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Research article

EXAMINING THE POSSIBILITY OF DETECTING *BRUCELLA CANIS* FROM TISSUE SAMPLES USING BRUCE-LADDER MULTIPLEX PCR ASSAY

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The goal of this study was to compare the results of serological and conventional bacteriological methods with the results obtained using multiplex PCR Bruce–ladder assay. Based on the obtained results, the usability of the assay was assessed in regard to rapid diagnosis of canine brucellosis directly from the samples of reproductive organs of infected dogs. Out of 225 blood samples, 33 (14.67%) had a positive agglutination reaction. In this study, out of the 225 assayed reproductive organs of dogs, *B. canis* was isolated from 3 samples (1.33%), while the PCR Bruce-ladder assay detected two positive samples (0.88%). Two dogs from which *B. canis* was isolated, an antibody titer of 1/200 was established in blood serums, and third dog from which *B. canis* was isolated was negative using the tube agglutination test. From a total of 225 assayed organ samples, a positive PCR reaction was obtained from two samples. The obtained results show that the tube agglutination method remains the first choice for the detection of dogs infected with *B. canis*. In addition, whenever possible, it is necessary to try isolation. It is desirable to attempt the detection of *B. canis* in tissues using PCR, but the results may not be treated as definitive and reliable.

Key words: *Brucella canis*, Bruce-ladder, dogs, reproductive organs

INTRODUCTION

Diagnosis of canine brucellosis is mainly carried out by using serological tests. What makes the circumstances more difficult is that when it comes to serological diagnosis of brucellosis, a cross-reaction is very common due to antigenic similarities of *Brucella canis* (*B. canis*), *Pseudomonas aeruginosa*, encapsulated strains of *Staphylococcus* spp. and *Bordetella bronchiseptica*. In addition, at the beginning of the infection, before

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the occurrence of active immunity, serological tests have no value. Commercial ELISA test kits for the diagnosis of brucellosis in dogs have not been developed and standardized yet. Given the shortcomings and possible inaccuracies of the results obtained through serological tests, classical isolation is preferred, as the most accurate and definitive confirmation of the presence of pathogens. However, due to the low sensitivity, and difficulties in identifying and differentiating *B. canis* from other *Brucella* species, application of classical bacteriological methods has limited value. The isolates must be sent to reference laboratories for definite identification, which significantly prolongs the duration of diagnosis and makes it more expensive. The introduction of molecular methods in clinical microbiological practice increased the possibility of rapid and accurate detection of *Brucella* species. Multiplex PCR Bruce-ladder assay [1,2] was recommended by the OIE (*World Health Organization for Animals*) as a quick and easy test for molecular identification and typing of more isolated *Brucella* species [3]. The inadequacy of the multiplex PCR Bruce-ladder assay is the impossibility to precisely identify *B. canis*, because it can be incorrectly identified as *Brucella suis* [2].

Based on our knowledge, there are no published papers on the attempts to identify *B. canis* directly from the tissues using Bruce–ladder assay. The goal of this study was to compare the results of serological and conventional bacteriological methods with the results obtained using multiplex PCR Bruce–ladder assay. Based on the obtained results, the usability of this assay was assessed in regard to rapid diagnosis of canine brucellosis directly from the samples of reproductive organs of infected animals.

MATERIALS AND METHODS

Sample collection

The test samples originated from a total of 225 stray dogs. Animals included in this study were neutered at the Faculty of Veterinary Medicine, University of Belgrade as part of the program for the control and reduction of the stray dog population in the city of Belgrade. Testes, uteri and blood samples were taken from the neutered dogs. The study was approved by Decision no. 323-07-03455/2015-05/1 of the Veterinary Department at the Ministry of Agriculture and Environment Protection of the Republic of Serbia.

Tube agglutination test (TAT)

Serum samples were tested using the method of slow serum agglutination. The sera were diluted 1/50, 1/100, and 1/200 with 3.5% NaCl and with the addition of formaldehyde saline solution and 2–mercaptoethanol (ICN Biomedicals Inc., Ohio). The antigen for slow serum agglutination was prepared from the reference *B. canis* RM 6/66 strain (received from The Military Medical Academy in year 2000.) according to the previously described procedure [4] and was kept at a temperature of +4 °C until its use. The interpretation of the results was carried out as recommended [4] so that the

serums with a positive agglutination reaction in titers of specific antibodies of 1/50 and 1/100 were denoted as suspicious, while positive agglutination with antibodies titer of 1/200 was considered a positive reaction [5]. As a positive control, serum of a definitely positive dog was used, in which *B. canis* was isolated (typified by the reference laboratory in France in 2004. – AFSSA – Agence Française de sécurité sanitaire des aliments) and in whose serum the presence of specific *B. canis* antibodies were found in a titer of 1/3200. As a negative control, serum derived from a healthy dog was used.

Bacterial isolation

Testis and uterus samples were homogenized in the Bag Mixer 400P apparatus (Interscience, France), measuring 8 strokes/s and diluted with sterile normal saline at a ratio of 1:2 (organs : normal saline). Thus, prepared samples were inoculated on *Brucella* agar (Torlak, Serbia) with the addition of *Brucella Selective Supplement* (Oxoid, UK) in Breed-Demeter bottles with Castaneda biphasic medium. The incubation was carried out at 37 °C in aerobic conditions for a period of 10 days. In order to confirm that the received isolates belong to the *Brucella* spp. the following were applied: rapid agglutination of colonies to the plate with serum positive to *B. canis*, production of H_2O_2 (catalase test), urease test on Christensen urea medium (Torlak, Serbia), acriflavine agglutination test (Sigma-Aldrich, USA), thionine resistance test (Sigma-Aldrich, USA) and basic fuchsin test (Sigma-Aldrich, USA), and H_2S production test. In addition, the API 20 NE (bioMerieux, France) was used.

DNA extraction

Extraction of DNA from the organ homogenate was performed using a commercial kit for the extraction GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). Extracted DNA samples were tested using multiplex PCR Bruce-ladder assay described by García-Yoldi et al. and López-Goñi et al. [1,2].

Multiplex PCR Bruce-ladder assay

After preliminary tests, the protocol was modified by adding 10 µl of extracted DNA in place of 1 µl DNA to the PCR mix, and the total amount of PCR mixture was 50 µl. The following primers were used: BMEI0998f, BMEI0997r, BMEI0535f, BMEI0536r, BMEII0843f, BMEII0844r, BMEI1436f, BMEI1435r, BMEII0428f, BMEII0428r, BR0953f, BR0953r, BMEI0752f, BMEI0752r, BMEII0987f, BMEII0987r (Metabion GmbH, Germany) (Table 1), in a final concentration of primers in the PCR mixture of 0.25 pmol/µl. The final concentration of dNTP (Thermo Scientific, USA) was 0.4 mM. *Taq* polymerase (Thermo Scientific, USA) was added in a volume of 0.5 µl. The program on which the PCR reaction was carried out included: an initial denaturation at 95 °C for 7 min followed by 25 cycles of denaturation of a template at 95 °C for 35 s, annealing at 64 °C for 45 s, extension of the primer at 72 °C for 180 s, and then a final extension step at 72 °C for 6 min. Visualization of obtained PCR products

was performed using the horizontal electrophoresis method in 1% agarose gel (Serva Electrophoresis GmbH, Germany) stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany). A commercial DNA marker – Mass ruler DNA 100 bp ladder (Thermo Scientific, USA) was used as molecular size marker. The following were used as positive controls: DNA extracted from the reference *B. canis* strain RM 6/66, *B. canis* SR - 1 isolates and *B. suis* WS 1 confirmed in the reference OIE laboratory (AFSSA, France 2004.).

Table 1. List of used primers

Primer	Sequence	DNA target
BMEI0998f BMEI0997r	ATC CTA TTG CCC CGA TAA GG GCT TCG CAT TTT CAC TGT AGC	Glycosyltransferase, gene wbo A
BMEI0535f BMEI0536r	GCG CAT TCT TCG GTT ATG AA CGC AGG CGA AAA CAG CTA TAA	Immunodominant antigen, gene bp26
BMEII0843f BMEII0844r	TTT ACA CAG GCA ATC CAG CA GCG TCC AGT TGT TGT TGA TG	Outer membrane protein, gene omp31
BMEI1436f BMEI1435r	ACG CAG ACG ACC TTC GGT AT TTT ATC CAT CGC CCT GTC AC	Polysaccharide deacetylase
BMEII0428f BMEII0428r	GCC GCT ATT ATG TGG ACT GG AAT GAC TTC ACG GTC GTT CG	Erythritol catabolism, gene ery C (Derythrulose-1-phosphatedehydrogenase)
BR0953f BR0953r	GGA ACA CTA CGC CAC CTT GT GAT GGA GCA AAC GCT GAA G	ABC transporter binding protein
BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	Ribosomal protein S12, gene rps L
BMEII0987f BMEII0987r	CGC AGA CAG TGA CCA TCA AA GTA TTC AGC CCC CGT TAC CT	Transcriptional regulator, CRP family

RESULTS

Out of 225 tested blood samples, 33 (14.67%) had a positive agglutination reaction. Of these, in 13 samples (5.78%) specific antibody titer of 1/50 was established, in 8 samples (3.55%) 1/100 and in 12 samples (5.33%) a titer of 1/200. In this study, out of a total of 225 assayed reproductive organs of dogs, *B. canis* was isolated from 3 samples (1.33%), while the PCR method detected two positive samples (0.88%) (Table 2).

Table 2. Results of the different diagnostic methods tested

Method	Titer/label of positive results		
TAT	1:50	1:100	1:200
	13	8	12
Isolation	3		
PCR	2		

B. canis was isolated from three out of the 225 samples of reproductive organs. Two dogs from which *B. canis* was isolated a serum antibody titer of 1/200 was established, and the third dog from which *B. canis* was isolated was negative using slow tube agglutination test (Table 3).

Table 3. TAT and PCR results of *B. canis* isolates from tissue samples

B. canis	**TAT**	**PCR**
Isolate 1	1:200	Positive
Isolate 2	1:200	Negative
Isolate 3	Negative	Negative

The results obtained through bacteriological testing are typical for members of the *Brucella* genus: positive catalase, oxidase and urease test, without H_2S production. Isolated bacteria were mucoid. For all three isolates for which API 20 NE (bioMerieux, France) was applied *Moraxella phenylpyruvica* profile was obtained. Preliminary results indicated that the isolated bacteria match the *B. canis* species. At the same time the characteristics of two positive controls were assayed: reference *B. canis* RM 6/66 strain, as well as *B. canis* isolate SR–1. Reference *B. canis* RM 6/66 strain showed sensitivity to basic fuchsine in a concentration of 1/50000 and 1/100000, and to thionine in a concentration of 1/50000 is sensitive, and in a concentration of 1/100000 *B. canis* RM 6/66 is resistant. Isolate *B. canis* SR-1 is Gram negative, oxidase and urease positive, H_2S negative coccobacilli with the R type colonies, sensitive to thionine at a concentration of 1/50000 and resistant to basic fuchsin. Isolates obtained in this study showed that they are sensitive to basic fuchsin and thionine at a concentration of 1/50000. All isolates were resistant to both basic fuchsin and thionin in dye concentration of 1/100000 (Table 4).

Table 4. Growth in the presence of dyes

B. canis	**Basic fuchsin**		**Thionine**	
	1:50000	**1:100000**	**1:50000**	**1:100000**
RM 6/66	—	—	—	+
Isolate 1	—	+	—	+
Isolate 2	—	+	—	+
Isolate 3	—	+	—	+

From a total of 225 assayed organ samples, positive PCR reaction was obtained from two samples (Figure 1). In both positive samples tested and in control *B. suis* and *B. canis* SR-1 strains, seven amplicons appeared (1682, 1071, 794, 587, 450, 272 and 152 bp), which based on Bruce-ladder assay corresponds to the *B. suis* DNA profile. Strain *B. canis* RM 6/66 showed absence of fragment size 794 bp and its profile based on Bruce-ladder method fits *B. canis* profile. One sample in which *Brucella* DNA was

detected through Bruce-ladder PCR came from a dog whose serum was positive in the agglutination reaction with the amount of antibody titer of 1/200 and from whom *B. canis* was successfully isolated. The second sample that was positive using PCR method came from the dog with suspicious serum in which the agglutination was positive for antibodies titer of 1/50, but from which *B. canis* was not isolated (Table 5). Eleven samples of tissue homogenate originating from dogs in which the titer of specific antibodies of 1/200 was found were negative using the PCR reaction. Also one sample of tissue homogenate derived from the dog from which *B. canis* was isolated was negative using PCR, but in which a titer of specific antibodies (negative agglutination) had not been detected (Table 3). Regardless of the Bruce-ladder assay modification and the increase of the amount of DNA tested in the PCR mixture, all of the amplicons in positive PCR reactions were faded and hardly visible.

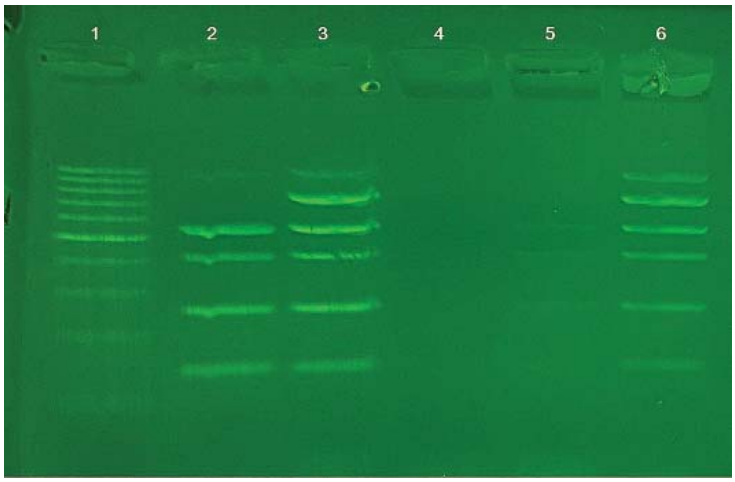

Figure 1. Multiplex PCR amplicons of *B. canis* from dog tissues
Lane 1 – 100 bp ladder; lane 2 – *B. canis* strain RM 6/66; lane 3 – *B. suis isolated from swine*; lane 4 – negative control; lanes 5, 6 – *B. canis DNA extracted* from reproductive organs of dogs

Table 5. TAT and bacterial isolation results of PCR positive tissue samples

Label of the PCR positive samples	TAT (titer)	Isolation
PCR positive sample 1	1:200	+
PCR positive sample 2	1:50	–

From the strains of *B. canis*, which were isolated from three dogs, DNA was also isolated and was tested using Bruce-ladder assay (Figure 2). In all three samples positive PCR reaction was obtained with 7 visible amplicons, actually, all samples had the 794 bp fragment which corresponds to the *B. suis* profile.

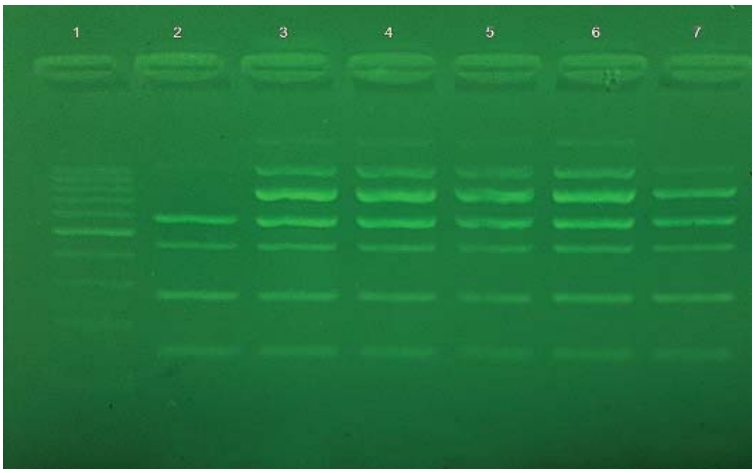

Figure 2. Multiplex PCR amplicons of *B. canis* isolates
Lane 1 – 100 bp ladder; lane 2 – *B. canis* strain RM 6/66; lane 3 – *B. suis isolated from* swine; lane 4 – *B. canis SR – 1*; lanes 5, 6, 7 – *B. canis* isolated bacteria from reproductive organs of dogs

DISCUSSION

Diagnosis of canine brucellosis is mainly based on several serological methods such as agglutination tests, indirect immunofluorescence, and the available literature describes homemade ELISA test kits and its variants. One study describes the dot-ELISA with two different antigens of *B. canis*. The test analyzed 40 serum samples that were previously tested using rapid and slow agglutination tests with 2-mercaptoethanol. From all the examined sera, 19 had agglutination titers >1/50. Using cytoplasmic proteins, 10 serums had positive reactions and using outer membrane proteins 7 serums tested positive. All sera positive in the dot ELISA test kits had agglutination titers >1/100. Unfortunately, serological diagnosis has not been internationally standardized and agglutination tests are used most frequently: screening - rapid slide agglutination, and slow - tube agglutination test (TAT). There are numerous studies that have used other types of serological tests for the diagnosis of brucellosis in dogs.

Due to the unreliability of the results obtained in each of the methods applied, accurate diagnosis of infections caused by *B. canis* can only be set based on the application of one of the above-mentioned methods. Also, simultaneous use of all three methods does not guarantee accurate diagnosis in the case of *B. canis*.

It is believed that stray dogs are a significant source of *B. canis*. In a retrospective study, which covered the period from 2004 to 2011, the results of agglutination tests of 193 serum samples originating from owned dogs (pets) were analyzed; specific antibodies to *B. canis* in th titre of 1/200 were found in 29 samples (15.03%). In 2011, 120 blood serum samples originating from stray dogs from Belgrade were assayed, of which the specific antibody titer of 1/200 was found in 8 samples (6.67%) [6]. Test results indicate a high seroprevalence of antibodies to *B. canis*, not only in stray dogs

but also in owned dogs prevalently from Belgrade. The use of parks and other green areas where owned dogs come into contact with each other and with stray dogs can be a reason for the relatively high prevalence of the disease. On the other hand, the tests conducted in rural parts of Serbia and smaller urban areas indicate that when it comes to dogs that are kept in closed spaces, backyards or within households, canine brucellosis has not been diagnosed and is only present in the stray dog population [7]. Taking into account the fact that canine brucellosis is a zoonosis, the diagnosis of this disease is also significant when it comes to public health.

In the first study of stray dogs in Belgrade, from 184 assayed sera, 49 (26.63%) had a titer of 1/50, 25 (13.58%) titer of 1/100, while 20 (10.87%) serums had a titer equal to or greater than 1/200. Reports indicated that *B. canis* has been continuously present for years in stray dogs as well as in owned dogs in Belgrade [8]. Data for countries in the region is largely missing, but it is known that brucellosis in dogs is a major problem in certain parts of the world, and that it is mostly related to the unresolved problem of stray dogs. This disease is a major problem in large commercial dog kennels where it is causing significant financial losses.

Molecular methods that are increasingly being applied in the diagnosis of infectious diseases also have great prospects when it comes to the diagnosis of brucellosis [9]. A small number of studies are based on the detection of *Brucella* spp. in tissue samples. In few studies a higher percentage of positive results were obtained from lymphoid tissue samples than from blood samples [10,11]. To prove brucellosis in 123 camels, Khamesipour et al. used molecular PCR method. The results show that 11.38% of the assayed blood samples and 13.01% of the lymph node samples were positive for *Brucella* spp.

In the current study, only one sample was positive when tested by all three methods: the blood serum determined an antibody titer for *B. canis* of 1/200, *B. canis* was isolated from the organs, and PCR was also positive. This sample came from the only dog that showed clinical symptoms – bilateral orchitis and fever.

The isolation of *B. canis* through conventional bacteriological techniques takes a long time, it's risky for the laboratory workers, and has low sensitivity due to the frequent contamination of materials. When the material is contaminated to a considerable extent, the isolation fails despite the use of selective media [12]. Contributing to the failure of isolation is the relatively slow growth of *Brucella* compared to contaminants and the fact that *Brucella* may be found in small numbers inside the examined material. Also, practical experience of laboratory workers is very important during the isolation. Bacteriological isolation is slow, costly and complicated, but it is recommended whenever possible in order to confirm the diagnosis with certainty.

The reason for the negative results in the Multiplex PCR Bruce-ladder assay can be very small undetectable amounts of DNA in tissue samples. In addition, tissue samples contain inhibitory components that interfere with the PCR reaction. There is also a

problem of *Brucella* distribution, i.e. DNA in the tissues sampled. All of the above could be the reason why false negative results are obtained using the PCR method.

All these results suggest that for the diagnosis of brucellosis choosing an adequate sample plays the most important role and that lymphoid tissue is certainly it. However, such a sample from dogs is often not available. Stage of infection can greatly affect the number and location of *Brucella* in the body. At the beginning of the infection, due to the fact that there is a prolonged bacteremia, blood should be sampled. Later, when it comes to chronic cases, the tissue of choice should be aborted fetuses, placenta, sperm and vaginal swabs.

The obtained results show that the slow agglutination method remains the first choice for the detection of dogs infected with *B. canis*. In addition, whenever possible, it is necessary to try isolation. For now, PCR remains the method of choice in genotyping, i.e. precise identification of isolated *Brucella* spp. strains. However, multiplex PCR Bruce-ladder assay for the time being has no advantage over the previously mentioned methods when it comes to the detection of *Brucella* directly from tissue samples. In other words, it is desirable to attempt the detection of *Brucella* in tissues using PCR, but the results may not be treated as definitive and reliable. Application of Bruce-ladder protocol requires further optimization in order to maximize its sensitivity for its use in clinical diagnostics.

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Authors` contributions

NS, DM, DB and KM carried out the molecular genetic studies, MM helped in molecular genetic studies, NS and DB collected the samples and together with SR carried out serological and bacteriological studies, interpreted the results and drafted the manuscript, SR conceived and designed the study, SR, DM, KM and MV helped in the result interpretation, critically revised the manuscript and helped to draft the manuscript.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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ISPITIVANJE MOGUĆNOSTI DETEKCIJE *BRUCELLA CANIS* U UZORCIMA TKIVA PRIMENOM BRUCE-LADDER MULTIPLEX PCR METODE

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Cilj ovog rada je bilo upoređivanje rezultata seroloških i konvencionalnih bakterioloških metoda sa rezultatima dobijenim primenom multiplex PCR Bruce-ladder metode. Na osnovu dobijenih rezultata, izvršena je procena upotrebljivosti metode u brzoj dijagnostici bruceloze pasa direktno iz uzoraka reproduktivnih organa inficiranih pasa. Od 225 ispitanih uzoraka krvnih seruma, kod 33 (14,67%) dobijena je pozitivna reakcija

aglutinacije. Od ukupno 225 ispitanih uzoraka reproduktivnih organa pasa, *Brucella canis* je izolovana iz 3 uzorka (1,33%), dok su PCR metodom otkrivena 2 pozitivna uzorka (0,88%). Kod dva psa kod kojih je izolovana *B. canis*, u krvnim serumima ustanovljen je titar antitela od 1/200, a treći pas kod koga je izolovana *B. canis* je bio negativan u testu spore serumske aglutinacije u epruveti. Pozitivna PCR reakcija je dobijena kod dva uzorka. Jedan uzorak bio je poreklom od psa kod koga je izolovana *B. canis* i čiji je serum u reakciji aglutinacije imao titar antitela od 1/200, dok je drugi uzorak bio poreklom od psa kod koga je aglutinacija bila pozitivna u titru 1/50 i kod koga nije izolovana *B. canis*. Dobijeni rezultati pokazuju da spora aglutinacija u epruveti ostaje metoda prvog izbora za otkrivanje pasa inficiranih vrstom *B. canis*. Osim toga, kad god je to moguće, potrebno je pokušati i izolaciju uzročnika. Poželjno je pokušati detekciju brucela u tkivima primenom PCR metode, ali dobijeni rezultati se ne smeju tretirati kao definitivni i pouzdani.