Accepted Manuscript

Title: Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the north region of Brazil

Author: Jenevaldo Barbosa da Silva Marcos Rogério André Adivaldo Henrique da Fonseca Cinthia Távora de Albuquerque Lopes Danillo Henrique da Silva Lima Stefano Juliano Tavares de Andrade Carlos Magno Chaves Oliveira José Diomedes Barbosa

PII: S0304-4017(13)00343-9
DOI: http://dx.doi.org/doi:10.1016/j.vetpar.2013.05.020
Reference: VETPAR 6858

To appear in: *Veterinary Parasitology*

Received date: 25-2-2013
Revised date: 22-5-2013
Accepted date: 24-5-2013

Please cite this article as: Silva, J.B., André, M.R., Fonseca, A.H., Lopes, C.T.A., Lima, D.H.S., Andrade, S.J.T., Oliveira, C.M.C., Barbosa, J.D., Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the north region of Brazil, *Veterinary Parasitology* (2013), http://dx.doi.org/10.1016/j.vetpar.2013.05.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the north region of Brazil

Jenevaldo Barbosa da Silva*, Marcos Rogério André, Adivaldo Henrique da Fonseca, Cinthia Távora de Albuquerque Lopes, Danillo Henrique da Silva Lima, Stefano Juliano Tavares de Andrade, Carlos Magno Chaves Oliveira and José Diomedes Barbosa

1Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias FCAV-UNESP, Vía de Acesso Prof. Paulo Donato Castellane s/n, 14884-900, Jaboticabal, SP, Brasil. E-mail: jenevaldo@hotmail.com
2Laboratório de Doenças Parasitárias, Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural de Rio de Janeiro (UFRRJ), Br 465, Km 7, 23890-000, Seropédica, RJ, Brasil. E-mail: adivaldo@ufrrj.br
3Instituto de Medicina Veterinária, Universidade Federal do Pará, Rodovia BR 316 Km 61, Bairro Saudade, 68740-970, Castanhal, PA, Brasil. E-mail: diomedes@ufpa.br

Abstract – Bovine babesiosis is a tick-borne disease caused mainly by *B. bovis* and *B. bigemina*, which are associated to considerable economic losses in cattle herds worldwide. Approximately 60% of buffalo herds in South America are located in Northern Brazil. Little is known about the impact of babesiosis on buffalo herds in Brazil. The present work aimed to verify the occurrence of *B. bovis* and *B. bigemina* in 542 water buffaloes in the state of Pará, Northern Brazil, using molecular and serological techniques. The percentage of seropositive animals for *B. bovis* and *B. bigemina* was 41.2% and 19.0%, respectively, by ELISA. *B. bovis* and *B. bigemina*
DNA were detected in 15 and 16% of sampled buffaloes, respectively. A high correlation (Kappa index of 0.9) 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. Significantly difference of positivity for serological and molecular assays was verified to localities and reproductive status of sampled animals, but not between buffalo breeds. The immune status of sampled buffaloes associated to the circulation of babesiosis agents in sampled population suggests that the studied area is at risk to clinical babesiosis outbreaks. Furthermore, this study demonstrated that this region can be classified as endemically unstable.

**Keywords:** Babesia, Brazil, ELISA, nPCR, Water buffaloes

1. Introduction

Brazil has the biggest water buffalo herds in the Latin America, where approximately 65% of animals is located in the Northern region of the country (IBGE, 2012). Since buffalo breeding has become an economically important activity, studies regarding the occurrence of pathogens circulating among buffaloes raised in Brazil are much needed.

Bovine babesiosis is a tick-borne disease caused by protozoa belonging to Phylum Apicomplexa, Order Piroplasmida, and genus Babesia, causing high morbidity and mortality worldwide (McCosker, 1981). Babesia bovis and B. bigemina show a high prevalence in tropical and subtropical areas, causing economic losses in cattle herds (Bock et al., 2004). On the other hand, the economic impact of babesiosis on buffalo herd has not been evaluated yet (Terkawi et al., 2011).
Although the observation of parasites in blood smears is routinely used in the babesiosis diagnosis, this technique shows a low sensitivity in subclinical and chronic phase of the disease (Terkawi et al., 2011). Molecular tools have shown a higher sensitivity and specificity. On the other hand, serological assays, such as Enzyme-Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test (IFAT), are used aiming to detect specific antibodies in carrier animals in epidemiological surveys. The combination of ELISA and Polymerase Chain Reaction (PCR) is considered a powerful tool in epidemiological surveys, showing high precision and sensitivity in babesiosis diagnosis (Terkawi et al., 2011). Our work aimed to verify the occurrence of *B. bovis* and *B. bigemina* in water buffaloes in state of Pará, Northern Brazil, using molecular and serological techniques.

### 2. Materials and Methods

#### 2.1. Sampling

Field samples of blood from water buffalo were collected from different farms in seven provinces in Marajó island and five provinces in Continent, in the northern region of Brazil in December 2011.

The number of samples to assess the prevalence of *B. bovis* and *B. bigemina* in the north region of Brazil was determined using the formula recommended by the Pan American Zoonosis Center (CEPANZO, 1979) for the study of chronic diseases:

\[ N = \frac{p(100-p)Z^2}{(d/p/100)^2} \]

where n, number of samples; p, expected prevalence; Z, confidence level, and d, error margin. An estimated prevalence of buffaloes positive for *B. bovis* and *B. bigemina* was 40%, confidence level of 95.0% and an error margin of 5.0%, were established. We estimated that 542 samples should be analyzed by ELISA.
Thus, we randomly selected a total of 382 buffaloes from Marajó Island and 160 buffaloes from Continent, being 299 and 243 of breed Murrah and Mediterranean, respectively. Regarding the reproductive period, 111 females were pregnant and 431 were non-pregnant. However, we only tested 272 buffaloes in PCR assays, because of high costs of performing molecular techniques in all sampled buffaloes.

Animal sampling was performed based on proportional stratification. Taking account that the Marajó Island and the continent have approximately herds of 1000000 and 400000 buffaloes, respectively, we select 2.5 times more animals in the island than that in the continent. While 38 buffaloes were sampled in 18 farms from Marajó island, a number of 160 buffaloes were sampled in 8 farms from continents. All sampled animals grazed on *Brachiaria decumbens* pastures, and were not treated with tick acaricides and anthelmintics. Despite of that, the majority of sampled animals showed a low infestation by ticks, predominantly represented by larvae and nymphs.

Whole blood samples were collected from caudal or jugular vein of individual water buffaloes. For serum samples, blood samples without EDTA were incubated at room temperature and then centrifuged at 3000 rpm for 15 min; the sera were collected. DNA was extracted from 200 µL of each 271 EDTA-whole blood sample using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, California, USA) according to the manufacturer’s instructions.

### 2.2. Enzyme-like immunosorbent assay (ELISA)

Microtiter plates (Immuron ®; Dynatech Laboratories Inc.) were coated overnight at 4 °C with each recombinant antigen at a concentration of 5 µg/ml
emulsified in a coating buffer. The assay was then performed as earlier described (Terkawi et al., 2011).

2.3 IFAT

Briefly, a 10µl field serum sample diluted in PBS (1:80) was applied as the first antibody on the fixed smears and then incubated for 1 h at 37 °C in amoist chamber. The assay was then performed as earlier described (Terkawi et al., 2011). The samples were tested at 1:80, 1:160, 1:320; 1:640 dilutions.

2.4. nested PCR

_A. marginale_ DNA was extracted from infected blood and the 18S rRNA gene were amplified by PCR as previously reported (Terkawi et al., 2011). 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).

2.5. Statistical analysis

The kappa coefficient was calculated to evaluate the agreement among the nPCR assay and ELISA. The chi-square test was used to evaluate significant differences (P < 0.05) of infection rate in animals of different breed, reproductive status, and locations. The operational procedures were done using the R statistical software (R Foundation for Statistical Computing, version 2.12.2, 2011).

3. Results
The prevalence of positive buffaloes in PCR assays for *B. bovis*, *B. bigemina* and both parasites was 15.1% (41/271), 16.2% (44/271) and 8.9% (24/271), respectively. The number of positive animals in PCR assays for *B. bovis* and *B. bigemina* was correlated to sampling area, breed and reproductive status. In Marajó island, the number of PCR positive buffaloes for *B. bovis* (25/191) and *B. bigemina* (26/191) was significantly lower (p=0.003 and 0.001, respectively) than the number of PCR positive buffaloes for *B. bovis* (16/80) and *B. bigemina* (18/80) in Continent. The number of positive buffaloes at *B. bovis* and *B. bigemina*-PCR assays did not show difference between Murrah and Mediterranean breeds (p=0.09 e p=0.07, respectively). Pregnant female buffaloes were more likely to show *B. bovis* (p=0.0001) and *B. bigemina* (p=0.0001) positive PCR results when compared to non-pregnant females.

IgG antibodies to *B. bovis* and *B. bigemina* was detected in 41.2% (223/542) and 19.0% (103/542) of sampled buffaloes, respectively. Furthermore, 18% (97/542) of sampled animals showed IgG antibodies to both parasites. Among seropositive buffaloes, 43.3% (97/229) showed seropositivity to both *B. bovis* and *B. bigemina*.

The percentage of seropositive buffaloes for *B. bovis* and *B. bigemina* in continent localities (46% and 50%, respectively) were higher (p=0.0001) than that found among animals from Marajó island (39% and 16%, respectively). Pregnant female buffaloes were more likely (p=0.0001) to show antibodies to Babesia sp. when compared to non-pregnant female buffaloes. The seropositivity for studied parasites did not shown significantly difference between Murrah and Mediterranean breeds.

Eighty-one (30%) and 75 (28%) out of 271 buffalo serum samples submitted to both ELISA and PCR assays showed positive results in at least one test for *B. bovis* and *B. bigemina*, respectively (Figure 1). The number of animals positive to ELISA or PCR
was higher (p=0.05 and p=0.04, respectively) than the number of positive animals in only one of the techniques utilized (Figure 1).

The number of samples positive to *B. bovis* and *B. bigemina* to both tests was 9.2% and 10.0%, number positive to PCR/negative to ELISA was 5.9% and 6.3%, number negative to PCR/positive to ELISA was 32.1% and 8.8% and number negative to both tests was 53.1% and 75.3% (Table 1). These results showed a high correlation between molecular and serological tests used in the present study (kappa index of 0.9).

### 4. Discussion

The combination between ELISA and PCR has been considered useful tool in epidemiological surveys regarding the occurrence of *Babesia* infections in buffaloes (Terkawi et al., 2011).

The present work showed a molecular occurrence of *B. bovis* and *B. bigemina* higher than that found in Thailand (1.7 and 4.1 times higher, respectively) (Terkawi et al., 2011) and China (24 and 28 times higher, respectively) (He et al., 2012). Herein, the percentage of co-positivity for *B. bovis* and *B. bigemina* (18.75%) was higher than that found among buffaloes from China (3.9%) (He et al., 2012).

In Argentina, an occurrence of *B. bovis*-PCR positive buffaloes ranging from 34% a 61% was observed (Ferreri et al., 2008). In India, buffaloes rarely show clinical signs of babesiosis when submitted to natural conditions because are refractory to infection due to their natural immunological resistance (Roychoudhury and Gautam, 1979). However, *B. bigemina* has been showing up as an agent of major concern in Indian cattle herds, parasitizing both cattle and buffaloes (Muraleedharan et al., 1984). In additional buffaloes might serve as reservoirs for the infections of cattle, since they are together in the pastures and continue to infect the tick vector (Bock et al., 2004).
The molecular occurrence of *B. bovis* and *B. bigemina* found in the present study was higher (1.3 and 4.4 times, respectively) than that found among buffaloes in Thailand (Terkawi et al., 2011). In Brazil, despite a high seroprevalence for *Babesia* sp. have been found among buffaloes from Rio of Janeiro, only 1% of sampled animals were positive at PCR targeting the studied agents (Côrrea, 2011). On the other hand, molecular surveys carried out among cattle in Brazil have found a molecular prevalence of 90% for *B. bovis* and *B. bigemina* (Oliveira-Sequeira et al., 2005).

A high correlation was observed between molecular and serological tests used in the present study (kappa index of 0.9). While these findings are similar to that found by Terkawi et al. (2011), they differed significantly from those found by Corrêa (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 infection among buffaloes in Northern Brazil.

The slightly higher rate of infection detected in pregnant female is most likely because the physiology of pregnancy and lactation period, which are associated with hormonal and immunological changes, and in a non-specific immunosuppression. The magnitude and timing of immunosuppression depend on many factors such as inadequate hygienic and sanitary management, inappropriate feed and housing, and genetic differences (Bonizzi et al., 2003).

5. Conclusion

This is the first epidemiological study investigating the occurrence of *B. bovis* and *B. bigemina* in water buffalo from Brazil using both molecular and serologic tests. These data provide information about the incidence of *B. bovis* and *B. bigemina* infections in water buffaloes and may guide future control programs of the disease.
The present work showed that water buffaloes from state of Pará, Northern Brazil, are exposed to bovine babesiosis agents, although do not show clinical signs of the disease. The lower rate of exposure to *Babesia* sp. verified among water buffaloes could be explained by the habitat where sampled animals live, where ticks show a lower rate of attachment to animal skin.

**Acknowledgements** - We are grateful to Dr. Rosangela Zacarias Machado by kindly supplied the antigen used. We also thank the Coordination for the Improvement of Higher Level of Education Personnel (CAPES) for financial support.

**Reference**


**Legend of tables and figures**

**Figure 1.** Serological and molecular detection of Babesia bovis and Babesia bigemina in water buffaloes from north region of Brazil.
Table 1. Summary of the molecular and serological detection of *B. bovis* and *B. bigemina* using nPCR assay and ELISA.
Table 1

<table>
<thead>
<tr>
<th>Agent</th>
<th>nPCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Babesia bovis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>41</td>
<td>25</td>
</tr>
<tr>
<td>(□)</td>
<td>231</td>
<td>87</td>
</tr>
<tr>
<td>(□)</td>
<td>272</td>
<td>112</td>
</tr>
<tr>
<td>Babesia bigemina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>44</td>
<td>27</td>
</tr>
<tr>
<td>(□)</td>
<td>228</td>
<td>24</td>
</tr>
<tr>
<td>(□)</td>
<td>272</td>
<td>51</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of positive and negative buffaloes in nPCR assays.

<sup>b</sup>Number of positive and negative buffaloes in both nPCR and ELISA assays.
Fig. 1

Percentage of positive water buffaloes (%)

- Babesia bovis
- Babesia bigemina

ELISA + nPCR
ELISA
nPCR

(p = 0.033)
(p = 0.053)
(p = 0.047)
(p = 0.022)
(p = 0.045)