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Brucella canis INFECTION IN DOGS ATTENDED IN VETERINARY CLINICS FROM PATOS, PARAÍBA STATE, BRAZIL

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ABSTRACT

To determine the frequency of anti-*Brucella canis* antibodies in dogs attended in veterinary clinics from Patos, Paraíba State, Brazil, as well as to identify risk factors and to isolate and identify the agent, 193 dogs were used. Agar gel immunodiffusion test (AGID) was used to detect *B. canis* antibodies in sera. Isolation of *B. canis* was carried out in blood and bone marrow from seropositive animals. Six animals tested seropositive in AGID, resulting in a frequency of 3.11%. *B. canis* was isolated from bone marrow of one seropositive animal, with confirmation by PCR. Lack of cleaning of the dog's environment was identified as risk factor (odds ratio = 7.91). This is the first report of isolation of *B. canis* in dogs from the Northeast region of Brazil.

Key words: Bacterial diseases, canine brucellosis, prevalence, risk factors, microbiological culture

Canine Brucellosis is an infectious disease of zoonotic potential whose etiologic agent is *B. canis*, a bacterium responsible for impairment of reproductive tract, primarily abortion and sterility in females and orchitis and epididymitis in males (1). Zoonotic potential of the disease should be taking into account due to complex and close relationship with human population, especially children.

Although clinical signs are usually related to disorders of the reproductive tract, in most cases, even it being a disease of systemic character, the animals are apparently healthy (asymptomatic), behaving as important sources of infection (1).

Etiological diagnosis is performed by detection of antibodies in blood serum or isolation of the agent from infected animals as well as molecular methods (6, 12). Bacterial isolation can be done by cultivation of blood and secretions of infected animals and although it provides a definitive diagnosis, this procedure is laborious and time-consuming and may produce false-negative results.

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Serological tests are more practical, quick and easy to implement enabling the execution of a considerable number of samples. However most serological tests are not specific to *B. canis* and can give false-positive results. Molecular methods such as polymerase chain reaction (PCR) have been widely used for the diagnosis of canine brucellosis enabling the detection of DNA of bacteria in several samples (8).

The aim of this work was to determine the frequency of anti-*B. canis* antibodies in dogs attended in veterinary clinics from Patos, Paraíba State, in the Northeast region of Brazil, as well as to identify risk factors and to confirm the infection by microbiological culture and PCR.

Dogs ≥ 3 months-old (n = 193) assisted in the Veterinary Medical Center Dr. Leonardo Torres and in the Clinic of Small Animals of the Veterinary Hospital of the Federal University of Campina Grande, Patos, Paraíba, Brazil, from July 2008 to April 2009, were used and selected with the consent of the owners. To identify risk factors, epizootiological questionnaires with closed questions were supplied to each dog owner. These questionnaires were administered by the same interviewer.

Blood (4 mL) was collected without anticoagulant from each dog. Blood samples were centrifuged (2000 X g for 15 min) and sera were stored at -20 ^{o}C prior to testing. From seropositive animals, blood and bone marrow samples were collected. Blood (4 mL) was collected from each animal by jugular venal puncture with sodium citrate as anticoagulant. An aliquot (2 mL) was submitted to bacterial isolation. Bone marrow was collected by iliac crest puncture and 2 mL were used for bacterial isolation.

Sera were tested by AGID test using *Brucella ovis* surface antigen, produced in the Instituto Tecnológico do Paraná (Tecpar, Paraná, Brazil). The tests were performed according to the laboratory recommendations, except for the substitution of agarose by 1% agar Noble (Difco, Detroit, MI, USA).

Risk factors analysis was performed in two steps: univariate and multivariate analysis. Univariate analysis was performed using the Chi-square test or Fisher's exact test (13), and those variables that presented $p \leq 0.20$ were used for multiple logistic regression. The multivariate analysis was then performed, using the stepwise forward method (5). The significance level in multivariate analysis was 5%. The tests were performed using the SPSS for Windows software package, version 13.0.

For blood and bone marrow cultures 2 mL of blood with sodium citrate and 2 mL of bone marrow were inoculated in Castañeda medium and incubated at aerobic atmosphere (37ºC for 30 days) (1). After growth, colonies were cultured on *Brucella* agar plates and incubated at aerobic atmosphere at 37ºC for five days for bacterial identification. Genus characterization was performed using Gram staining and identification of the biochemical profile: catalase, oxidase, citrate, nitrate reduction, motility, H_2S production and urease (7).

DNA extraction of isolated bacteria was performed by boiling method (10, 11). A 2.3 x 10^9 bacteria/mL suspended in 1000 µL of sterile bi-distilled water, corresponding to 8 on the MacFarland scale, was heated for 10 min to 99 ^{o}C, and further used for PCR assay. DNA obtained was stored at –20 ^{o}C till amplification.

For the reaction primers B4 (5'-TGGCTCGGTTGCCAA TATCAA-3') and B5 (3'-CGCGCTTGCCTTTCAGGTCTG-5') were used to amplify a 223 bp sequence of the gene encoding the periplasmic immunogenic protein BCSP31 specific for *Brucella* spp. The amplification reaction mixture was prepared in a volume of 50 µL containing 200 µM of each deoxynucleoside tryphosphate, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl2, 0.5 mM of each primer, 1.5 U platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 µL of template DNA. The reaction was performed in a DNA thermal cycler (MJ Research PTC 200 DNA engine, Watertown, MA, USA) without mineral oil. Ultrapure water was used as negative control and *Brucella abortus* strain ATCC 544 as positive control. After an initial denaturation at 95^{o}C for 2 min, the PCR profile was set as follows: 30s of template denaturation at 95^{o}C, 30s of primer annealing at 62^{o}C and 30s of primer extension at 72^{o}C, for a total of 40 cycles, with a final extension at 72^{o}C for 5 min. The samples were analyzed by electrophoresis in a 2% agarose gel and then stained with ethidium bromide (0.5 mg/mL). The DNA bands were visualized under UV light.

Six animals tested positive at AGID test, resulting in a frequency of 3.11%. In the univariate analysis to determine risk

factors for *B. canis* infection, variables cleaning of the dog's environment and walking with the dogs were selected (Table 1). Risk factor identified in multivariate analysis by logistic regression was lack of cleaning of the dog's environment (odds ratio = 7.91, 95% CI = 1.50 - 41.72, p = 0.015), which is plausible from the biological standpoint, since the routine cleaning of the environment with common disinfectants may reduce the survival of bacteria eliminated by infected dogs in the environment, especially in case of parturition or abortion. Therefore, it is suggested that this practice must be commonly adopted in order to avoid exposure of other animals and humans to the risk of infection.

Table 1. Risk factors analysis to *Brucella canis* seroprevalence in 193 dogs from the Patos municipality, State of Paraíba, in the Northeast region of Brazil.

Variables	Sample size	Univariate analysis		Multivariate analysis		
		Seropositive (%)	p	Odds ratio	IC 95%	p
Owner education						
Illiterate	2	0 (0.0)				
1st – 8th grade	82	4 (4.9)				
Secondary	67	1 (1.5)				
Higher	42	1 (2.4)	0.666			
Sex of the dogs						
Female	83	2 (2.4)				
Male	110	4 (3.6)	0.701			
Age (months)						
3 – 6	14	0 (0.0)				
6 – 12	46	2 (4.3)				
12 – 24	44	2 (4.5)				
> 24	89	2 (2.2)	0.669			
Breed						
Mixed	110	3 (2.7)				
Pure	83	3 (3.6)	1.000			
Access to street						
No	140	5 (3.6)				
Yes	53	1 (1.9)	1.000			
Food						
Commercial	71	2 (2.8)				
Prepared at home	64	1 (1.6)				
Scraps	58	3 (5.2)	0.510			
Contact with other dogs						
No	93	4 (4.3)				
Yes	100	2 (2.0)	0.431			
Contact with wildlife						
No	181	6 (3.3)				
Yes	12	0 (0.0)	1.000			
Dog's environment						
Soil	106	4 (3.8)				
Cement	87	2 (2.3)	0.692			
Cleaning of the dog's environment						
Yes	169	3 (1.8)		1		
No	24	3 (12.5)	0.026	7.91	1.5 – 41.72	0.015
Abortion destination						
Throw away	189	6 (3.2)				
Burying/burning	4	0 (0.0)	1.000			
Walk with the dogs*						
No	72	4 (5.6)				
Yes	121	2 (1.7)	0.198			
Contact with ponds						
No	164	6 (3.7)				
Yes	29	0 (0.0)	0,594			

*Variables selected and used in the multivariate analysis

Bacteria with morphostaining and biochemical characteristics similar to *Brucella* spp. was isolated from bone marrow of one seropositive animal. *Brucella* DNA was extracted from isolated colonies using the boiling procedure and the extracted DNA was amplified using genus-specific primers for *Brucella* spp.

In this study, the agent was isolated from the bone marrow of one seropositive animal. Although blood is considered the material of choice for the isolation due to long period of bacteremia, *B. canis* can also locate in other lymphoid organs, so that it can be recovered by bone marrow aspirates in the absence of positive blood cultures (6).

The biochemical profile of the isolate was: catalase positive, oxidase positive, citrate negative, nitrate reduction positive, motility negative, H_2S production negative and urease positive. The results of biochemical tests are consistent with those obtained in other studies (3, 4, 7) with the exception of urease positive. Several studies of isolation of *B. canis* found some variations when biochemical tests were performed as Flores Castro et al. (2), which had samples that did not reduce nitrate. Larsson and Costa (9) examined 27 dogs, and 3 had positive blood cultures. When biochemical tests were performed the strains showed production of H_2S positive.

The findings described above indicate the presence of *B. canis* infection among pet dogs from Patos, Paraíba State, Brazil, and this work is the first to report the isolation of *B. canis* in dogs in the Northeast region of Brazil, with confirmation by PCR. Since human infection with *B. canis* has been reported (12), attention should be paid to possible human infection with this zoonosis through pet dogs and preventive measures must be taken to prevent the transmission. It is suggested that routine cleaning of the dog's environment should be adopted in order to avoid exposure of other animals at risk of infection.

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