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BRUCELLA SPP. ISOLATION FROM DOGS FROM COMMERCIAL BREEDING KENNELS IN SÃO PAULO STATE, BRAZIL

Lara B. Keid[1]*; Rodrigo M. Soares[1]; Zenaide M. Morais[1]; Leonardo J. Richtzenhain[1]; Sílvio A.Vasconcellos[1]

[1]Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil.

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ABSTRACT

Dogs from 12 commercial breeding kennels were submitted to clinical investigation and laboratorial tests for diagnosis of *Brucella* spp. infection. The sampling was carried out between April 2000 and February 2002 and the laboratorial tests employed were agar gel immunediffusion test (AGID) and blood culture. From 171 dogs examinated, 39 (22.8%) showed at least one clinical sign compatible with brucellosis, 58 (33.91%) were AGID positive and 24 (14.03%) were positive by blood culture. Gram negative bacterial cells with a biochemical pattern compatible with that of bacteria belonging to genus *Brucella* were isolated from blood specimens of 24 animals. According to *Kappa* index and McNemar test, the association between AGID and blood culture (k=0.360 with 95% of confidence interval; X^2=25.93, p=0.000), between AGID and clinical test (k=0.248 with 95% of confidence interval; X^2=6.11, p=0.013), and between blood culture and clinical examination (k=0.442 with 95% of confidence interval; X^2=6.76, p=0.009) were not statistically significant. Qui-Square test indicated no association of sex and the results of clinical examination (X^2=1.35 and p=0.2447), AGID (X^2=1.58 and p=0.2086) or bacterial isolation (X^2=1.48 and p=0.2230). Within 12 kennels, seven had at least one dog positive by blood culture and nine had at least one animal positive by AGID. The association of epidemiological data with direct and indirect methods of diagnosis is necessary to perform a definitive diagnosis of *Brucella* infection in dogs, as positive results by AGID can be consequence of non-specific reactions and must be confirmed by blood culture. Negative results by AGID must also be confirmed using direct methods of diagnosis or repeating the serologic test after 30 days, because of the low sensitivity of this test.

Key words: dogs, brucellosis, *Brucella canis*, diagnosis, isolation

INTRODUCTION

Canine brucellosis caused by *Brucella canis* is one of the major infectious causes of reproductive disorders in dogs. (4,5,6,7,13,15,16,29,35). Enlargement of lymph nodes, uveitis, diskospondylitis, polyarthritis, glomerulonephritis, osteomyelitis and pyogranulomatous dermatitis are clinical signs other than reproductive failure often associated with brucellosis in dogs (5,29). It is important to emphasize that some infected animals may be asymptomatic, being considered important sources of infection (16).

When brucellosis is introduced in a confined population, the infection spreads rapidly, leading to economic losses and risks for public health (17,25,30,32,35).

In Brazil, epidemiological surveys of brucellosis in dogs from animal shelters of zoonosis control divisions or companion pets have been often assessed by using slide agglutination test (SAR) or agar gel immunediffusion test

*Corresponding author. Mailing address: Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. Av. Prof. Dr. Orlando de Marques Paiva, 87. 05508-000, São Paulo, SP, Brasil. Tel.: (+5511) 3091 7704, Fax: (+5511) 3091 7928. E-mail: larakeid@yahoo.com.br

(AGID) (2,3,8,11,12,19,21,22,25,26,27,28,33,34). However, results of application of these methods for the diagnosis of brucellosis in commercial breeding kennels are rare.

Larsson *et al*. (19) tested 164 dogs from commercial breeding kennels in São Paulo, SP and observed 9.1% of positive animals by SAR and 2.4% by tube agglutination test (TAT). In four breeding kennels with history of abortion in metropolitan region of Botucatu, SP, Megid *et al*. (25) related seropositivity for *Brucella* infection in 12.0%; 4.6%; 31.0%; and 41.4% by AGID test. Mólnar *et al.* (27) testing 236 dogs from animal shelters of zoonosis control divisions and companion animals from urban and rural area from Belém, PA, found 45.37%; 38.56% and 46.14% of *Brucella* positive dogs, using AGID, complement fixation (CF) and ELISA respectively. The frequencies of positive dogs from commercial kennels were 40.32% by AGID, 25.80% by CF and 41.93% by ELISA.

The two rough species of *Brucella, Brucella canis* and *Brucella ovis,* share surface lipopolyssacharides (LPS) antigens, which can be used for the diagnosis of both canine and ovine brucellosis. Actually, the AGID test employing *B. ovis* LPS antigen has been extensively used to diagnose canine brucellosis. (4,7,25,27,28,34).

However, the tests mentioned above often give false positive results because the surface antigens are also common to other bacteria species such as *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Bordetella bronchiseptica* (4,6,7,16, 24,29,36). Also, AGID and SAR are tests of low analytic sensitivity (23,26), so they can fail to detect both early and chronic infections (4,29).

As a consequence of the inaccuracy of serological tests, bacteriological methods should always be used to confirm the diagnosis of *Brucella canis* infection. Several samples can be employed for the direct detection of *Brucella* in dogs with blood samples being the best specimens to be chosen because: (i) *Brucella* infected dogs usually have a prolonged period of bacteremia; (ii) this kind of sample is rarely contaminated by other microorganisms because venal puncture is a procedure less prone to contamination; and (iii) blood culture allows the diagnosis of early infections, when sera antibody levels are not yet detectable by serological tests (4,6,7,16,29). However, the success of *Brucella* isolation depends on the viability of the microorganism and also on the phase of the infection, so that negative results in these tests do not exclude the possibility of infection (4,7,16).

In Brazil, only few reports of *Brucella canis* isolation from dogs have been published. In Belo Horizonte, MG, Godoy *et al.* (12) observed a *B. canis* positive blood culture from a female stray dog presenting vaginal discharge. Larsson and Costa (18) testing 27 dogs by TAT and blood culture found five animals positive by agglutination test and three positive by blood culture. Vargas *et al.* (35) isolated *B. canis* from tissues of placenta, aborted and neonatal fetuses from two female dogs from a kennel in Uruguaiana, RS. Gomes *et al*. (13) isolated successfully *Brucella canis* from genital organs from a dog presenting orchitis and epydidimitis,

The objective of the present work is to report the occurrence of Canine brucellosis in commercial breeding kennels localized in São Paulo State in which the animals were submitted to clinical examination and laboratorial diagnosis using AGID and blood culture.

MATERIALS AND METHOD

Animals

A *Brucella* infection study was conducted in a total of 50 male and 121 female dogs of several breeds from 12 commercial breeding kennels localized in São Paulo State, Brazil (Table 1). Sampling and obtention of clinical data occured between April 2000 and February 2002.

Clinical examination

The dogs were submitted to clinical examination and the owners were asked to answer a questionnaire to record the following clinical data: abortion, conception failure, vaginal discharge, whelping of dead puppies, neonatal death, orchitis, epididymitis, lymph nodes enlargement and uveitis. Canine brucellosis was suspected if an animal presented at least one of the clinical signs.

Table 1. Characteristics of the dogs from commercial breeding kennels in São Paulo State, according to the location of the kennel and sex of the animals.

Kennel	Municipality	Number of dogs	Number of males	Number of females
1	Osasco/SP	17	17	0
2	Campo Limpo Pta. /SP	22	1	21
3	São Paulo/SP	18	5	13
4	Cotia/SP	14	4	10
5	Campo Limpo Pta. /SP	4	1	3
6	Jaú/SP	9	1	8
7	São Paulo/SP	10	7	3
8	Mogi das Cruzes/SP	15	2	13
9	Itu/SP	37	8	29
10	São Bernardo do Campo/SP	12	0	12
11	São Paulo/SP	5	2	3
12	São Paulo/SP	8	2	6
Total		171	50	121

Serologic diagnosis

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

Blood culture

Blood samples were collected in sodium citrate anticoagulant. The culture were performed as described by Alton *et al*. (1). Briefly, 2 mL of blood with sodium citrate was inoculated in Castañeda medium and incubated at aerobic atmosphere, at 37ºC for 30 days. After growth, colonies were cultured on *Brucella* agar plates and incubated at aerobic atmosphere at 37ºC for five days for bacterial identification. The genus characterization were performed using Gram staining and identification of the biochemical profile: catalase, oxidase, citrate, indole, nitrate, motility, fermentation in TSI medium and urease. For identification of *Brucella* at species level, tests for H_2S production and growth of colonies in the presence of thionin and basic fucsin stains diluted 1:50,000 and 1:100,000 from 0.1% stock solution were performed.

Statistical Analysis

The agreement between AGID test and blood culture was determined by *Kappa* indexes. The association between the results of each laboratorial test with the clinical data was evaluated by McNemar test. Qui-square was used to evaluate the association of the results obtained with clinical or laboratory examinations with the sex of the animal. The statistical analises were performed using the Dag_Stat software (20).

RESULTS

Among the 171 examinated dogs, *Brucella* spp. were detected in blood specimens of 24 animals (14.62%), 58 were (33.91%) AGID positive and 39 (22.80%) presented clinical signs of the disease. The frequencies of seropositive dogs observed in the commercial kennels ranged from 40% to 77.77% (Table 2). When blood cultures were performed, the frequencies of positive results ranged from 2.70 % to 54.54 % (Table 2).

The biochemical profile of the 24 isolates was: catalase positive, oxidase positive, nitrate reduction positive, H_2S production negative, fermentation in TSI media negative, indole negative, citrate negative, motility negative, urease positive. The growth pattern of the isolates in the presence of thionin and basic fucsin is presented in Table 4.

The concordance between AGID and blood culture using *Kappa* index, with 95% confidence interval indicated a fair agreement ($k = 0.360$). The association between AGID and blood culture results were not statistically significant ($X^2 = 25.93$, $p = 0.000$) as determined by McNemar test.

The association between the results of the laboratorial tests and clinical data were analyzed by McNemar test and *Kappa* index. The McNemar test indicated no concordance between the observation of clinical signs and the AGID results ($X^2 = 6.11$ and $p = 0.013$), nor between the presence of clinical signs and blood culture results ($X^2 = 6.76$ and $p = 0.009$). *Kappa* index indicated moderated agreement between clinical evaluation and blood culture (k=0.442, 95% confidence interval) and fair agreement between clinical evaluation and AGID (k=0.248, 95% confidence interval).

Using Qui-Square test, the sex of the animal could be associated neither with the results obtained with clinical examination nor with results of laboratory tests. The p values and X^2 of the statistical test for the associations of sex of the animal with results of clinical examination, AGID and blood culture were respectively: $p = 0.2447$, $X^2 = 1.35$ for clinical examination, $p = 0.2086$ and $X^2 = 1.58$ for AGID, and $p = 0.2230$ and $X^2 = 1.48$ for blood culture.

Table 2. Positivity for *Brucella* according to the method of detection.

Kennel	*Number of dogs*	*Clinically positive*[1]	*AGID positive*[2]	*Blood culture positive*[3]
1	17	0 (0 %)	7 (41.17 %)	0 (0 %)
2	22	13 (59.09 %)	16 (72.72 %)	12 (54.54 %)
3	18	7 (38.88 %)	14 (77.77 %)	5 (27.77 %)
4	14	4 (28.57 %)	1 (7.14 %)	1 (7.14 %)
5	4	1 (25.00 %)	2 (50 %)	0 (0 %)
6	9	1 (11.11 %)	6 (66.66 %)	0 (0 %)
7	10	4 (40.00 %)	4 (40.00 %)	0 (0 %)
8	15	6 (40.00 %)	6 (40.00 %)	4 (26.66 %)
9	37	1 (2.70 %)	0 (0 %)	1 (2.70 %)
10	12	1 (8.33 %)	0 (0 %)	1 (8.33 %)
11	5	0 (0 %)	2 (40.00 %)	0 (0 %)
12	8	1 (12.50 %)	0 (0 %)	0 (0 %)
Total	171	39 (22.80 %)	58 (33.91 %)	24 (14.03 %)

1: Number and frequency of dogs with at least one clinical sign compatible with *Brucella* infection; 2: Number and frequency of dogs positive by agar gel immunediffusion test (AGID); 3: Number and frequency of dogs positive by blood culture.

DISCUSSION

With regard to the resistance to thionin and basic fucsin, 18 from the 24 isolates had a biochemical pattern similar to *B. canis* of Canadian or Mexican origin, as described by Forbes and Pantekoek (10)

and by Gomes *et al*. (13). The remaining isolates had a biochemical pattern compatible with *Brucella canis* RM6/66 reference strain (1), which was also observed by Larsson and Costa (18) and Godoy *et al.* (12) (Table 4).

Although *Brucella* species have some degree of host specificity and most of the investigated kennels presented positive dogs by AGID for rough species, the likelihood of other species of *Brucella* being responsible for the infections should not be discarded, specially in those populations where positive AGID results were not observed (9,13). Moreover, thionin and fucsin resistance should not be taken as a gold standard to classify *Brucella* at the species level, as some strains of *B. suis* and *B. abortus* could present growth patterns similar to *B. canis* (1).

In three from 12 kennels, there was at least one animal presenting clinical signs of brucellosis, one animal AGID positive and none blood culture positive (Table 3). Because of the low accuracy of the serological tests, the absence of microbiological isolation in these kennels leads to two possible conclusions: either the occurrence of non-specific reactions of AGID or the presence of chronically infected but abacteremic dogs, that shelter the bacteria in other organs (4,6,16,24,29). In kennels having at least one AGID positive and no blood culture negative dog, *Brucella* infection can not be confirmed, with or without clinical signs (Table 3).

In two from 12 kennels, there was at least one AGID positive animal, but neither *Brucella* spp. could be isolated nor clinical signs compatible with brucellosis could be assigned (Table 3). In this case, the results can also be a consequence of either animals presenting chronic and abacteremic infection or non-specific AGID results (4,7,16,29).

Four kennels presented dogs showing clinical signs of brucellosis and with positive AGID and blood culture (Table 3). Even though the isolation of microorganism from at least one animal from a suspected population of dogs confirms *Brucella* infection, it does not mean that all AGID positive but blood culture negative dogs were really infected mainly because of the low specificity of the serological test (4,7,16,29,36). In a situation like that, it is imperative to considerate the herd management, as the kennels in which the dogs are kept in collective pens or whose dogs have frequent external mating, the risk of dissemination of the infection is higher when compared with populations in which the animals are kept in individual pens and the exchange of dogs is not practiced frequently (6,25).

In all kennels where *Brucella* spp. was isolated from at least one dog (6/12), clinical signs of the infection were observed at least once, irrespectively the results of AGID (Table 3). However statistical correlation between results of clinical examination and blood culture revealed no association (McNemar test) and a moderate agreement (*Kappa* index). This is indicative that the presence of clinical signs is not enough to evidence *Brucella* infection at a herd level.

From the six kennels in which *Brucella* were isolated, two have no AGID positive animals. This information suggests that the AGID test lacks sensitivity and the AGID negative/blood culture positive dogs were possibly in early infection, when sera antibodies did not achieve detectable level (4,7,16).

Although AGID is considered a good screening test to be applied for diagnosis of canine brucellosis at the population level, the results presented here indicate that negative results

Table 3. Distribution of the kennels according to the positivity for *Brucella* spp. by the three detection methods.

Results of clinical and laboratory investigation[1]	*Number of kennels*
AGID positive, blood culture positive, with clinical signs	4
AGID positive, blood culture positive, without clinical signs	0
AGID positive, blood culture negative, with clinical signs	3
AGID positive, blood culture negative, without clinical signs	2
AGID negative, blood culture negative, without clinical signs	0
AGID negative, blood culture positive, with clinical signs	2
AGID negative, blood culture positive, without clinical signs	0
AGID negative, blood culture negative, with clinical signs	1
Total	12

1: Key for considering a kennel positive by AGID, blood culture or the presence of clinical signs compatible with brucellosis; AGID positive: kennel with at least one animal positive by AGID; AGID negative: kennel with all animals negative by AGID; blood culture positive: kennel with at least one animal positive by blood culture; blood culture negative: kennel with all animal negative by blood culture; with clinical signs: kennel with at least one dog showing at least one clinical sign compatible with *Brucella* infection; without clinical signs: kennel with no dog showing no clinical sign compatible with *Brucella* infection.

Table 4. Growth pattern of 24 *Brucella* spp. isolates in medium containing thionin and basic fucsin.

Number of *Brucella* isolates	Thionin		Basic Fucsin	
	1:50,000	1:100,000	1:50,000	1:100,000
18	positive	positive	positive	positive
6	positive	positive	negative	negative

obtained with AGID test must be carefully interpreted, specially in kennels where clinical signs of brucellosis are observed. (7,29,31). From the results, it may be inferred that AGID has not a satisfactory performance to be chosen as a screening test for the detection of *Brucella* infection in dogs.

As a proposal to control *Brucella* infection in commercial kennels, we suggest careful analysis of the results obtained with serodiagnosis, bacteriological assays and clinical examination of the dogs, so that the presence of a single blood culture positive dog confirms the infection in a suspected population (16). In a case such like that, the remaining dogs (AGID positive/blood culture negative) should also be considered infected and removed from the kennel, with the aim of minimizing the risk of transmission and reducing the infection prevalence in the population. However, it should be in mind that a considerable number of non infected animals can be eliminated from the population.

Negative results obtained by AGID test should also be confirmed, because of the low sensitivity (23,26) and consequently the low accuracy to detect early infections (4,7,16). Again, it is stressed the importance of the concurrent use of direct and indirect methods for the diagnosis of *Brucella* infections. Nevertheless, we emphasize that the absence of positive dogs by bacterial isolation in kennels where serologically positive dogs are observed do not mean that *Brucella* infection is absent. In a situation like that, serological and bacteriological tests must be repeated each 30 days for infection surveillance.

When brucellosis is detected in a kennel, quarantine and monthly serological and bacteriological monitoring for four to five months, associated with rigorous disinfection are essential to control the infection (16).

Considering the difficulties mentioned above, it is clear that the association of direct and indirect laboratorial tests with clinical and epidemiological data is essential to perform a definitive diagnosis of brucellosis.

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RESUMO

Detecção de *Brucella* em cães provenientes de canis comerciais do estado de São Paulo, Brasil

Cães provenientes de 12 canis comerciais do estado de São Paulo foram submetidos à investigação clínica e a provas laboratoriais para o diagnóstico de infecção por *Brucella* spp. A colheita de amostras foi realizada entre os meses de abril de 2000 e fevereiro de 2002 e os exames laboratoriais empregados foram a imunodifusão em gel de ágar (IDGA) e a hemocultura. De 171 cães examinados, 39 (22,80 %) apresentaram pelo menos um sinal clínico compatível com brucelose, 58 (33,91%) foram positivos pela IDGA e 24 (14,03%) pela hemocultura. Bactérias Gram negativas com perfil bioquímico compatível com o gênero *Brucella* foram isoladas das 24 amostras de sangue positivas pelo isolamento bacteriano. De acordo com o coeficiente *Kappa* e o teste de McNemar, não foi observada concordância entre os resultados obtidos na hemocultura e IDGA (k=0,360 com intervalo de confiança de 95%; X^2=25,93, p=0,000), entre resultados da IDGA e do exame clínico (k=0,248 com intervalo de confiança de 95%; X^2=6,11, p=0,013) e entre os resultados da hemocultura e do exame clínico (k=0,442 com intervalo de confiança de 95%; X^2=6,76, p=0,009). A associação dos resultados obtidos pelos exames clínicos e laboratoriais com o sexo dos animais não foi estatisticamente significante (Qui-Quadrado), sendo observado X^2=1,35 e p=0,2447 para o exame clínico, X^2=1,58 e p=0,2086 para IDGA e X^2=1,48 e p=0,2230 para hemocultura. Dos 12 canis examinados, sete apresentaram pelo menos um animal positivo pela hemocultura e nove pelo menos um animal positivo pela imunodifusão. A associação de dados epidemiológicos com testes laboratoriais diretos e indiretos deve ser enfatizada para o diagnóstico definitivo da brucelose canina. Resultados positivos pela imunodifusão em gel de ágar podem ser conseqüência de reações inespecíficas e devem ser confirmados pela hemocultura. Os resultados negativos obtidos pela imunodifusão também devem ser confirmados utilizando-se métodos diretos de diagnósticos ou repetindo-se o teste sorológico com 30 dias de intervalo, devido à baixa sensibilidade desse teste diagnóstico.

Palavras-chave: cães, brucelose, *Brucella canis*, diagnóstico, isolamento

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