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Detection of *Brucella abortus* DNA in the reproductive tract of buffalo (*Bubalus bubalis*) cows

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ABSTRACT. *Brucella* spp. are the pathogens responsible for brucellosis, which is a cosmopolitan and serious disease for animals and humans. Recent studies have revealed the presence of *Brucella* DNA in the reproductive tract tissues of buffaloes based on microbiological cultures collected from vaginal swabs. We tested for *Brucella abortus* DNA in the uterus, perivaginal lymph nodes, and uterine mucus of buffalo cows naturally infected with *Brucella* spp., as defined by a positive complement fixation test. Uterine tissue, uterine mucus, and perivaginal lymph node samples from 16 *Brucella* seropositive female buffaloes were analyzed. *Brucella* DNA was found in five of the uterine tissue samples, eight of the perivaginal lymph node samples, and 14 of the uterine mucus samples. Furthermore, Bayesian inference analysis indicated that the DNA sequences obtained from these tissues and mucus grouped with corresponding sequences of the *B. abortus* species.

Key words: Diagnostics; PCR; Bacterium; Zoonosis; *Brucella*

INTRODUCTION

A recent study described the difficulty in controlling brucellosis in buffaloes within the Amazon biome and showed that vaccination is not efficient in preventing the disease in this region (Sousa et al., 2017). Therefore, it is very important to assess all the characteristics of brucellosis epidemiology in cattle and buffaloes to implement an efficient control program in the region. The etiological agent of brucellosis has been found in northern Brazil, specifically in Pará state, with varying prevalence rates. Furthermore, this region of Brazil is home to the largest buffalo herds in the country (Silva et al., 2014). Another state in the same region of Brazil, Maranhão, also had *Brucella* spp. infections detected via serological testing of the animals; the bacteria were isolated from vaginal swabs and the species confirmed through PCR analysis (Santos et al., 2017). Although the infection begins in the oral, nasopharyngeal, and mucosal regions of the conjunctiva and throughout the skin, the bacteria are transported by macrophages to the lymph nodes, where they can reproduce (Sousa et al., 2015a). The bacteria can then spread to other lymph nodes, mammary glands, and other organs containing many phagocytic mononuclear cells (Sousa et al., 2017). *Brucella* spp. also have a strong tropism for the uterus and the trophoblast during the last trimester of gestation (Xavier et al., 2009). Furthermore, *B. abortus* can be detected from the first to the fourth month of pregnancy in the amniotic fluid, allantoic fluid, and uterus of female buffaloes. By the fourth month of pregnancy, *B. abortus* DNA is also detected in the parotid gland, prescapular lymph node, mediastinal lymph nodes, precrural lymph node, and mammary glands of the mother. As early as the fifth month of gestation, *B. abortus* DNA is more frequently detected in the amniotic fluid, allantoic fluid, and placenta, but less frequently in the cotyledons and uterus. It is also possible to detect *B. abortus* DNA in fetal tissues (including the heart, spleen, and kidney) at the fifth month of gestation (Sousa et al., 2015a; 2015b).

We used conventional PCR to detect *B. abortus* in the uterus, perivaginal lymph node, and uterine mucus of buffalo cows that tested positive for *Brucella* seropositivity in a complement fixation test.

MATERIAL AND METHODS

The experimental protocol used to collect these data was submitted to the Ethics Committee on the use of animals of the Federal Rural University of Amazonia. There was no objection since the research does not involve the manipulation of live animals, following the guidelines of the National Council for Control of Animal Experimentation of Brazil (CONCEA).

Animal samples

A total of 2200 breeding age buffaloes (24 - 60 months), destined for export, were monitored at three properties located in three cities in the State of Pará, Brazil (Nova Timboteua, n = 330; Moju, n = 550; and Tailândia, n = 1320). All animals were submitted

to the Rose Bengal Test, and those that reacted positively were reevaluated by the complement fixation test, of which only 16 were classified as positive according to the National Program for the Eradication of Brucellosis and Tuberculosis. Tests were performed by the technicians in charge of the export company.

After testing, the 16 animals were sent to a slaughterhouse in Castanhal, Pará, Brazil, registered, and inspected by the State Animal Inspection Service of the Pará State Agricultural Defense Agency (ADEPARÁ). Of the 16 animals from which samples were collected, one was 30 days pregnant, four were 60 days pregnant, six were between 100 and 120 days pregnant, and five were not pregnant. The complete reproductive tract was collected from each of the animals and macroscopic evaluation followed. Uterine mucus was collected for diagnostic tests, and perivaginal lymph node and uterine tissue samples were individually collected for diagnostic tests and to be dissected, identified, packed, and transported in a cooled expanded polymer box to the laboratory where they were stored at -20°C until being processed for molecular analyses.

Laboratory procedures

For analysis, 200 mg of each harvested tissue sample was macerated individually using a sterile mortar and pestle. The macerated tissue was transferred to a 2-mL microtube containing 800 µL of extraction solution (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, and 400 mM NaCl), 100 mL of 10% SDS, and 20 µL of Proteinase K (10 µg/µL). The homogenate was then incubated at 65°C overnight. After incubation, proteins and cellular debris were precipitated by the addition of 300 µL 6 M NaCl, maintained at 4°C for 15 min, and then centrifuged at 14,000 rpm for 20 min at 20°C. The supernatant (500 µL) was transferred to a clean and labeled microtube. Next, 250 µL of 8 M guanidine hydrochloride (pH 8.0) and 250 µL of 0.49 M ammonium acetate solution were added to the supernatant and the mixture was kept under gentle agitation for 90 min. Nucleic acids were precipitated by the addition of 500 µL of 100% cold isopropyl alcohol and centrifugation at 11,000 x *g* for 5 min. The supernatant was discarded and 400 µL of 70% isopropyl alcohol was added. After drying, the pellets were resuspended in 50 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 g/mL RNase) (Biase et al., 2002).

After DNA extractions were complete, the DNA samples were subjected to agarose gel electrophoresis (1.5% agarose) in 1x TAE application buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) using 2 µL of extracted DNA and 2 µL of the Blue/GelRed mix (1:1) at 90 V for approximately 30 min.

Polymerase chain reactions were run based on the IS711 gene sequence of the *Brucella* genome, for which we used the oligonucleotide sequences described in Ning et al. (2012) (Forward 5'-GAGAATAAAGCCAACACCCG-3' and Reverse 5'-GATGGACGAAACCCACGAAT-3') to amplify a sequence of 317 bp. The final reaction volume was 25 µL under the following reagent conditions: 2.5 µL of 10× buffer, 2 mmol $MgCl_2$, 1.25 mmol of each dNTP, 10 mmol of both primers (forward and reverse), 2 U of *Taq* DNA polymerase (Invitrogen, Fortaleza, CE, Brazil), and 50-100 ng of extracted genomic DNA. The reactions were performed in a Mastercycler Personal Thermocycler (Eppendorf, Hamburg, Germany). The temperature conditions established for the cycles were: initial denaturation at 94ºC for 5 min, 30 cycles of amplification characterized by denaturation at 94ºC for 1 min, annealing at 58°C for 1 min, and extension at 72ºC for 1

min, and then a final extension at 72ºC for 10 min. PCR products were subjected to horizontal gel electrophoresis in 1.5% agarose gels stained with GelRed (Biotium, California, USA) and run at 90 V for 30 min. Positive PCRs for *Brucella* DNA from uterine tissues, uterine mucus, and perivaginal lymph nodes were purified with Illustra ExoProStar 1-Step (GE Healthcare, London, UK) and subsequently sequenced in both the sense and antisense directions in an AB3500 Genetic analyzer (Applied Biosystems, Carlslad, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen, Carlslad, CA, USA) and BigDye® XTerminator Purification Kit (Invitrogen, Carlslad, CA, USA). All kits were used according to manufacturers' guidelines.

Sequence analyses

Consensus sequences obtained using the BioEdit program (v7.0.5.3) were aligned with representative sequences from eight species of the genus *Brucella* selected at Genbank (http://www.ncbi.nlm.nih.gov/genbank). The nucleotide substitution model was selected with the jModelTest program (v2.1.10) and phylogenetic analyses were developed by Bayesian inference in the MrBayes program (v3.2.2, K2P + I model, 500,000 generations, and two runs). The distribution of the likelihood values was evaluated in the Tracer program (v1.6), and the final phylogenetic tree, with maximum credibility among the data, was obtained with the FigTree program (v1.4.2). In addition, nucleotide identity calculations were developed using Geneious software (v8.1.3).

Statistical analyses

The data were processed in spreadsheets and submitted to the statistical program SAS (University edition), where the frequencies of positive PCRs in the uterine tissue, uterine mucus, and perivaginal lymph node samples were submitted to Fisher's statistical test (5%), and the concordance between CFT and PCR methods was tested by the Kappa test.

RESULTS

Uterine tissue, uterine mucus, and perivaginal lymph node samples from 16 *Brucella* seropositive females were analyzed (Table 1). The presence of *Brucella* DNA was confirmed using agarose gel electrophoresis, in which an amplification band of 317 bp corresponded to the IS711 region of the *Brucella* genome (Figure 1).

Table 1. PCR detection of *Brucella abortus* in uterine tissue, perivaginal lymph node samples, and uterine mucus of *Brucella* seropositive buffalo cows.

Samples (n=16)	Positive	Kappa test	
		Replicability*	P-value
Perivaginal lymph node	8[b]	Good	0.0005
Uterine mucus	14[a]	Excellent	0.0001
Uterine tissue	5[b]	Low	0.0075

Different letters in the same column differ statistically by Fisher's Exact Test ($P < 0.05$). *according to Rosner (2006).

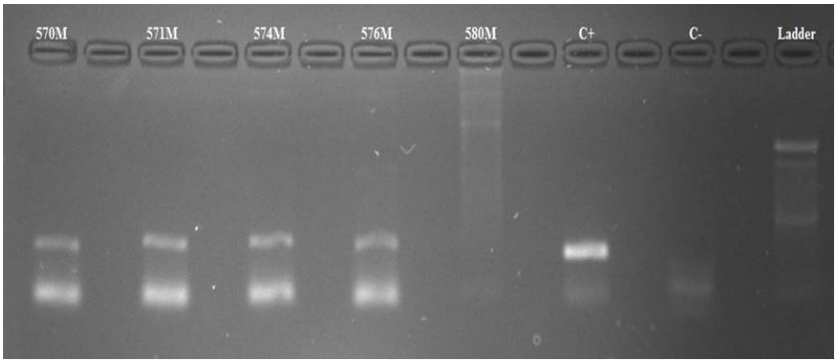

Figure 1. Horizontal gel electrophoresis in 1.5% agarose, stained with GelRed (Biotium, California, USA), run at 90 V in 30 min, and visualized in UV light with a photo documentation system (Biosystem). The 317 bp PCR product of the prime *Brucella* fragment found in uterine mucus is shown.

The uterine mucus samples were positive for *Brucella* spp. DNA than the other samples ($P < 0.05$). Nucleotide sequencing and phylogenetic analysis was completed on 10 randomly selected samples positive for *Brucella* DNA. Analysis via Bayesian inference indicated that the sequences obtained from the perivaginal lymph node, uterine mucus, and uterus tissue samples of buffalo cows grouped next to reference sequences of the *B. abortus* species (Figure 2).

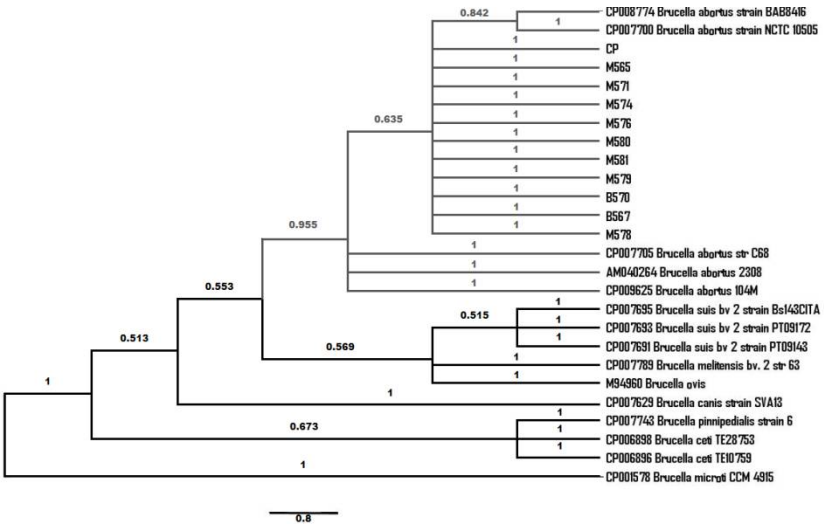

Figure 2. Phylogenetic tree obtained by Bayesian inference of a partial sequence of the *wboA* gene (311 bp) of the genus *Brucella*. The probability values are expressed in the sequences of the species represented in gray (565, 571, 574, 576, 578, 579, 580, 581, 567, 570; Positive control = PC). The calculation of the identity matrix indicated that all the samples obtained in our study had a nucleotide identity percentage of 99 - 100% when compared to each other.

DISCUSSION

The results showed that PCR can be highly sensitive and specific in the detection of sequences that include the IS711 region of the genome in *Brucella* spp. This conclusion

agrees with previous studies using buffaloes experimentally inoculated with *B. abortus* (Sousa et al., 2015a; 2015b), and studies of bovine abortions caused by *Brucella* spp. that can invade host epithelial cells and allow infection through mucosal surfaces (Poester et al., 2013 and Antoniassia et al., 2016).

It was possible to detect *Brucella* DNA from swab samples of the uterine mucus, similar to what has been observed in dogs, where the use of a swab is minimally invasive, avoiding stress in the animals (Fernandes et al., 2013). Thus, the use of a swab to detect *Brucella* in the reproductive tract of buffaloes may be an excellent tool to confirm diagnosis of the pathogen.

The detection of *B. abortus* DNA in the perivaginal lymph nodes of our study may be related to the fact that these lymph nodes drain the areas common for *Brucella* spp. infection or lesions and are therefore are more likely to come into contact with the bacteria. Furthermore, there is a high level of *B. abortus* detected in animals during the gestational period as demonstrated by the high concentration of erythritol degradation products (Sousa et al., 2015b).

The non-detection of *B. abortus* DNA in some perivaginal lymph nodes of our study can be explained by the fact that after bacteremia, *Brucella* spp. are phagocytosed by macrophages and multiply in the regional lymph nodes, and then invade the uterine lumen and chorionic villi, where there is greater availability of substrates for their multiplication (Sousa et al., 2015a; 2015b). The results of Sousa et al. (2015b), together with those our study, indicate that starting in the second month of gestation *B. abortus* DNA can be detected by PCR in the uterus, amniotic fluid, and allantoic fluid of pregnant buffalo cows. The *B. abortus* infection may be present in the uterus of infected buffalo cows within the first, second, and third months of gestation (Sousa et al., 2015b).

In conclusion, the highest detection rate of *Brucella* DNA was in the uterine mucus samples compared to the other sample types analyzed. The DNA in the reproductive tract of *Brucella* seropositive animals diagnosed by serology in the State of Pará, Brazil was confirmed as that of *B. abortus*.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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