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# Comparison of different direct diagnostic methods to identify *Babesia bovis* and *Babesia bigemina* in animals vaccinated with live attenuated parasites

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**Abstract**

Blood smear examination, flow cytometry, duplex Polymerase Chain Reaction (PCR), and duplex nested PCR (nPCR) were evaluated for detection of *Babesia bigemina* and *Babesia bovis* infections in cattle vaccinated with live attenuated strains. Two groups of four cattle were immunized with either *B. bigemina* (*Bi*) or *B. bovis* (*Bo*). On day 23 post inoculation (PI), *Bi* cattle were vaccinated with *B. bovis* (*BiBo*) and *Bo* cattle were vaccinated with *B. bigemina* (*BoBi*). *Babesia bigemina* was first detected by blood smear examination 7.5 ± 3.5 days PI in the *Bi* group and 32.2 ± 1.7 days PI in the *BoBi* group. The first occurrence of *B. bovis* in blood smears was 8.0 days PI in the *Bo* group and 36.0 ± 2.6 days PI in the *BiBo* group. Flow cytometry detected parasitized erythrocytes on day 1.7 ± 1.5 and 2.2 ± 1.5 PI in the *Bi* and *Bo* groups, respectively, but did not discriminate between the two *Babesia* spp. Duplex PCR detected *B. bigemina* on day 4.0 ± 0.8 and 26.0 ± 0.8 PI in the *Bi* and *BoBi* groups, respectively, and *B. bovis* on day 4.0 and 25.3 ± 0.5 PI in the *Bo* and *BiBo* groups, respectively. The duplex nPCR detected *B. bigemina* on 3.0 ± 0.8 and 25.0 ± 0.0 days PI in the *Bi* and *BoBi* groups, respectively, and 4.7 ± 1.7 and 27.7 ± 6.2 days PI in the *Bo* and *BiBo* groups, respectively. Duplex nPCR outperformed the other tests in terms of specificity and sensitivity, indicating that it is the most useful method for identifying *Babesia* spp. in cattle following vaccination.


*Keywords:* *Babesia bigemina*; *Babesia bovis*; Live vaccine; Diagnosis; nPCR

## 1. Introduction

Babesiosis is an important hemoparasitosis caused by intra-erythrocytic protozoa of the genus *Babesia*. The two species affecting bovines in tropical and

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subtropical areas are *Babesia bovis* and *Babesia bigemina* (Böse et al., 1995).

Control methods such as chemotherapy, premunition and vaccination with attenuated parasites are used to avoid economic losses caused by *Babesia* infection. The live vaccine for bovine babesiosis was first developed and tested in Australia and has been subsequently used in South Africa and South America (Sacco et al., 2001; Standfast et al., 2003). However, it may happen that some animals do not get infection after inoculation requiring new vaccination, while others may develop excessive parasitemia and other symptoms, requiring treatments. The application of a reliable laboratory test, able to identify early infections with *Babesia* spp., is thus imperative to allow these animals to be re-vaccinated or treated as quickly as possible (Shkap et al., 2005).

Blood smear examination is considered to be the ''gold standard'' for diagnosis of babesiosis, however, parasite visualization may be difficult in cases when small numbers of parasites are present in the peripheral blood (Böse et al., 1995). New and improved diagnostic tests for bovine babesiosis have therefore been developed and alternative methodologies proposed, such as the flow cytometry (Wyatt et al., 1991; Bittar, 2002), the Polymerase Chain Reaction (PCR) and the nested PCR (nPCR) (Figueroa et al., 1993).

In this work, we comparatively evaluated four distinct laboratory techniques, and identified the best diagnostic method to monitor animals that had been vaccinated with live attenuated *B. bigemina* and *B. bovis*.

## 2. Materials and methods

### 2.1. Vaccination and monitoring protocols

Eight cross-breed steers (Holstein × Zebu), six-months old and negative for *Babesia* infection by the Indirect Fluorescent Antibody Test (IFAT), duplex PCR, duplex nPCR and flow cytometry, were used in the study. The steers were divided into two groups of four animals each and vaccinated with live attenuated *B. bigemina* and *B. bovis* (Souza et al., 2000). On day 0, the *Bi* group was intravenously vaccinated with $5.0 \times 10^6$ *B. bigemina*-infected red blood cells (RBCs), while the *Bo* group received $1.6 \times 10^8$ *B. bovis*-infected RBCs. On the 23rd day post inoculation (PI), *Bi* steers were intravenously vaccinated with $3.2 \times 10^8$ *B. bovis*-infected RBCs and designated as the *BiBo* group. Similarly, *Bo* steers were inoculated with $1.0 \times 10^7$ *B. bigemina*-infected RBCs and subsequently named *BoBi* group. Venous blood samples were collected daily from day 0 to day 44 PI and were monitored by examination of stained blood smears

Thin blood smears were prepared using peripheral blood and stained with a panoptic kit (Laborclin, Pinhal, Brazil). The percentage of infected erythrocytes was determined and recorded after examination of 100 microscopic fields (IICA, 1987). Venous blood samples were used for determination of packed cell volume (PCV), for flow cytometry, as well as for DNA extraction for use in PCR and nPCR.

### 2.2. Flow cytometry

A 100 μl aliquot of diluted blood (1:200, v/v in 0.9% saline) was mixed with 100 μl hydroethidine (Polysciences, Warrington, USA), previously solubilized at a concentration of 10 mg/ml in DMSO (Sigma Chemical, St. Louis, USA) and then diluted to 50 μg/ml in 0.9% saline, to yield a final hydroethidine concentration of 25 μg/ml. The mixture was incubated and processed as described by Bittar (2002). The final result was expressed initially as the percentage of fluorescent positive erythrocytes (%FPE), and the absolute values of hydroethidine-labeled RBCs obtained by multiplying the %FPE by the number of RBCs per $mm^3$, obtained from the packed cell volume (McCosker, 1975).

Blood samples collected during five consecutive days from four uninfected cattle (negative control) and from *Babesia* spp. positive animals (by blood smear examination) were used to calculate the cut-off by the receiver operating curve (MedCalc Statistical package).

### 2.3. Duplex PCR and nPCR

Blood DNA extraction was performed using the Wizard® Genomic Purification kit (Promega, Madison, USA). Amplification of *B. bovis* and *B. bigemina*