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Usefulness of Urine as a Sample for Detection of *Brucella* Spp in Male Canines

Boeri EJ[1*], Lopez G[2], Guido GG[1], Ciocca M[1], Elena SC[3] and Wanke MM[4]

[1]*Zoonosis Institute Dr. Luis Pasteur, Av. Diaz Velez 4821 (1405) Buenos Aires, Argentina*

[2]*Faculty of Agricultural Sciences, National University of Lomas Zamora, Ruta 4 Km 2,5, Pcia Buenos Aires, Argentina*

[3]*National Health Service and Food Quality (SENASA-DILAB) OIE/ Brucellosis Reference Laboratory Talcahuano 1660 (1640), Martínez, Buenos Aires, Argentina*

[4]*Faculty of Veterinary Science, Theriogenology Area, Chorroarín 280 (1427) Buenos Aires, Argentina*

[*]**Corresponding author**: Eduardo J Boeri, Zoonosis Institute Dr. Luis Pasteur. Av Diaz Velez 4821 Ciudad Autónoma de Buenos Aires República, Argentina, Tel: +54 11 4958-9941; E-mail: eduardoboeri@hotmail.com

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Abstract

Urine was used as a sample and Sensitivity (S), Specificity (Sp) and the positive Likelihood Ratio (LR+) of molecular and serological methods, combined with epidemiology and the clinical symptoms for detection of *Brucella spp*., were compared in blood and urine samples from 241 male canines. The rapid slide agglutination test together with 2-mercaptoethanol (2-ME RSAT) were used as a screening test, followed by confirmation using an indirect immunoenzymatic assay (iELISA) and bacteriological culture. Results were as follows: Test a) PCR (Polymerase Chain Reaction) of blood compared to blood culture: S 80%, Sp 92%, LR+ 10.32 (CI 5.27-19.20) test b) iELISA compared to blood culture: S 100%, Sp 94%, LR+: 16.57 (CI 9.97-27.53), test c) PCR of urine compared to urine culture: S 100%, Sp 93% (CI 8.36-21.56), LR+: 13.64 (CI 8.36-21.56) test d) iELISA compared to urine culture: S 100%, Sp 93%, LR+: 14.5 (CI 9.03-23.26). We conclude that molecular and serological tests in conjunction with epidemiology are both useful for diagnosis and that both blood and urine samples should be assayed together.

Keywords: *Brucella canis*; Urine PCR; *Brucella spp* urine detection; Male canine urine; *Brucella canis* epidemiology; Serology iELISA

Introduction

Brucellosis is an infectious bacterial disease produced by gram-negative coco bacilli belonging to the genus *Brucella* with a sub-acute to chronic course. Although canines can be affected by various species of *Brucella*, the most specific and epidemiologically important is *Brucella canis*. *Canine brucellosis* is characterized by infertility with abortions and genital discharges as the main symptoms in females, and epididymitis and orchitis in males. General symptoms in both sexes include disco spondylitis, uveitis and generalized lymphadenopathy. Transmission may be venereal (during mating), through the placenta or by ingestion or contact with infected materials such as abortions and secretions. Routes of elimination are: urine [1-4], especially in males, semen, abortions and pathological vaginal discharges.

Laboratory diagnosis includes: a) indirect tests based on the detection of antibodies in serological tests such as 2-mercaptoethanol Rapid Slide Agglutination Test (2-ME-RSAT), indirect Enzyme-Linked Immuno Sorbent Assay (iELISA) and agar gel immune-diffusion (AGID) which are all quick and relatively easy to perform; b) direct tests such as bacterial isolation and molecular methods such as Polymerase Chain Reaction (PCR).

This disease has been studied in depth in females, since abortion is the most obvious and evident sign. In addition, postpartum vaginal fluids are the most common source of human and animal infection due to the large number of germs present (10^{10} bacteria/ml). The zoonotic potential has been reported in immunosuppressed patients and in family outbreaks [5,6]. In addition, the risk of infection with *Brucella canis* has been considered fairly high for people who handle dogs in breeding kennels or who are exposed to infected animals [7]. The prevalence and clinical importance of *B. canis* has been underestimated so far because of the difficulties in primary isolation and differentiation [6].

After the initial work of Serikawa and Carmichael on the importance of urine in environmental dissemination of *B. canis*, only one study on brucellosis in humans and two in canines (one of which was from Argentina) referring to the presence of bacteria in urine have been published [8-10].

Male dogs shed *B. canis* in semen and urine, in quantities oscillating between 10^6 and 10^3 to 10^6 bacteria/ml, respectively. This shows the importance of male urine as a source of infection in the human environment. The literature recommends bladder puncture to obtain urine for cultures [1,11]. However, sometimes this is not possible either because of the resistance of the owners to have this technique performed on their pets or because of animal health and welfare recommendations [12].

Reports comparing serological, bacteriological and molecular tests in blood, semen and vaginal swabs for diagnosis of canine brucellosis have been published [13,14], but no similar studies have been carried out in urine. Because of the importance of using this type of sample for diagnosing the disease, the aim of this study was to compare the Sensitivity, Specificity and positive Likelihood Ratios of molecular and serological testing combined with Epidemiology, between urine and blood samples used to detect *Brucella spp*. in male canines.

Materials and Methods

An observational comparative cross-sectional study was undertaken between August 2012 and September 2014. Dogs that were taken to a mobile Surgery Service or taken for surgery to the Luis Pasteur Zoonosis Institute (IZLP) of the City of Buenos Aires or to the Anti-rabies Center of Lomas de Zamora (a suburb of Buenos Aires) were studied. Samples were taken from a total of 241 male canines, between 8 months to 7 years of age, which were either clinically healthy or showed clinical symptoms compatible with canine brucellosis. All dog owners gave a written consent for the taking and processing of the samples. Any canines under antibiotic treatment or without clinical signs compatible with brucellosis were not included. Samples were taken from every male canine that met the inclusion criteria, in order of arrival to the establishments.

Epidemiological data

A file card was prepared for each animal indicating: name, address and telephone number of the owner or responsible holder, and the length of tenure. Data on the pet included: age, breed, habits (domiciliary: stayed at home or left only on a leash; peri-domiciliary: left the home under supervision; vagabond: had no owner and was loose on the streets), symptoms compatible with brucellosis and records of mating. Samples were taken after completing the physical examination and the animals were anesthetized for surgery, thus ensuring a correct restraint and minimal discomfort. Canines with any epidemiological link were considered at risk for canine brucellosis (e.g. a history of untested mating, peri-domiciliary habits, vagabonds, dogs adopted from the street, originating from infected breeding kennels or animal shelters, with a history of cohabitation with positive animals) or with clinical signs such as orchitis, epididymitis, scrotal dermatitis, testicular atrophy or spinal pain.

Sampling

Blood was obtained from the external jugular vein (5 ml) and after changing the needle for a new sterile one, first 2 ml were placed, under cover of a flame to minimize the risk of contamination, in a *Brucella* tryptose phosphate broth with the addition of 2% sodium citrate and kept at room temperature. Next, for serology, 2 ml were placed in a clean dry tube and left to coagulate for 20 minutes to avoid hemolysis. The sample was then centrifuged to separate the serum, which was kept at -20ºC until processing. Finally, 1 ml was placed in an RNAase-free tube with 200 µl of 2.5% sodium citrate to prevent clotting and was chilled until processing for PCR. Urine samples (5 ml) were obtained using a sterile K-30 catheter, placing 2 ml in a sterile Falcon tube for uroculture and 3 ml were placed in RNAase-free tubes and maintained in a refrigerator (4ºC) until processing for PCR.

Diagnostic methods

Serological tests: BPA (buffered plate antigen test) to detect anti-SLPS *Brucella spp*. antibodies (*B. abortus*/S1119-3) according to the standard procedure described by Angus and Baton in 1984 [15].

2-ME RSAT (rapid slide agglutination test) as a screening test to detect anti-*Brucella* rough strain antibodies [16]. The assay is produced by DILAB-SENASA and is carried out according to George and Carmichael using the *B. canis* M-strain [17].

Indirect ELISA (indirect Enzyme-linked immune-sorbent assay) used as a confirmatory test, carried out according to Lucero et al. [18] considering a sample positive when optical density cut off values were equal to or greater than 0.28.

Molecular tests: Whole blood and urine were used for DNA extraction using extraction columns for PCR. DNA was obtained using a High Pure PCR Template Preparation Kit Version 2.0 content version (October 20, Roche Diagnostics, GmbH Roche Applied Science, Germany) following the manufacturer's instructions. Detection of *Brucella spp*. was performed by PCR amplifying the region BCSP31 since this locus is highly conserved in all *Brucella species* [19,20]. The master mix reaction was carried out as follows: 15 µl ultra-pure water, 200 µM desoxynucleoside tryphosphate, 5 µl Colorless GoTaq® Reaction Buffer containing 1.5 mM $MgCl_2$ (final concentration), 0.5 µM of each primer B4/B5 (Forward 5'-TGGCTCGGTTGCCAATATCAA-3'/Reverse 5`-CGCGCTTGCCTTTCAGGTCTG-3´) and 1U GoTaq® DNA Polymerase (Promega). DNA of *B. canis* RM6/66 was used as the positive control and ultrapure water as the negative control. The reaction was performed using a thermal cycler (Techne, model number TC3000, Bibby Scientific Ltd., United Kingdom). After initial denaturation at 95ºC for 5 min, the PCR protocol was: 60 s of template denaturation, 60 s of primer annealing at 65ºC, and 60 s of extension at 72ºC (total 35 cycles), with a final extension at 72ºC for 10 min. Samples were analyzed by electrophoresis in a 1.5% agarose gel using a TAE (Tris acetate edta buffer) stained with ethidium bromide (0.5 µg/ml) and 223 bp DNA bands were visualized under UV light. PCR Mini beta actin was used as an internal control, whose band was 86 pb Forward 5'- GAG ACC TTC AAC ACC CCA G-3'/Reverse 5'- ATC ACG ATG CCA GTG GTA C-3' [21].

Microbiological cultures: Bacterial isolation was used in this study as the gold standard for confirmation of definitive diagnosis of *Brucella* spp.

Blood cultures: incubated for 30 days at 37ºC in an atmosphere supplemented with 10% CO_2 and transferred weekly to solid media [22].

Urine cultures: One aliquot of urine (0.1 ml) was spread onto solid media and the other aliquot (0.1 ml) was inoculated into liquid medium; then both aliquots were incubated in the same conditions as the blood cultures.

Diagnostic methods combined with epidemiological data

Due to the relevance of epidemiological data, diagnostic criteria were combined as detailed in Tables 1 and 2.

Epidemio	PCR blood	PCR urine	Diag *Brucella*
Pos	Pos	Pos	Pos
Pos	Neg	Pos	Pos
pos	Pos	Neg	Pos
Pos	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Pos	Mon
Neg	Neg	Pos	Mon
Neg	Neg	Pos	Mon

Table 1: Epidemiology plus symptoms/molecular tests, Canines with a combination of PCR positive and epidemiology were considered positive, since the presence of DNA in the sample tested could indicate contact with *Brucella* spp. Animals whose samples had only a positive PCR diagnosis were considered for monitoring.

Epidemio	RSAT	iELISA	Diag *Brucella*
Neg	Neg	Neg	Neg
Neg	Pos	Pos	Pos
Neg	Pos	Neg	Neg
Neg	Neg	Pos	Pos
Pos	Pos	Neg	Pos

Pos	Neg	Pos	Pos
Pos	Neg	Neg	Neg
Pos	Pos	Pos	Pos

Table 2: Epidemiology plus symptoms/serological test, Canines with a combination of at least two of the three positive factors and those with only a positive iELISA were considered positive since this test was confirmatory.

Statistical analysis

Data were entered into a database (Microsoft Excel 97) and were then analyzed with the statistical package (SPSS 19, VCC stat Epidat 2.0 and 3.1). Estimates of the 95% confidence interval (CI 95) were carried out. Significance level was set at $P<0.05$. To estimate the usefulness of the diagnostic methods, screening values were estimated: Sensitivity (S), Specificity (Sp), positive Likelihood Ratio (LR+) with their respective Receiver Operating Characteristics (ROC) curves and estimates of the area under the corresponding curves, using the DeLong test. LR+ was estimated as the sensitivity divided by 1 minus the specificity (LR+=S/ (1-Sp)) [23]. Kappa coefficient and Altman's level of concordance classification were used [24,25].

Results

Of the 241 male canines tested, 30 were reactive to at least one of the different tests carried out, showing an overall prevalence of 12.44% (CI: 8.54-17.30). Individual results of each test were as follows: 73.3% (22/30) were positive to 2Me RSAT, 66.6% (20/30) were positive to iELISA, 13% (4/30) were isolated using blood culture, 10% (3/30), were isolated using urine culture, 23% (7/30) were positive to blood PCR and 70% (21/30) were positive to urine PCR. An epidemiological link was observed in 80% (24/30) and only 13% (4/30) had symptoms consistent with canine brucellosis. Results can be seen in Table 3.

CAN	BPA	2me-RSAT	iELISA	Blood Culture	Urine Culture	Blood PCR	Urine PCR	EPI	Symptoms
1	Neg	Pos	0.87	Neg	Neg	Pos	Pos	Yes	Ws
2	Neg	Neg	0.12	Neg	Cont	Pos	Pos	Yes	Ws
3	Neg	Pos	0.99	Pos	Neg	Neg	Pos	Yes	Ws
4	Neg	Pos	0.80	Pos	Neg	Pos	Pos	Yes	Ws
5	Neg	Pos	0.34	Neg	Neg	Neg	Pos	Yes	Ws
6	Neg	Pos	0.99	Neg	Neg	Neg	Pos	Yes	Ws
7	Neg	Pos	0.16	Neg	Neg	Neg	Pos	Yes	Ws
8	Neg	Pos	0.29	Neg	Neg	Neg	Pos	No	Ws
9	Neg	Neg	0.12	Neg	Neg	Neg	Pos	Yes	Ws
10	Neg	Neg	0.18	Neg	Neg	Neg	Pos	Yes	Ws
11	Neg	Pos	0.99	Neg	Neg	Neg	Neg	No	Ws
12	Neg	Neg	0.10	Neg	Neg	Neg	Pos	Yes	Ws
13	Neg	Pos	0.99	Neg	Neg	Neg	Neg	Yes	Ws
14	Neg	Pos	0.40	Cont	Cont	Neg	Neg	Yes	Ws
15	Neg	Pos	0.99	Neg	Neg	Pos	Pos	Yes	Ws
16	Neg	Pos	0.16	Neg	Neg	Neg	Neg	Yes	Ws
17	Neg	Pos	0.90	Neg	Pos	Pos	Pos	Yes	Ws
18	Neg	Pos	0.99	Pos	Pos	Neg	Pos	Yes	À t
19	Neg	Pos	0.13	Cont	Neg	Neg	Neg	No	Ws
20	Neg	Neg	0.15	Neg	Neg	Neg	Pos	Yes	Ws
21	Neg	Pos	0.40	Neg	Neg	Pos	Neg	Yes	Ws
22	Neg	Pos	0.99	Pos	Pos	Neg	Pos	Yes	E
23	Neg	Pos	0.89	Neg	Neg	Neg	Neg	No	Ws

24	Neg	Pos	0.50	Neg	Neg	Neg	Pos	Yes	At
25	Neg	Pos	0.32	Neg	Neg	Neg	Pos	Yes	Ws
26	Neg	Pos	0.40	Neg	Neg	Neg	Neg	No	Ws
27	Neg	Neg	0.15	Neg	Cont	Neg	Pos	Yes	Ws
28	Neg	Pos	0.32	Neg	Neg	Neg	Neg	No	Ws
29	Neg	Neg	0.18	Neg	Neg	Pos	Pos	Yes	Ws
30	Neg	Neg	0.29	Neg	Neg	Neg	Pos	Yes	E

Table 3: Serological, Bacteriological and Molecular results of reagents of these test plus epidemiology and symptoms

Anti-SLPS *Brucella spp.* antibodies were ruled out, as all animals studied were negative to the BPA test.

Table 4 shows the values of LR +, S and SP by PCR in blood, urine and serology, contrasted with urine and blood cultures showed similar values of S, Sp and LR +. LR + was greater than 10 in all cases studied (Table 4 and Figure 1).

Test		**Blood culture**			**Test**		**Urine culture**		
	S	**SP**	**LR+**	**n=237**		**S**	**SP**	**LR+**	**n=235**
Blood PCR	80%	92%	10.31		Urine PCR	100%	93%	13.64	
CI 95	28-100	88-95	5.52-19.23		CI 95	29-100	89-96	8.36-21.56	
iELISA	100%	94%	16.57		iELISA	100%	93%	14.05	
CI 95	48-100	90-97	9.97-27.53		CI 95	29-100	89-96	9.03-23.26	

Table 4: Results from PCR and serology compared to blood and urine culture results. The values of S, SP, LR+ are shown with their respective CI. Note that 6 urines and 4 contaminated blood cultures were not included.

Discussion

When considering a presumptive diagnosis in any infectious disease, compatible clinical signs and symptoms are always taken into account. In our study only 13% of positive animals had compatible clinical symptoms, which is consistent with previous studies from our group [26,27]. In fact, none of the symptoms of brucellosis are pathognomonic, but can be due to other diseases and many cases of Brucellosis cannot be diagnosed with only the history or the physical examination [11]. *Brucella* excretion is intermittent in some dogs [3] therefore urine cultures in whole animals (non-castrated) are useful in animals in which the blood culture was negative. Due to the characteristics of the sample, urine should be processed quickly and the use of enriched media is necessary [25]. Other authors have reported that the bacteria can be isolated in urine despite not finding it in blood samples [7,11,28], which is consistent with some of our results (Table 3). One interesting finding of this study was the small number of contaminated samples obtained by catheterization, which would contradict the need for bladder puncture recommended by various different authors. This finding could trigger future research.

Our study also found PCR reactive samples taken from genital discharges in dogs with negative serology and, in some cases also with negative blood cultures. These findings are consistent with previous [26] studies comparing traditional diagnostic methods with PCR (Table 3) and could be due to the presence of bacterial DNA in animals that have been in contact with sick animals but have not received a sufficient dose of bacteria to develop the disease and form detectable antibodies. In these cases, to establish a definitive diagnosis it is necessary to perform a longitudinal analysis to verify the evolution of the results of different tests. While a PCR result does not indicate the viability of the microorganism (as this test only detects DNA) detection of bacterial presence in urine would suggest that the animal is eliminating live or dead bacteria, or at the very least, traces of bacterial DNA.

Conclusions

The values of S, Sp and LRV+ in urine and blood tests show overlapping CI, therefore we can say that both samples are useful for diagnosis of canine brucellosis. The index of agreement between molecular tests of blood and urine was weak; therefore, with the results from our study, we cannot state that either of the two samples was useful on its own; hence we recommend the use of both types of samples together for brucellosis testing. We conclude that our study provides evidence of the usefulness of the urine sample for the diagnosis of canine brucellosis as much as that of blood samples.

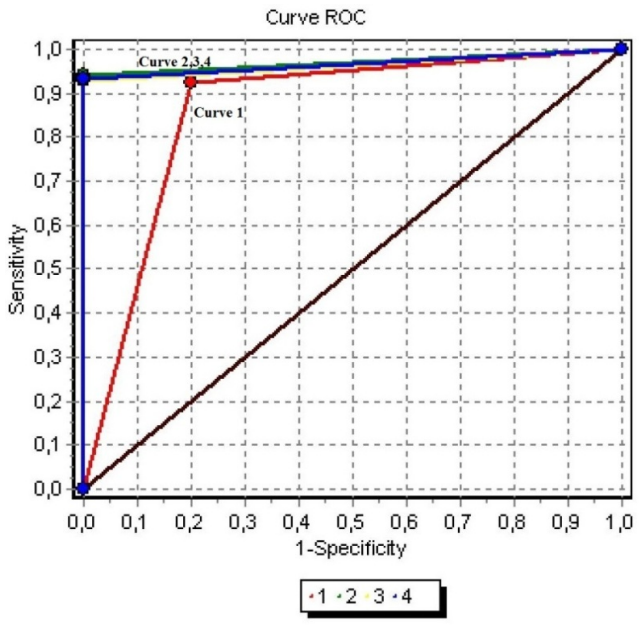

Figure 1: This curve shows the LR+ of all diagnostic tests. Comparison of these four curves shows that the area under ROC curve is higher in number 2,3,4 than number 1.

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