Detection of *Brucella* spp. in dogs at Pantanal wetlands

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Abstract

Canine brucellosis is an infectious disease that produces reproductive disease in both males and females. Although *Brucella canis* is more common, the infection by *Brucella abortus* is more frequent in dogs sharing habitats with livestock and wild animals. We decided to investigate the role of dogs in the maintenance of *Brucella* spp. in the Pantanal wetland. Serum and whole blood samples were collected from 167 dogs. To detect antibodies against *B. abortus* and *B. canis*, buffered acidified plate antigen (BAPA) and agar gel immunodiffusion (AGID) tests were performed. To detect *Brucella* spp., *B. abortus* and *B. canis* DNA, PCR was performed using the *bcs31*, *BruAb2_0168*, and *BR00953* genes, respectively. To confirm the PCR results, three *bcs31* PCR products were sequenced and compared with sequences deposited in GenBank. The seropositivity rates of 7.8% and 9% were observed for the AGID and BAPA tests, respectively. Positivity rates of 45.5% and 10.8% were observed when testing *bcs31* and *BruAb2_0168*, respectively, while there was no positivity for *BR00953*. The sequenced products had 110 base pairs that aligned with 100% identity to *B. abortus*, *B. canis*, and *B. suis*. Considering our results, dogs may be acting as maintenance hosts of *Brucella* spp. in the Pantanal region.

Keywords *Brucella* spp. · Reservoir host · Serology · Molecular tests · Pantanal

Introduction

Canine brucellosis is an infectious disease that produces late abortions in females, epididymitis, and prostatitis in males, as well as nonspecific symptoms, such as fever, lymphadenitis, lameness, uveitis, polyarthritis, discospondylitis, paresis, and ataxia [1]. The etiological agent *Brucella canis* is transmitted through direct contact with genitourinary secretions of infected dogs, aborted placental and fetal material, and vaginal, prostate and seminal fluids [1].

Outbreaks of canine brucellosis have been recently reported around the world, resulting in economic losses for kennel owners [2–6]. Moreover, the occurrence of *B. canis* in humans suggests that this infectious agent is an emergent zoonosis, with veterinarians and animal handlers being particularly at risk [7–12].

Although *B. canis* is typically associated with dogs, three other species have been reported to infect dogs, *B. melitensis*, *B. suis*, and *B. abortus* [13–18]. Of these additional species, *B. abortus* is most commonly found in dogs, especially in enzootic environments where domestic animals are in close contact with livestock and wildlife species [19, 20].

The Pantanal is the largest wetland ecosystem in the world and is a region in which cattle ranching is the principal economic activity, with an estimated cattle population of four million [21, 22]. In this region, distinctive flood and dry seasons occur that greatly vary in intensity from year to year [21]. With approximately 160,000 ha in the core of South America, this region shelters a rich and dense wild mammalian fauna that shares the same habitat with domestic animals [22]. Cases of brucellosis caused by *B. abortus* have been reported in the Pantanal region that affect some species of domestic animals

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and wildlife [23–27]. Although Furtado et al. (2015) [27] previously reported that dogs are exposed to *Brucella* spp. in the Pantanal, as determined by serology, the role of this domestic species in the enzootic persistence of brucellosis in this region remains unknown. In this sense, we aimed to detect *Brucella* spp. in dogs in the Pantanal wetland using serological and molecular tests, identifying the role of dogs in the maintenance of *Brucella* spp.

Material and methods

Samples

Between August 2013 and July 2014, we collected serum and whole blood samples from 167 dogs from the Nhecolândia subregion (18° 59' 15" S; 56° 37' 03"), of the southeastern Pantanal. The sample size was determined by a non-probabilistic method. All procedures were performed in accordance with the Ethics Committee for Animal Use of the Universidade Católica Dom Bosco (001/2013).

Serology

Agar gel immunodiffusion (AGID) and buffered acidified plate antigen (BAPA) tests were performed to detect antibodies against *B. canis* and *B. abortus*, respectively. The antigen used in the AGID test consisted of proteins and lipopolysaccharides from *B. ovis* (strain Reo 198), and the antigen used in the BAPA assay consisted of inactivated *B. abortus* (strain 1119–3), both produced by the Instituto de Tecnologia do Paraná (TECPAR®). Both techniques were performed according to the manufacturer's instructions.

Molecular tests

The DNA extraction was performed according to Sambrook and Russel (2001) [28]. The concentration and purity of the extracted DNA were determined using a spectrophotometer (Biodrop® Denville Scientific Inc. Holliston, MA 01746) at an absorbance of 260 nm and by electrophoresis. PCR was performed using the primers listed in Table 1. As positive controls, we used *B. abortus* (strain S2308) and *B. canis* (strain SG 771) genomic DNA. The products were subjected to electrophoresis at 100 V for 40 min on a 1.5% gel agarose, which was then stained with ethidium bromide, visualized under an ultraviolet transilluminator, and documented.

Sequencing and phylogenetic analysis

For sequencing, we used the amplified products from the *bcspl31* PCR due to the greater number of base pairs

present compared to the *BruAb2_0168* products. Three PCR products with high concentrations and absence of nonspecific bands were purified with Kit Qia Quick PCR purification columns (Qiagen® Hilden, Alemanha) and sequenced with the same forward and reverse primers used in the initial PCR amplification. A Big Dye terminator cycle sequencing kit version 3.1 (Applied Biosystems® Foster, California, EUA) was used according to the manufacturer's instructions.

To determine if the amplified products matched *Brucella* spp. sequences, a basic local alignment search tool (BLAST) was used, comparing the sequenced samples to sequences deposited in GenBank. An alignment of the *bcspl31* amplified sequences with some *Brucella* species, including *B. abortus* (CP008774.1), *B. suis* (CP009096), and *B. canis* (CP007629), was performed using Clustal W (<ftp://ftp.ebi.ac.uk/pub/software/clustalw2/>).

Statistical analysis

Cohen's kappa statistic was used to evaluate the degree of concordance between all diagnostic assays with positive results using R version 3.3.1 program [32].

Results

We observed a 7.8% (13/167) seropositivity rate using the AGID test (CI = 2.24–9.55%) and a 9% (15/167) seropositivity rate using the BAPA test (CI = 5.29–14.64%) (Table 2). All samples testing positive by AGID tested negative using BAPA and vice versa. Regarding the molecular tests, using the *B4/B5* primer pair, 45.5% (76/167) (CI = 37.85–53.37%) of dogs tested positive, while using the primers to amplify *BruAb2_0168*, 10.8% (18/167) (CI = 6.69–16.74%) of dogs tested positive (Fig. 1). There were no positive results using the primers to amplify *BR00953*.

Comparisons among the different diagnostic tests showed a slight agreement between the *B4/B5* and *BruAb_0168* PCRs (κ 0.07, CI = –0.09–0.23), but no agreement between the BAPA test and the *BruAb_0168* PCR (κ –0.04, CI = –0.37–0.29) or between the AGID test and the BAPA test (κ –0.06, CI = –0.47–0.34).

The three sequenced *B4/B5* PCR products had 110 bp that aligned with 100% of identity to the *bcspl31* genes of *B. abortus*, *B. canis*, and *B. suis* (best hits nr access | e-value 3e-49) (Fig. 2).

Discussion

We demonstrated that, in addition to reports of dogs having been exposed to *Brucella* spp. in the Pantanal wetland [27],

Table 1 Primers used in molecular tests to detect different *Brucella* species in dogs from the Pantanal region

Primer	Species	Sequence (5'–3')	Amplicon	Region	References
<i>BruAb2_0168f</i> <i>BruAb2_0168r</i>	<i>B. abortus</i>	GCACACTCACCTTCCACAACAA CCCCGTTCTGCACCAGACT	81 bp	Chromosome II, <i>BruAb2_0168locus</i>	[29]
<i>B4f</i> <i>B5r</i>	<i>Brucella</i> spp.	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCCTTTCAGGTCTG	223 bp	<i>bcs p 31</i>	[30]
<i>BR00953f</i> <i>BR00953r</i>	<i>B. canis</i> , <i>B. suis</i> e, <i>B. neotomae</i>	GGAACACTACGCCACCTTGT GATGGAGCAAACGCTGAAG	272 bp	Chromosome I, <i>polA</i>	[31]

they can also present bacteria in the bloodstream, as shown by the positive results from the molecular methods used in this study. Furthermore, this is the first study that reports the sequencing of the *Brucella bcs p 31* gene from dogs in the Pantanal region.

This gene codifies the *Brucella* cell surface 31 kDa protein and has been used as a target since the end of the 1980s to detect *Brucella* spp. [30, 33], providing high diagnostic sensitivity and specificity [34, 35]. *Brucella* spp. show a high degree of phylogenetic similarity to a free-living genus of saprophytes *Ochrobactrum* spp. [36], and the *B4/B5* primers can hybridize to the genomes of these species [37]; however, false-positive PCR results can be discarded when examining natural infections, since this bacterium does not replicate within eukaryotic cells [38].

The difference in the detection of *Brucella* by *B4/B5* (45.5%) and *BruAb2_0168* (10.7%) PCRs may be explained by their specificity. Indeed, unlike the *B4/B5* primer set, the *BruAb2_0168* primers detect a region of a transposon insertion site that is only present in *B. abortus* strains [39]. Considering that *BR00953* detect *B. canis*, *B. suis*, and *B. neotomae* [31, 40], and no positive results were generated with this primer, dogs in the studied area may be infected by another species of *Brucella*.

Although our results demonstrated an alignment between *B. abortus*, *B. canis*, and *B. suis* (Fig. 2), it is not enough to discriminate *Brucella* species because different species of *Brucella* present a high degree of similarity (98 to 99%) [41]. Nevertheless, our sequencing analysis confirmed that *Brucella* spp. was found infecting dogs in the Pantanal region.

Table 2 Frequency of dogs testing positive in different diagnostic methods. Results are expressed by the number of positive animals followed by the percent testing positive

Diagnostic test	Positivity	Confidence interval
AGID	13/7.8%	(CI = 2.24–9.55%)
BAPA	15/9%	(CI = 5.29–14.64%)
<i>B4/B5</i>	76/45.5%	(CI = 37.85–53.37%)
<i>BruAb2_0168</i>	18/10.7%	(CI = 6.69–16.74%)

The lack of agreement between the serological and molecular diagnostic methods, as demonstrated by the Kappa test ($p > 0.05$), is acceptable in natural infections because detectable amounts of IgG in the serological tests are only expected during the chronic phase of infection. Thus, during the acute phase, which is characterized by bacteremia and an absence of detectable IgG, positive PCR results and negative serological results are expected. In contrast, because of the absence of bacteria in the bloodstream of infected animals during the chronic phase, negative PCR results and positive serological results would be expected. As our statistical analysis showed a lack of agreement between the serological and molecular tests, the diagnosis of *Brucella* spp. infections must be done by a combination of both methods.

In the case of *B. canis* infections, our results showed that seropositive dogs are chronically infected, since they did not test positive by PCR using the *BR00953* oligonucleotides. Although whole blood is considered a good template for the isolation and molecular detection of *B. canis* due to the prolonged initial bacteremia produced by this species, the sampling of animals during the chronic phase may cause false-negative results. After an initial oronasal exposure, dogs are bacteremic for 7–30 days (acute phase); in the first 3–4 months, the bacteremia declines, and the organisms become localized in the targeted reproductive tissues, with intermittent bacteremic

Fig. 1 Electrophoresis of the PCR product in a 2% agarose gel. Lane 1: 100 bp molecular marker (Invitrogen®); lanes 2 to 5: positive samples of *BruAb2_0168* (*B. abortus*); 6: positive control; 7: negative control

Fig. 2 Multiple sequence alignment of *bscp31* *Brucella* genes (from samples 03, 09, and 11, and *B. canis*, *B. abortus*, and *B. suis* from GenBank) using the MEGA 6.0 program

<i>bscp31</i> GENE	CGGGTAAAGC	GTCGCCAGAA	GGCGCAAATC	TTCCACCT-T	GCCCTTGCCA
<i>bscp31</i> <i>B. canis</i>					
<i>bscp31</i> <i>B. suis</i>					
<i>bscp31</i> <i>B. abortus</i>					
Animal 09	..A..C...	T..		A..	..G.....
Animal 03					
Animal 11					G.....
<i>bscp31</i> GENE	TCATAAAGGC	CGGTGCCGTT	ATAGGCCCAA	TAGGCAACGT	CTGACTGCGT
<i>bscp31</i> <i>B. canis</i>					
<i>bscp31</i> <i>B. suis</i>					
<i>bscp31</i> <i>B. abortus</i>					
Animal 09					
Animal 03					
Animal 11					
<i>bscp31</i> GENE	AAAGCCGGAC	T			
<i>bscp31</i> <i>B. canis</i>		.			
<i>bscp31</i> <i>B. suis</i>		.			
<i>bscp31</i> <i>B. abortus</i>		.			
Animal 09		.			
Animal 03		.			
Animal 11		.			

episodes that can last for years (chronic phase) [42]. As described above, the interpretation of the diagnostic results should be made with an attempt to yield a precise diagnosis.

The infection of dogs by *B. abortus* in the Pantanal wetland could occur via the oral route for two reasons. First, dogs are fed raw meat from slaughtered cattle, and strains of *B. abortus* produce urease that protects the bacteria during its passage through the stomach [16, 20, 43]. Second, since antibodies against *B. abortus* S19, which is used to immunize livestock [44], can be detected via a BAPA test [45], we did not rule out the presence of this strain in the sampled animals because vaccinated cattle can act as a source of *B. abortus* S19 contamination of the environment [46, 47]. This is especially the case during periods of flooding, when domestic animals and wildlife come into close contact [22], favoring the horizontal transmission.

In the Pantanal wetland, *B. abortus* has already been identified in crab-eating foxes (*Cerdocyon thous*), brown-nosed coatis (*Nasua nasua*), and pampas deer (*Ozotoceros bezoarticus*) [25, 26], and its occurrence in cattle was reported [23, 24]. Thus, the contact of dogs with wildlife and cattle may promote the transmission of *B. abortus* among them, as was reported in Egypt [20]. Since infected dogs can eliminate *Brucella* spp. through secretions and excretions, especially during recurrent episodes of bacteremia [42], this domestic host species may have an important role in the spread of *Brucella* spp. to livestock in the Pantanal region.

Considering our results, dogs in the Pantanal wetland may be acting as maintenance hosts for *Brucella* spp. in the natural environment. The dogs in this region would likely be primarily infected by *B. abortus* due to close contact with infected livestock and wildlife, which often occurs during the wet season.

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Compliance with ethical standards

All procedures were performed in accordance with the Ethics Committee for Animal Use of the Universidade Católica Dom Bosco (001/2013).

Conflict of interest The authors declare that they have no conflict of interest.

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