Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 74 (2010) 658–662
Theriogenology
www.theriojournal.com

Detection of *Brucella canis* from inguinal lymph nodes of naturally infected dogs by PCR

Z. Aras*, U. S. Uçan

Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, Campus, 42 075, Konya, Turkey

Received 30 December 2009; received in revised form 15 March 2010; accepted 25 March 2010

Abstract

The aim of this study was to standardize and evaluate a PCR assay for the detection of *Brucella canis* (*B. canis*) in lymph node samples of naturally infected dogs. The performance of the PCR was compared with the results of bacteriological culture as reference method. Forty-eight inguinal lymph node samples were collected from 48 dogs (18 males and 30 females) that died in the city's pound in the years 2007–2008 and were examined by microbiological culture and the PCR assay. *B. canis* was isolated from 4 (8.3%) of 48 lymph node samples. Forty-four (91.7%) of the samples were bacteriological culture negative. *B. canis* DNA was directly detected from all culture positive lymph node samples (n = 4) by PCR. All of the culture negative samples were confirmed as negative by PCR. When the culture method was used as a gold standard, sensitivity and specificity of the PCR assay were found to be 100%. The limit of PCR detection of *B. canis* DNA was 1.4×10^1 CFU/g at least. In conclusion, the PCR assay has been shown to have a diagnostic performance equal to bacteriological culture for detection of *B. canis.* By a non-hazardous protocol for laboratory workers, the assay can be performed in one day.

Keywords: Canine brucellosis; PCR; Microbiological culture; Lymph node.

1. Introduction

Canine brucellosis is caused by *Brucella canis* (*B. canis*) and was first recognized in 1966. It has been acknowledged as the cause of great economic loss in kennels [1,2]. *B. canis* can cause abortions and stillbirths in pregnant dogs and epididymitis and testicular atrophy in male dogs. Generalized lymphadenitis in both sexes can occur and some dogs remain chronically infected for years [3]. *B. canis* is probably found throughout most of the world; however, New Zealand and Australia appear to be free of this disease [2].

Clinical signs of infection are variable. Dogs with brucellosis may show mild or no clinical signs of infection [1]. Lymphadenitis is a common finding in both genders, affecting most lymph nodes in the body. Examination of aspirates or biopsies of lymphatic ganglions generally reveals lymphatic hyperplasia with a large number of plasmatic cells [2]. The only definitive method of diagnosis is by isolation of *B. canis* from blood, vaginal discharges, lymph nodes, fetal tissues, or semen [1,2]. Serological tests can be used for diagnosis of infection. However, cross-reactions between *B. canis* and other Gram-negative bacteria can occur in some tests, particularly agglutination tests. Nonspecific agglutination reactions also occur in some dogs [4–6].

The polymerase chain reaction (PCR) is an alternative to bacteriological isolation for direct diagnosis of canine brucellosis. It is a rapid, highly sensitive, very

* Corresponding author. Tel.: +90 332 223 36 15; fax: +90 332 241 00 63.
E-mail address: zekiaras@hotmail.com (A. Zeki).

0093-691X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.03.023

specific, and inexpensive technique to detect *Brucella* DNA [7,8]. Detection of *Brucella* spp DNA has already been performed in vaginal swabs [8], blood [9], and semen [10,11] of dogs, with good sensitivity and specificity. The diagnosis of *Brucella* spp DNA was performed in bovine, goat, and ovine lymphoid tissue samples [12–15]. Because of the absence of studies describing the direct detection of *Brucella* spp by PCR in lymphoid tissue sample from dogs, a PCR method for the detection of *Brucella* spp in lymph nodes of naturally infected dogs was evaluated in this stufy. The performance of the test was also measured.

2. Materials and methods

2.1. Dogs

Samples were collected from 48 dogs (18 males and 30 females) that died of unknown causes in the city's pound of Konya province, Turkey in the years 2007–2008. Breeds included Boxer (2), Golden Retriever (1), Kangal (3), and mongrel (42). The ages of the dogs were between 1–10 yr.

Clinical signs that suggested canine brucellosis were investigated in these dogs by post mortem examination and questionnaire forms that were obtained from the Veterinarian of the city pound. These data stated that generalized lymphadenitis, scrotal dermatitis, epididymitis, orchitis, testicular atrophy, metritis, vaginal discharge, abortion, stillbirth, osteomyelitis, uveitis, lethargy, decreased appetite, weight loss, and hyperthermia were seen and some of the dogs were reported to have received antibiotics.

Aseptic precautions were taken during collection of samples and different disposable gloves were used in the collection of each sample. After necropsies of the animals, spleen, liver, mesenterial and inguinal lymph nodes were aseptically removed with a sterile scalpel and immediately placed in sterile containers. All materials were cooled on ice during transport to the laboratory. The samples were placed in 20% glycerol with Brucella Broth (Sigma, St. Louis, MO, USA) and were kept at −20 °C until tests that were carried out every 2 mo. Lymph node samples from 5 male dogs that were previously diagnosed as negative to canine brucellosis by clinical, serological, and culture examinations were contaminated with *B. canis* NCTC 10854 (supplied by Refik Saydam Hifzissihha Institute, Ankara, Turkey) to determine the detection limit of the PCR assay from a lymph node.

2.2. Bacteriological examinations

Specimens were processed using the method described by Alton et al [1]. Briefly, a 5 g portion of tissue samples was placed in a stomacher bag with 10 mL of sterile phosphate-buffered saline (pH 7.2) and homogenized for 5 min. The homogenate was divided into 2 mL aliquots. One of the aliquots was used for DNA extraction and the other for bacteriological cultures. One mL each of tissue homogenate was immediately cultured onto duplicate Blood Agar Base (Oxoid, Hampshire, UK) containing 5% defibrinated sheep blood with *Brucella* Selective Supplement (Oxoid, SR0083A, Basingstoke, UK), MacConkey Agar (Oxoid, Hampshire, UK) and Blood Agar Base containing 5% defibrinated sheep blood. The cultures were incubated at 37°C under aerobic conditions for 7 d. *B. canis* were identified using standard classification tests including colonial morphology, Gram stain, acriflavine aglütination, catalase, oxidase, urease, citrate utilisation, indole production, nitrate reduction, Voges–Proskauer, motility, H_2S production, CO_2 requirement, serum requirement, growth in the presence of thionin (20 μg/mL), basic fuchsin (20 μg/mL), and safranin O (100 μg/mL) and agglutination with monospecific A, M, and R antiserum [1].

2.3. DNA extraction

Brucella DNA from lymphoid node samples was extracted using the protocol described by Leal-Klevezas et al [16]. Four hundred μL of the homogenate sample were added to 400 μL of lysis solution (2% Triton-X 100, 1% SDS, 100 mm NaCl, 10 mm Tris-HCl pH 8.0) and 10 μL of Proteinase K (10 mg/mL), thoroughly mixed, and incubated for 30 min at 50 °C. Four hundred μL of saturated phenol were added, mixed thoroughly, and centrifuged for 5 min at 8,000 × g. After transferring the aqueous layer to a fresh tube, an equal volume of chloroform-isoamyl alcohol was added (24:1); the tubes were mixed thoroughly and centrifuged for 5 min at 8,000 × g. The upper layer was again transferred to a fresh tube and 200 μL of 7.5 M ammonium acetate was added and mixed thoroughly. Samples were kept on ice for 10 min, and then centrifuged for 5 min at 8,000 × g and aqueous content transferred to a fresh tube. Two volumes of 95% ethanol or 1 volume of isopropanol were added, mixed, and stored overnight at −20°C. DNA was recovered by centrifuging the samples for 5 min at 8,000 × g; pellets were rinsed with 1 mL of 70% ethanol, air-dried, and resuspended in 50 μL of TE buffer. The samples were

stored at −20 °C until use as templates for amplification.

2.4. PCR assay

The *Brucella* specific primer pairs were used in PCR for amplification of the 16S–23S rRNA interspace region of *Brucella* spp [9]. The sequence of forward primer was 5′-ACATAGATCGCAGGCCAGTCA-3 and reverse primer was 5′-AGATACCGACGCAAACGCTAC-3′. Specific primers showed a single amplicon, the size of which was 214 bp.

The PCR assays were performed in a total reaction volume of 50 μL containing 5 μL of 10 × PCR buffer, 1.5 mM $MgCl_2$, 250 μM each of the four dNTPs (Fermentas, Vilnius, Lithuania), 1.25 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 0.5 μM of each primer (IDT, USA), and 5 μL of template DNA. The amplifications were performed in a thermalcycler (Ependorf, Mastercycler *gradient,* Germany) with the following steps: 1 × 4 min at 95 °C, 40 × 30 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C, and a final extension at 72 °C for 5 min. DNAs extracted from samples contaminated with *B. canis* NCTC 10854 and nuclease free water was served as positive and negative controls, respectively. The PCR products (10 μL) were then analyzed by electrophoresis on 2% agarose gel, and the gel was stained with ethidium bromide (1.5 μg/mL) and photographed.

2.5. Determination of detection limit of the PCR

The detection limit of the PCR assay in lymphoid node samples was estimated using *B. canis* NCTC 10854 reference strain. The suspension of the 72 h culture of *B. canis* was prepared in sterile saline and 10-fold dilutions (10^{-1} to 10^{-10}) were made in order to determine the colony-forming unit (cfu). One hundred microliters of the suspension were inoculated onto two

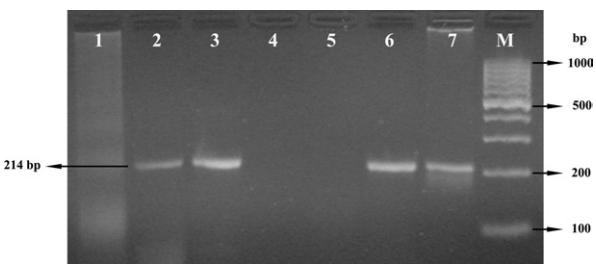

Fig 1. Agarose gel electrophoresis of PCR assay for detection of *B. canis* from lymph nodes. The figure shows a single band 214 bp DNA fragment. M: 100 bp marker (Fermentas, 0241). Line 1: negative control; lines 2,3,6: positive lymph node samples; lines 4,5: negative lymph node samples; line 7: *B. canis* NCTC 10854 (positive control).

Table 1
Diagnostic sensitivity and specificity of tissue PCR assay.

	Culture		
	Positive dogs	Negative dogs	Total
Tissue PCR assay			
Positive	4	0	4
Negative	0	44	44
Total	4	44	48

Diagnostic sensitivity and specificity of tissue PCR assay was 100%.

Blood Agar Base plates and incubated at 37 °C for 5–7 days, after which the colonies on the plates were counted. The concentration of undiluted *B. canis* culture was estimated as 1.4×10^7. A 1 g of lymphoid tissue samples was placed in a stomacher bag with 1 ml of sterile saline and homogenized for 5 min. Decreasing numbers of bacteria (methanol killed and washed but not lysed) were added to the lymphoid node homogenate samples (obtained by 5 non-infected dogs) to determine the sensitivity of the assay. The final concentrations of the organism in the mixture was 1.4×10^7, 1.4×10^6, 1.4×10^5, 1.4×10^4, 1.4×10^3, 1.4×10^2 and 1.4×10^1 cfu/g. *B. canis* (NCTC 10854) DNA was extracted from all dilutions of the mixture and processed by PCR as described above.

3. Results

Brucella was isolated from 4 (8.3%) of 48 samples of inguinal lymph nodes. All *Brucella* isolates were identified as *B. canis* by morphological, cultural, and biochemical characteristics. Forty-four (91.7%) of the samples were culture negative. All isolates were grown on blood agar and Brucella selective agar, but were not done on MacConkey agar.

B. canis DNA was directly detected from all culture positive lymph node samples by PCR (Fig 1). All of the culture negative samples were confirmed as negative by PCR. When the culture method was used as a gold standard, both sensitivity and specificity of the PCR assay were found to be 100% (Table 1).

Of the 4 infected dogs, 3 were female and 1 was male. Ten of the 48 dogs had hyperthermia, decreased appetite, weight loss, and cough and been given antibiotics (enrofloxacine or amoxicillin for respiratory disease etc.) before they died. These dogs were negative by culture and PCR. Four of the 48 dogs examined had clinical signs suggestive of brucellosis. One of the 4 dogs had hyperthermia, testicular atrophy, hepatomegaly, and generalized lymphadenitis. *B. canis* was isolated from some of the tissues (spleen, liver, mesente-

rial and inguinal lymph nodes) of the male dog (noted as bacteremic). The other 3 dogs had atrophic inguinal lymph nodes and the isolation of *B. canis* was made only from inguinal lymph nodes of these female dogs (accepted as chronic brucellosis). These 4 dogs tested positive by direct PCR. The remaining 34 dogs had no clinical signs of brucellosis and had negative PCR and culture.

Decreasing numbers of bacteria were added to the lymph node samples to investigate sensitivity of the assay. A positive PCR result was always obtained with different aliquots containing at least 1.4×10^1 CFU/g of lymph node samples. Sensitivities of 1.4×10^1 and 1.4×10^2 CFU/g were achieved after agarose gel electrophoresis. We determined the limit of PCR detection of *B. canis* DNA to be 1.4×10^1 CFU/g at least.

4. Discussion

Asymptomatic infections are common in canine brucellosis. The most important feature of the disease is a prolonged period of bacteraemia that may be intermittent during the chronic stage [1]. After a bacteraemia, the infection becomes established in various lymph nodes and organs. Lymphadenitis, especially involving the retropharyngeal and inguinal nodes, appears in both genders, although generalized lymphadenitis and follicular hyperplasia of the spleen are also commonly detected [2]. The agent of infection can be isolated from aspirates or from biopsies of lymphatic ganglions of dogs that have especially chronic or asymptomatic infection. However, the isolation method also has its own limitation. Selective media are necessary for the isolation of *Brucella* strains. Using Farrell's medium for R-type *Brucella* isolation seem to be a bit controversial. Some researchers have reported that Ferrell's medium might not be suitable for the isolation of R-type *Brucella* agents, given its high inhibitory effect on the *B. canis* and *B. ovis* [17,18]. Marin et al [19] have suggested the medium in question might be inhibitory on some strains of *B. melitensis* and *B. ovis.* However, the researchers did not check the effect of the medium on another R-type species of *B. canis.* Tudor and Togoe [20] have reported that the best medium for isolation of *B. canis* was that in which the ingredients match those of Farrell's medium. In the present study, Farrell's medium and blood agar were simultaneously used to isolate the bacteria and similar results from each culture were obtained.

PCR may be a choice of detection of pathogens from biopsy or necropsy samples of lymph nodes. On the other hand, PCR of vaginal swab samples can detect positivity in a dog regardless of the viability of the organism [8]. Taken together, in asymptomatic and/or chronic *B. canis* infections, the best source of the organisms in which the organism can likely be found viable is the lymph nodes, especially those draining retropharyngeal and inguinal areas. In a parallel study, one dog showed clinical Brucellosis signs (bacteremic) and 3 were diagnosed with evidence of chronic infection (presence of atrophic lymph nodes). Regardless of serology, the fact that bacteremic or chronic stage of infection were diagnosed by PCR in this work suggested that the dogs with unknown history in the pounds where *B. canis* is common could be tested for the disease using lymph node samples. A full agreement between the PCR assay and the isolation of *B. canis* was found as stated in Table 1. Diagnostic sensitivity and specificity of the present PCR assay is also high when compared to those from the same tissue from different species [12–15]. These differences of sensitivity and specificity of studies may be a result of variations among DNA extraction protocols, target genes, gold standard tests, and even higher concentrations of DNA that could inhibit the test performance.

By extraction from semen samples of noninfected dogs, a PCR assay has been reported to detect as few as 3.8 fg of *B. canis* DNA experimentally diluted [11]. A similar detection limit for *B. canis* by PCR in vaginal swabs of bitches has also been noted [8].

The ITS66 and ITS279 pair of primers which was directed to the 16S–23S rRNA interspace region of *B. canis* DNA was used in the present PCR assay to detect *B. canis* in lymphoid tissue samples of dogs. Its diagnostic sensitivity and specificity were 100%. The same primers were reported to assess different PCR assays for detection of *B. canis* in canine blood [[9], semen [11], and vaginal swabs [8]. The diagnostic sensitivity and specificity of those PCR were reported to be the same as in the present work [8,9,11].

Sero-prevalence of canine brucellosis has been documented by several studies so far in Turkey [6,21–24]. This study revealed that the infection is of a major concern, at least in the animal shelter in which roughly 1210 stray dogs are kept. The people in the region are encouraged to adopt a dog from this shelter by authorities and civil organizations. A recent study examining occurrence of *B.canis* infection in a human risk group from the region revealed that sero-prevalence was 9.2 [5]. Because it is a zoonosis, and the agent can be shed intermittently by secretions of the infected dog, a regular check from samples including inguinal lymph

nodes is worth of doing before releasing a dog from the shelter.

In conclusion, a PCR assay from the inguinal lymph node has been developed, showing a diagnostic performance similar to bacteriological culture for detection of *B. canis*. This assay has the advantage of being able to identify *B. canis* directly from the lymph node samples in one day. It is also a useful tool for confirmation of cultures and has high specificity. Fast and accurate diagnosis of canine brucellosis is very important for public health and achievement of the control and eradication programs. Because it is a much less hazardous method compared with bacteriologic culture on diagnosis, the PCR protocol evidenced here can be recommended for diagnostic labs.

References

[1] Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory, INRA, Paris. 1988.
[2] Wanke MM. Canine brucellosis. Anim Reprod Sci 2004;82/83: 195–207.
[3] Carter GR, Chengappa MM. Essentials of Veterinary Bacteriology and Mycology, Lea & Febiger, London. 1991.
[4] Lucero NE, Escobar GI, Ayala SM, Lopez G. Sensitivity and specificity of an indirect enzyme-linked immunoassay for the diagnosis of *Brucella canis* infection in dogs. J Med Microbiol 2002;51:656–60.
[5] Köylü O, Aras Z, Uçan US. Seroprevalence of *Brucella canis* infection in a riscy human population in province Konya. Infek Der 2009;23(2):73–7.
[6] Uçan US, Aras Z, Zorlutuna M. Detection of canine brucellosis by a rapid agglutination test using *Rhizobium tropici* as antigen. Revue Méd Vét 2010;161(2):51–56 (in press).
[7] Bricker BJ. PCR as a diagnostic tool for brucellosis. Vet Microbiol 2002;90:435–46.
[8] Keid LB, Soares RM, Vasconcellos SA, Chiebao DP, Salgado VR, Megid J, Richtzenhain LJ. A polymerase chain reaction for detection of *Brucella canis* in vaginal swabs of naturally infected bitches. Theriogenology 2007a;68:1260–70.
[9] Keid LB, Soares RM, Vieira NR, Megid J, Salgado VR, Vasconcellos SA, Da Costa M, Gregori F, Richtzenhain LJ. Diagnosis of canine brucellosis: comparison between serological and microbiological tests and a PCR based on primers to 16S–23S rDNA interspacer. Vet Res Commun 2007c;31:951–65.
[10] Kim S, Lee DS, Suzuki H, Watari M. Detection of *Brucella canis* and *Leptospira interrogans* in canine semen by multiplex nested PCR. J Vet Med Sci 2006;68:615–8.
[11] Keid LB, Soares RM, Vasconcellos SA, Chiebao DP, Megid J, Salgado VR, Richtzenhain LJ. A polymerase chain reaction for the detection of *Brucella canis* in semen of naturally infected dogs. Theriogenology 2007b;67:12030–10.
[12] Fekete A, Bantle JA, Halling SM. Detection of *Brucella* by polymerase chain reaction in bovine fetal and maternal tissues. J Vet Diagn Investig 1992;4:79–83.
[13] Gupta VK, Verma DK, Singh K, Kumari R, Singh SV, Vihan VS. Single-step PCR for detection of *Brucella melitensis* from tissue and blood of goats. Small Rumin Res 2006;66:169–74.
[14] O'Leary S, Sheahan M, Sweeney T. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res Vet Sci 2006;81:170–6.
[15] Ilhan Z, Aksakal A, Ekin IH, Gülhan T, Solmaz H, Erdenliğ S. Comparison of culture and PCR for the detection of *Brucella melitensis* in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. Lett Appl Microbiol 2008; 46:301–6.
[16] Leal-Klevezas DS, Martinez-Vazquez OI, Lopez-Merino A, Martinez-Soriano JP. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. J Clin Microbiol 1995;33:3087–90.
[17] Corbel M, Banai M. Genus I. Brucella Meyer and Shaw 1920. In: Bergey′s Manual of Systematic Bacteriology Volume Two - The Proteobacteria, Garrity GM, Brenner DJ, Krieg NR (Ed.), Springer, New York, 2005, pp. 370–86.
[18] Brown GM, Ranger CR, Kelley DJ. Selective media for the isolation of *B. ovis*. Cornell Vet 1971;61:265–80.
[19] Marin CM, Alabart JL, Blasco JM. Effect of antibiotics contained in two *Brucella* selective media on growth of *Brucella abortus, B. melitensis*, and *B. ovis*. J Clin Microbiol 1996;34(2): 426–8.
[20] Tudor LA, Togoe I. Evaluation of classic methods for *Brucella canis* isolation and identification. Vienna 2008 ISCFR Symposium and EVSSAR Biannual Meeting, 9 July 2008, Vienna.
[21] İstanbulluoğlu E, Diker S. *Brucella canis* üzerinde serolojik incelemeler. Ankara Üniv Vet Fak Derg 1983;30:14–18.
[22] Diker KS, Aydin N, Erdeger J, Özyurt M. A serologic survey of dogs for *Brucella canis* and *Brucella abortus* and evaluation of mercaptoethanol microagglutination test. Ankara Univ Vet Fak Derg 1987;34:268–76.
[23] Öncel T, Akan M, Sarıeyyüpoğlu B, Tel OY, Ciftci A. Seroprevalence of *Brucella canis* infection of dogs in two Provinces in Turkey. Turk J Vet Anim Sci 2005;29:779–83.
[24] Tanrıtanır P, Ceylan E, Berktaş M. The seroprevalence of canine brucellosis in shepherd dogs. Kafkas Üniv Vet Fak Derg 2009;15:155.