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DETECTION OF *BRUCELLA* SPP. IN BLOOD SAMPLES OF WATER BUFFALO (*BUBALUS BUBALIS*) FROM MARAJÓ ISLAND, AMAZON, BRAZIL

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ABSTRACT

Brucellosis is a global zoonotic infectious disease with great public health and economical importance, which affects the buffalo herds worldwide. Diagnostic methods for brucellosis usually depend on serological tests and direct culture, which may lack sensitivity. Thus, we aim to detect *Brucella* spp. using the conventional polymerase chain reaction (PCR) method in blood samples water buffaloes from five municipalities on Marajó Island, Eastern Amazon. A total of 97 male buffalos were sampled during slaughter and blood submitted to PCR. Among the 97 samples analyzed, 33% were positive and 36% were from Chaves, 27% from Cachoeira do Arari, 18% from Soure, 12% from Santa Cruz do Arari and 3% from the municipality from Salvaterra. It appears that *Brucella* spp. it is highly prevalent in the blood of male domestic water buffaloes from Marajó Island.

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INTRODUCTION

The domestic water buffalo (*Bubalus bubalis*) has its origin in the domestication of Indian wild buffaloes in Mesopotamia, in the Hindu valleys and in China during the third and second millennium BC. In Brazil, the buffalo were first introduced to Marajó Island in 1895, with herds of water buffalo from the Mediterranean breed from Italy (Brasil, 1998). The *Bubalus bubalis* species has adapted very well to the topographic characteristics of Brazil, specifically in the Amazon region, in addition to its rusticity and dual purpose, in milk and meat, it presents itself as a good option for protein animal in the region (Nadir Jr *et al*., 2012, Vale *et al*., 2013). The state of Pará currently holds the largest herd of buffalo in Brazil, with 491,290 heads (40% of the national herd) and Marajó Island has the largest water buffalo herd in Brazil (Brasil, 2019). Because of the peculiar characteristics of the island and its relative simplicity and barriers to gene flow, molecular epidemiology studies on the socioeconomically relevant diseases (Acevedo *et al*., 2013), such as brucellosis, mycobacteriosis, and others zoonotic disease, are important.

Brucellosis is a highly contagious disease with worldwide distribution, characterized as an important zoonotic disease (Paula *et al*., 2015). Its agents, bacteria of the genus Brucella, can adapt to different hosts (Mirnejad *et al*., 2013). Among the known species of the genus *Brucella* are six "classic" species: *B. abortus*, *B. melitenses*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Newby *et al*., 2013). Four new species have been described: *B. pinnipedialis*, *B. ceti*, *B. microti and B. inopinata* (Saegerman *et al*., 2011). Bacteria belonging to the genus Brucella infect a large number of animals, including cattle, buffaloes, goats, sheep, canines and other domestic and wild, terrestrial and marine mammals (Brasil, 2006, Batista *et al*., 2020). Humans can also be infected with this bacterium (Mirnejad *et al*., 2013). The illness presents itself as an occupational disease, being more present in people whose activity is directly related to animals or their products and derivatives (Quinn *et al*., 2005).The main methods for the diagnosis of buffalo brucellosis in the state of Pará have been

the serological methods, mainly, the buffered acidified plate antigen test (Silva *et al*., 2014; Casseb *et al*., 2015). The polymerase chain reaction (PCR) assay has been used for the detection of *Brucella* spp. DNA, in different tissue samples for the diagnosis of bovine and buffalo brucellosis (Sousa *et al*., 2015; Santos *et al*., 2017). Thus, we evaluated the presence of DNA from *Brucella* spp. in blood samples from male buffalo from different locations on Marajó Island, State of Pará, in northern Brazil.

MATERIALS AND METHODS

A total of 97 water buffaloes whole blood samples were collected, all male, aged 36 months or older, from five municipalities on the Marajó Island, belonging to the Arari Microregion located in the Marajó mesoregion, in the state of Pará, Eastern Amazon and Northern Brazil: Chaves, Cachoeira do Arari, Salvaterra, Santa Cruz do Arari e Soure (Figure 1) slaughtered in a abattoir between August and November of 2015. All samples used in this study were collected in full compliance approved by the Research Ethics Committee with Experimental Animals at the Federal University of Pará (CEPAE 07-2015). The blood was collected, during bleeding in the slaughter flowchart, in tubes with ethylenediamine tetraacetic acid (EDTA) anticoagulant. After sampling the whole blood was kept under refrigeration (-80) for further processing.

The DNA of the samples and vaccine B-19 containing *B. abortus* (positive control) were extracted using the phenol-chloroform method (Sambrook *et al*., 1989). The chosen primer pair previously described and established by Baily *et al*. (1992), it is specific genus and delimit a fragment of 223 bp of gene that encodes a 31 kDa membrane protein B4 (Forward 5'-TGGCTCGGTTCCAATATCAA-3') AND B5 (Reverse 5'-CGCGCTTGCCTTTCAGGTCTG-3'). For the amplification of the DNA of *Brucella* spp. a conventional PCR was performed in a volume of 25 µL, containing: 15,8 µl of ultrapure water, 2,5 µl of 10^x buffer, 1,0 µl of dNTP, 0,5 µl of MgCl2, 1,0 µl of each primer (B4 e B5), 0,2 µl of Taq DNA polimerase (invitrogen®) and of the 3,0 µl of DNA sample extracted. The PCR reaction occurred under the following conditions: initial denaturation of 93°C at 5 minutes, followed by 40 cycles of 90°C at 1 minute (denaturation), 60°C at 1 minutes (annealing) and 72°C at 1 minute (extension) with a final extension 72°C at 10 minutes. After PCR amplification, the amplified product was loaded into wells in 1.5% agarose gel, containing SYBR® Safe DNA nucleic acid staining reagent and the electrophoresis equipment was set to run at 130 V for 50 min. Then the gel reading was performed on under UV light. The size of the amplified fragment (223bp) was estimated by comparing the 1000 bp and / or 1 Kb molecular weight markers (DNA ladder Invitrogen®). The data were tabulated and statistically treated by simple percentages and the differences between the percentages were compared using the chi-square test, assuming statistical significance for $p < 0.05$ and for values less than 5 the G test, implemented in the programs by Microsoft Excel and BioEstat 5.3.

RESULTS

Among the total of 97 samples, it was observed that 33% were positive in detecting the DNA fragment of *Brucella* spp. All tested municipalities showed positive results, with the following results: Chaves with 36% of positive animals, followed by the municipalities of Cachoeira do Ariri, 27%, Soure, 18%, Santa Cruz do Ariri, 12% and the Salvaterra, with 3% of the animals were positive for Brucella spp (Figure 1). In relation to cities, the municipalities of Chaves and Cachoeira do Arari were more prevalent than the other municipalities ($p < 0.01$).

DISCUSSION

The technique used in the study assist in the diagnosis of brucellosis as it presents high levels of sensitivity when detecting immunogenic protein of 31 KDa present in the genus *Brucella*. Guido (2003) obtained significant values when carrying out the research of *B. abortus* in milk of experimentally contaminated buffalo when detecting the same target region. In our study, a high frequency of positive animals was observed in the PCR test for *Brucella* bacteria in the animals' whole blood. The result can be explained by the high index of seropositivity found in this region where brucellosis is endemic (Casseb *et al*. 2015). Sousa *et al*. (2015) in his study detected DNA for *B. abortus* in 11.57% of 95 samples of buffalo lymph nodes from the island of Marajó. Some behavioral characteristics of buffaloes, for example, staying for long periods in mud holes and weirs, facilitates exposure to certain microorganisms, such as Brucella, which is able to survive for weeks or months in water, urine, feces, moist soil, and manure under favorable humidity and temperature conditions (Wray 1975, Borriello *et al*. 2013).

The National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), started in 2001 in Brazil, recommends that brucellosis serodiagnosis in buffalo females be performed from 24 months of age on, in calves vaccinated with sample B19 between 3 and 8 months of age, using the evidence from the rose Bengal plate agglutination test, 2-mercaptoetanol (2-ME) and/ or complement fixation test (CF) (Brasil, 2016). However Pereira *et al*. (2015) recommends an earlier serological test in buffalo females vaccinated with the B19 sample, because they show a down drop in antibodies after vaccination when compared to bovine females vaccinated with the same vaccine, showing differences in the humoral immune response in these species. Our study demonstrates the possibility of using a conventional PCR test of blood samples from male water buffalo, which are not vaccinated against brucellosis in Brazil, therefore, the positiveness of the samples cannot be due to the use of the vaccine. One limitation of the study was the lack of sequencing of positive samples due to absence of equipment. It is possible that the *Brucella* species, if isolated, may be from *B. abortus*, because according to a study carried out in Brazil, brucellosis in buffaloes and cattle is caused predominantly by *B. abortus* biotype 1 (Megid *et al*., 2005, Ayala *et al*. 2019). Another study also on the island of Marajó, with lymph node samples, carried out by the Real Time PCR technique, found *B. abortus* (Sousa *et al*., 2015). However, it is necessary to carry out genetic sequencing of these samples to confirm or reject this hypothesis, as we cannot rule out the possibility of another species already known or a new species not known to science. Further studies are needed to molecularly characterize the species of *Brucella* sp. in buffaloes from Marajó island. Draws attention the high positiveness index of Brucella spp. in blood samples of the analyzed species, because according to Corrêa & Corrêa (1992) bacteremia in cattle is usually low, or establishing bacteremia around one to two weeks (Santellano-

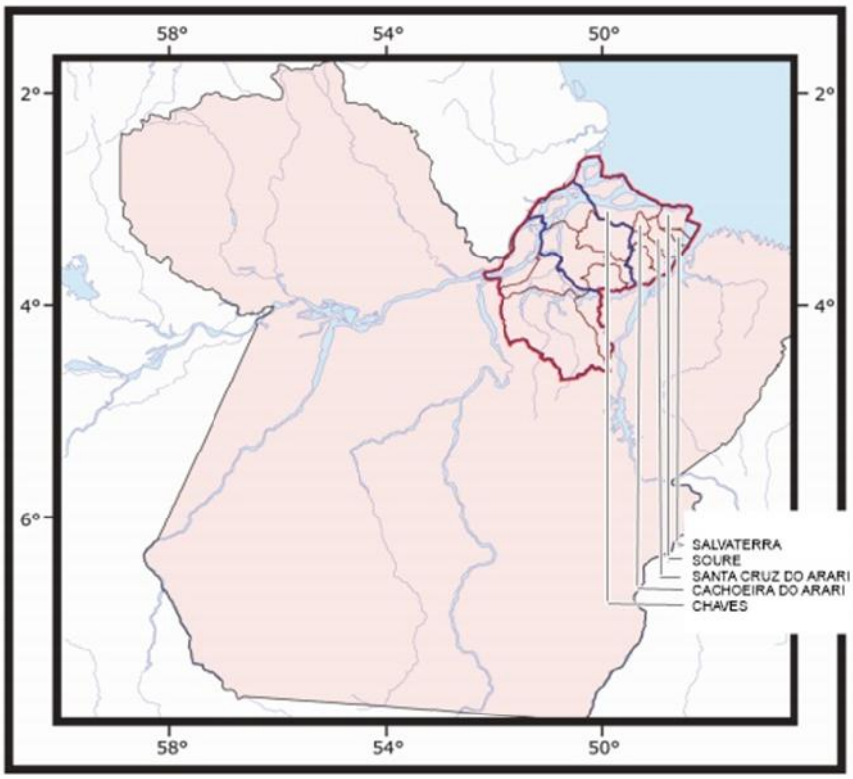

Figure 1. Map of the State of Pará, with emphasis on the municipalities of Salvaterra, Soure, Santa Cruz do Arari, Cachoeira do Arari and Chaves, places of origin of the Water Buffalo, belonging to the Arari micro-region, on the Marajó Island, Amazon Oriental, North Region of Brazil

Estrada *et al*., 2004), however, the degree of bacteremia in water buffalo species is not known, therefore needing a more in-depth study regarding this species. The high rate of frequency in the detection of agent DNA in buffalo can increase the risk of contamination through human exposure. The transmission of the bacteria to humans can occur through direct contact with sick animals, through the ingestion of raw milk and its derivatives, the consumption of contaminated animal meat, which can have serious consequences for public health (Schlafer & Miller, 2007). Another important point was the presence of *Brucella* spp. in blood samples, perhaps making these animals potential contaminants of human beings on Marajó Island, especially when handling these animals for blood collection to perform serological tests for brucellosis or other diseases, making it essential to use personal protective equipment, a fact little seen during the handling of these animals by veterinarians and keepers on the Island.

Brucella abortus infection is preferably located in the genital organs of females and males, causing abortion, orchid, epididymitis and reduced productivity (Aguiar *et al*., 2001, Xavier *et al*. 2009), however the chronic nature of the disease leads to difficult observation of clinical signs in males, so the vast majority of males with brucellosis are asymptomatic (Brasil, 2016), favoring the lack of diagnosis resulting in the permanence of positive animals , making them a source of infection for the herd where they live (Junqueira Júnior *et al*., 2013). If not mistaken, our study is the first to demonstrate DNA for *Brucella* spp. in blood samples of male water buffalo raised in the Amazon ecosystem, on Marajó Island, where one third of the animals were positive. The results demonstrate a need for deepens studies, mainly, from the genetic sequencing of isolated blood samples to elucidate the possible species or species of Brucella that may be present in these water buffaloes raised in the Amazon ecosystem.

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