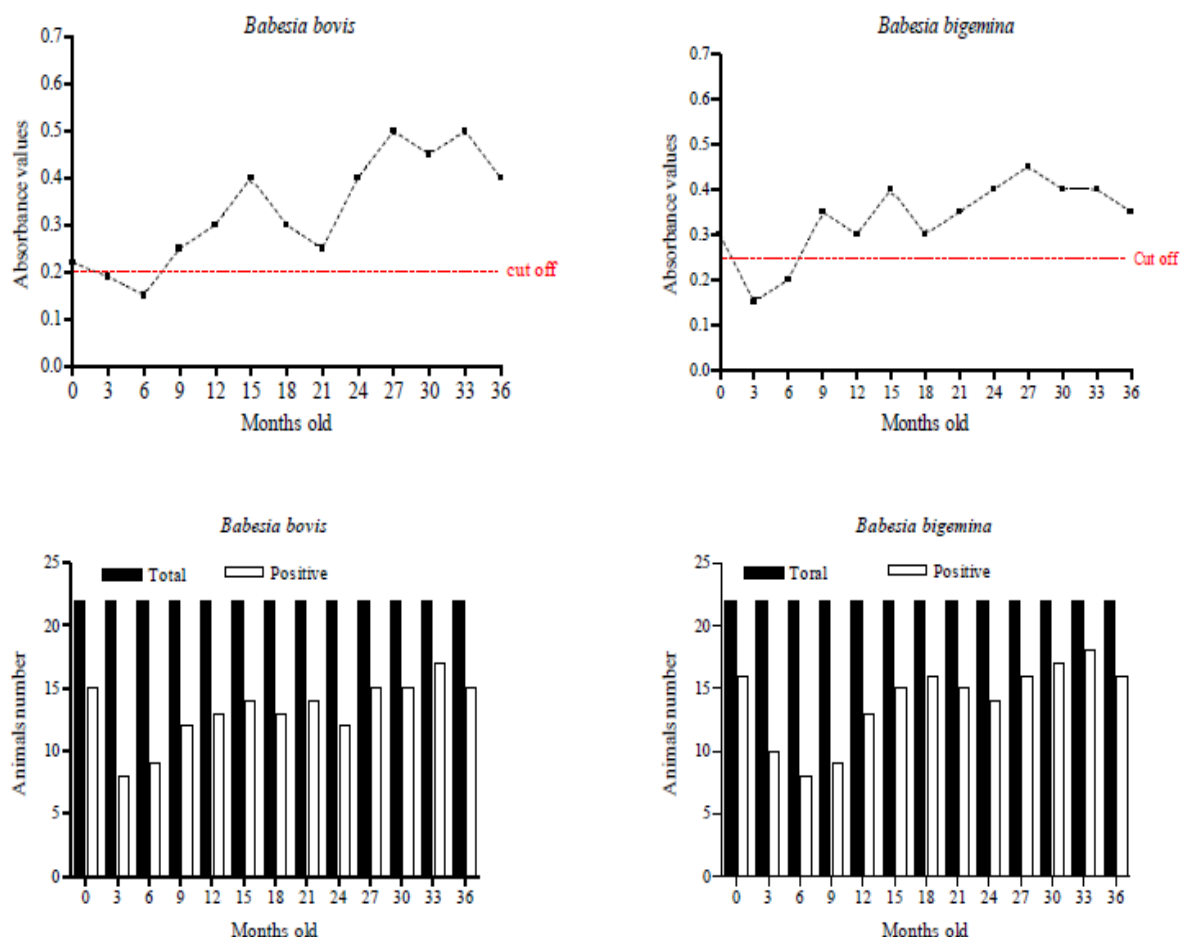
Results and Discussion

The fluctuation of the prevalence of *B. bovis* and *B. bigemina* was compared on the basis in animals of different months old (Figure 1). Analysis of serological data for *B. bovis* revealed a prevalence of 51, 60% and 70% among animals of age ≥ 1 to

≤ 12 months, > 12 to ≤ 24 and over 24 months, respectively. For *B. bigemina* the seroprevalence was 50%, 68% and 76% for the three age groups

examined. In a study on dairy cattle, Tembue et al. (2011) observed that animal less than 1 year old and those having ticks present on them were more infected with *B. bovis* and *B. bigemina*.

Figure 1. Serological detection of *Babesia bovis* and *Babesia bigemina* in cattle from Rio de Janeiro, Brazil. Twenty two calves were evaluated by ELISA every three months during the three year of life.

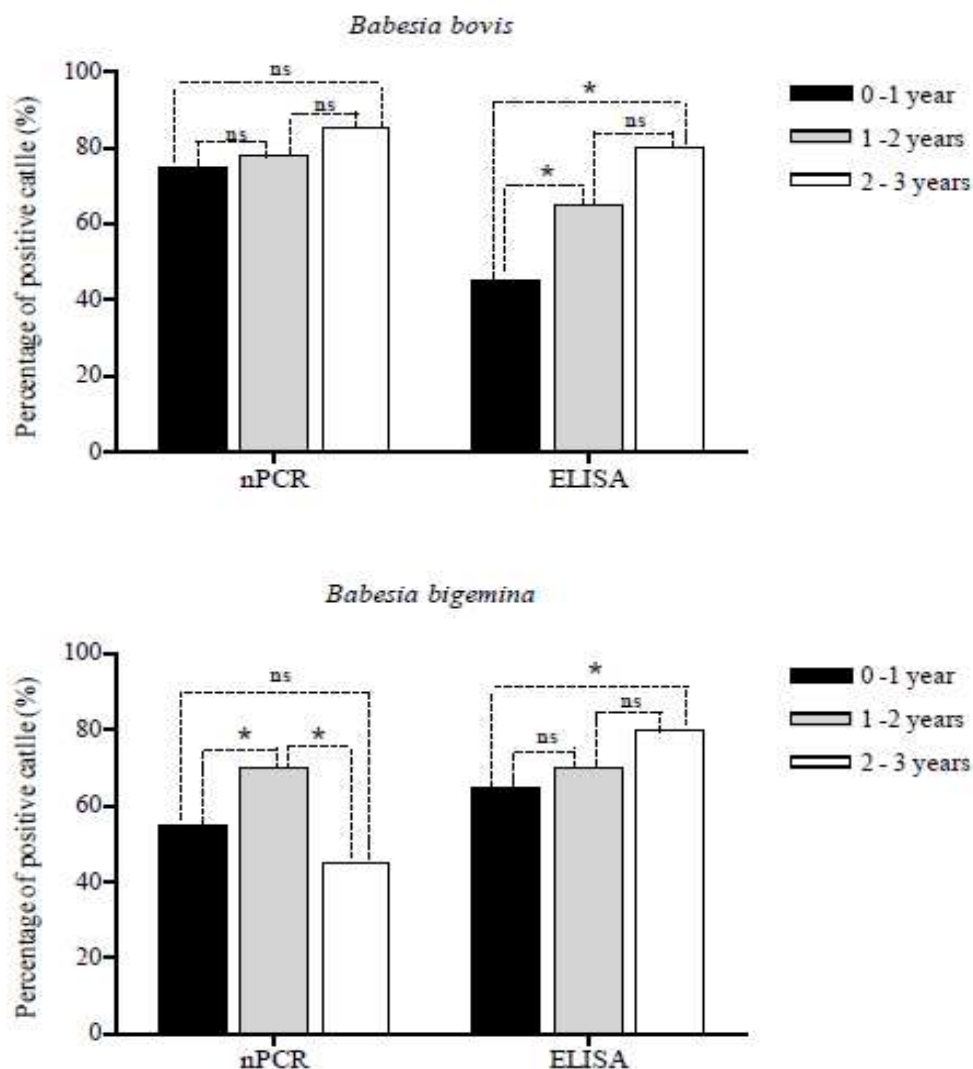


Source: Elaboration of the authors.

Statistically significant differences ($P < 0.05$) were essentially observed in aged cattle for both parasitic infections for both agents (Figure 2). This was consistent with related studies that noted an increase in the prevalence in older cattle (APPLEWHAITE; CRAIG; WAGNER, 1981;

SUKANTO; PAYNE; PARTOUTOMO, 1993). In general, young and adults are susceptible to babesiosis, while calves are naturally resistant due to the strong innate immune response with high concentration of nitric oxide in the spleen (ZINTL et al., 2005).

Figure 2. Comparisons of the prevalence serological (ELISA) and molecular (nPCR) of *Babesia bovis* and *Babesia bigemina* infections on the basis of age of calves in state of Rio de Janeiro, Brazil. Each asterisk indicates a significant difference (* $p < 0.05$).

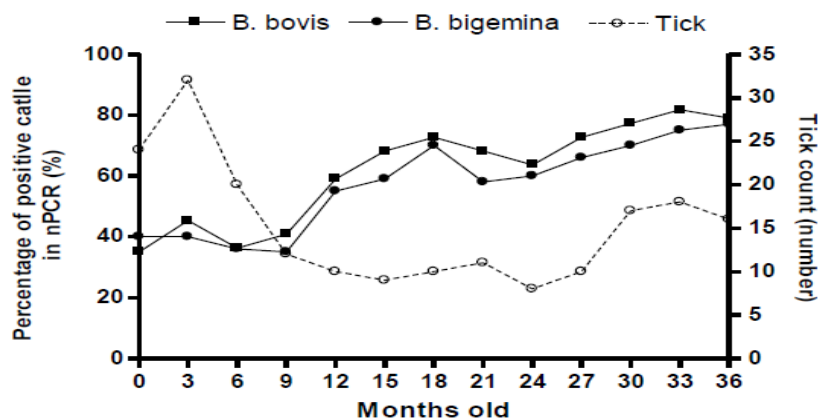


Source: Elaboration of the authors.

In this study the tick infestation showed to be an important risk factor for babesiosis (Figure 3). Regarding animal age, the results from the present study are agree with what was observed by Andrade et al. (1998), who noticed a linear increase in the number of ticks as the animals became older. Conversely, highly specialized susceptible cattle from low endemicity areas are subject to great

risk when introduced to high endemicity or places where tick populations fluctuate during the year (JONSSON; BOCK; JORGENSEN, 2008). It is possible that in the studied herds in a low vector population by insufficient for maintaining constant transmission of *B. bovis* and *B. bigemina*. In this case, an imbalance caused by the parasite-host relationship due to infrequent transmission creates an artificial endemic instability condition.

Figure 3. Comparisons of the prevalence molecular (nPCR) of *Babesia bovis* and *Babesia bigemina* with *Rhipicephalus microplus* count in cattle of Rio de Janeiro, Brazil.



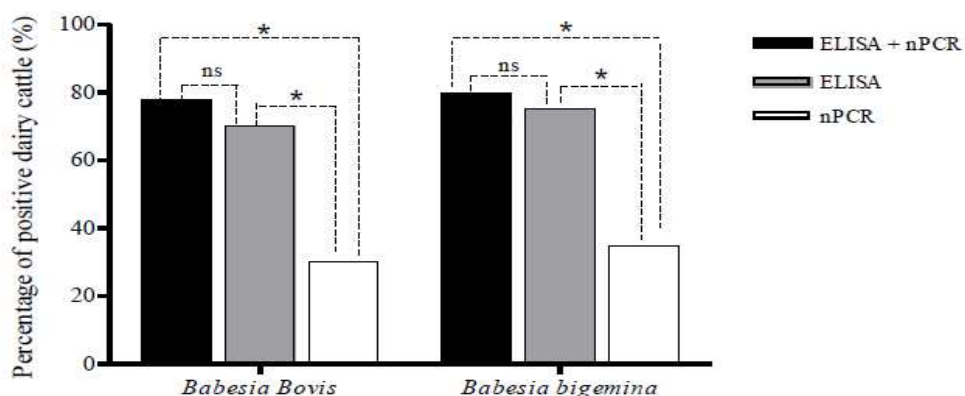
Source: Elaboration of the authors.

Although this serological assay detected higher positive rates of both parasites than the molecular detection method, the results obtained from the nPCR assay were highly concordant with this of ELISA, as determined by the kappa values, which were calculated to be 0.65 and 0.70 for *B. bovis* and *B. bigemina*, respectively (Figure 4). While these findings are similar to that found by Terkawi et al. (2011). In a nutshell, our results showed that a combination of both molecular and serological techniques is useful tool in for epidemiological

investigations with high accuracy in the diagnosis of *Babesia* sp. in cattle.

The present work showed that water cattle from state of Rio de Janeiro, Brazil, are exposed to bovine babesiosis agents, although do not show clinical signs of the disease. The PCR and ELISA diagnosis of *B. bovis* and *B. bigemina* utilizing blood as a source to obtain template DNA proved efficient in detecting hemoparasites in asymptomatic cattle bred in the regions studied.

Figure 4. Molecular and serological detection of *B. bovis* and *B. bigemina* using nPCR assay and ELISA test. The results of nPCR were cross-tabulated with these of ELISA. Twenty two calves were evaluated by ELISA every three months during the three year of life.



Source: Elaboration of the authors.

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