Comparison of a Serological Method, a Bacteriological Method and 16S rRNA *Brucella canis* PCR for Canine Brucellosis Diagnosis

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ABSTRACT

The objective of the study was to evaluate the sensitivity, specificity and the positive and negative predictive value of a PCR assay for canine brucellosis diagnosis using 16S rRNA specific primers compared to serology, 2 mercaptoethanol-microtiter plate agglutination tests (2ME-MPAT) and a blood culture test. A sample of 48 dogs was divided into three groups, according to the results of blood culture tests and 2ME-MPAT. Group 1was comprised of infected *Brucella canis* dogs, who were positive to both a blood culture test and 2ME-MPAT (n=16). Group 2 contained non-infected *B. canis* dogs, who were negative to both a blood culture test and 2ME-MPAT (n=16). Group 3 contained suspected infected *B. canis* dogs, who were negative to a blood culture test but positive to 2ME-MPAT (n=16). Samples in Groups 1 and 2 were used to calculate the diagnostic sensitivity, specificity, positive predictive value and negative predictive value of PCR and the results performed in Group 3 were also discussed. The diagnostic sensitivities and specificities of PCR were 100%. The positive and negative predictive values and accuracy of PCR were 100%. In conclusion, the results revealed that the PCR was an effective technique for the diagnosis of canine brucellosis in blood samples, especially in dogs suspected of being positive by 2ME-MPAT, but negative by a blood culture test.

Key words: *Brucella canis,* serological method, bacteriological method, polymerase chain reaction, 16S rRNA

INTRODUCTION

Brucella canis, the causative pathogen of canine brucellosis, is the most common clinical manifestation of late abortion, embryonic death, conception failure, epididymitis, orchitis, sperm abnormalities and infertility in dogs (Wanke, 2004; Greene and Carmichael, 2006; Keid *et al.,* 2007a, b, c; Keid *et al.,* 2009). In addition, *B. canis* is a contagious bacterial zoonosis transmittable to humans (Wanke, 2004; Corbel, 2006; Greene and Carmichael, 2006).

Currently, canine brucellosis is extensively diagnosed by serological and bacteriological laboratory tests (Nimri, 2003; Corbel, 2006). However, the limitations of serology are the lack of diagnostic specificity or diagnostic sensitivity and a high frequency of false-positive reactions due to cross-reactions between *B. canis* and other gram-negative bacteria,

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such as the mucoid strains of *Pseudomonas aeruginosa, Bordetella bronchiseptica, Actinobacillus equuli, Streptococcus,* and *Staphylococcus* (Greene and Carmichael, 2006; Keid *et al*., 2007a). Therefore, false-positive reactions are very common and positive samples should be tested additionally using a more sophisticated, specific diagnostic method (Corbel, 2006; Greene and Carmichael, 2006). Strategically, the definitive diagnosis of *B. canis* infection should be a direct method of diagnosis, such as microrganism isolation (Nimri, 2003; Corbel, 2006; Keid *et al*., 2009). The gold standard of this disease is bacterial isolation, followed by bacteriological identification (Bricker, 2002).

Although the isolation of *Brucella* from blood culture is considered as the diagnostic standard for canine brucellosis, the PCR assay is a good method to confirm the diagnosis to prove major fastidious or slowly growing bacteria (Al Dahouk *et al*., 2003; Greene and Carmichael, 2006; Keid *et al*., 2007a). Microbiological culture has the disadvantage of being time-consuming because it takes most colonies about 10 to 14 days or longer to be detected for characterization of the etiological agent of canine brucellosis (Greene and Carmichael, 2006; Keid *et al*., 2007a). In addition, bacterial isolation depends on bacterial growth and viability (Keid *et al*., 2007c). Thus, there is a need to develop and evaluate the performance of fast, sensitive and specific diagnostic techniques for canine brucellosis diagnosis, when compared to conventional culture methods (Bricker, 2002; Greene and Carmichael, 2006). Currently, the PCR assay is used as a veterinary diagnostic tool to detect canine brucellosis because it has been confirmed to have high diagnostic sensitivity and specificity (Bricker, 2002; Keid *et al*., 2009). Furthermore, it is possible to make a rapid diagnosis from blood specimens and infected organs of canine brucellosis using the PCR-based assay (Bricker, 2002; Greene and Carmichael, 2006).

The objective of this study was to evaluate the performance of the sensitivity, specificity, and the positive and negative predictive value of the PCR assay for canine brucellosis diagnosis using 16S rRNA specific primers by comparing them to the 2 mercaptoethanol-microtiter plate agglutination test (2ME-MPAT) serological method and a blood culture test.

MATERIALS AND METHODS

Samples

Forty-eight dogs were divided into three groups, according to the results of blood culture tests and 2ME-MPAT. Group 1 contained infected *B. canis* dogs, who were positive to both a blood culture test and 2ME-MPAT (n=16). Group 2 consisted of non-infected *B. canis* dogs, who were negative to both a blood culture test and 2ME-MPAT (n=16). Group 3 contained suspected infected *B. canis* dogs, who were negative to a blood culture test but positive to 2ME-MPAT (n=16). Samples in Groups 1 and 2 were used to calculate the sensitivity, specificity, and the positive and negative predictive value of the PCR assay (Keid *et al*., 2007a, b, c). Blood samples were collected from 48 dogs suspected to be infected. A total of 3-4 ml of blood was collected from each dog by cephalic vein puncture, using EDTA as an anticoagulant. Two to three milliliters were immediately submitted for bacterial isolation and the remaining 1 ml of the sample was stored at -20°C for later use for the PCR assay (Keid *et al*., 2007a, b, c).

Bacterial isolation and growth conditions

Blood samples (2-3 ml) were cultured in brucella broth (Difco, Detroit, USA) at 37°C under aerobic conditions for 45 days (Corbel, 2006). Blood cultivation was performed at the Kampaeng Saen Veterinary Diagnostic Laboratory, Kasetsart University. Subcultures were performed on brucella agar every three days and plates were

incubated at 37°C under aerobic atmospheric conditions for a further 72 h. Presumptive identification of *B. canis* colonies were based on morphological and cultural properties, and biochemical characteristics (Keid *et al.,* 2007c).

Serological tests

For the serological diagnosis, 3 ml of blood was collected without anticoagulant from each dog by cephalic vein puncture. Blood samples were centrifuged (4,000Xg for 15 min) and 48 canine sera were examined by 2ME-MPAT to detect antibodies against *B. canis* (Al Dahouk *et al.*, 2003; Keid *et al.,* 2007a, b, c).

DNA extraction and nucleic acid amplification

DNA extraction of *B. canis* was carried out using the phenol-chloroform extraction method as described by Sambrook and Russell (2001). Finally, the DNA pellet was air-dried and resuspended in 20 μl of TE buffer (pH 8). DNA concentration and purity were determined by spectrophotometer (Beckman CoulterTM DU® 530, Life Science UV/Vis, USA) by reading the optical densities at A_{260} and A_{280}. Samples were aliquotted and stored at -20°C for further use (Sambrook and Russell, 2001).

Primers

The genus-specific oligonucleotide primer pair, primers F4 and R2, designed for the detection of *Brucella* spp. were derived from the 16S rRNA sequence of *B. abortus* (EMBL accession number X13695) (Romero *et al.*, 1995; Bricker, 2002). The expected size of the amplification product from *B. canis* was 905 bps (Table 1).

Polymerase chain reaction

Twenty microliters of the amplification reaction mixtures were composed of 200 μmol each of deoxynucleoside triphosphate (dNTP), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9), 1.5 mmol/L $MgCl_2$, 0.5 μmol of each primer, 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 μl of DNA template. The reaction was performed in a DNA thermal cycle (MJ Research PTC 200 DNA engine, Watertown, MA, USA). After an initial denaturation at 95°C for 5 min, the PCR profile was set as follows: 30 s of template denaturation at 95°C, 90 s of primer annealing at 54°C and 90 s of primer extension at 72°C, for a total of 35 cycles, with a final extension at 72°C for 6 min. The DNA extracted from blood spiked with the DNA of *B. canis* strain KPS was used as a positive control in each set of samples. DNA extracts from blood samples of 48 dogs were tested. Ten microliters of the reaction mixture was analyzed by electrophoresis at 100V for 23 min using 1.0% (wt/vol) agarose gel containing 1X TAE (40 mM Tris-acetate (pH 8.0), 1mM EDTA), stained with ethidium bromide (0.5 μg/ml). DNA bands were visualized under UV light. Each sample was analyzed three times in separate independent experiments (Romero *et al.*, 1995; Keid *et al.,* 2007a, b, c). The PCR products were used to ligate with plasmids pGEM-T (Promega) and the ligation reaction was used to transform *E. coli* strain DH-5α. The purified plasmid was sequenced using ABI Prism® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystem) at the DNA Sequencing Laboratory, Faculty of Medicine, Mahidol University. DNA sequence comparisons and alignment were performed with the GenBank

Table 1 PCR specific primers for targeting the 16S rRNA gene.

Primers	Sequence (5′ → 3′)	Location[a]	Products(bps)
Forward - F4	TCG AGC GCC CCG CAA GGG G	63-79	905 bps
Reward - R2	AAG GAT AGT GTC TCC ACT AA	947-966	

[a] Based on the nucleotide sequence of the *B. abortus* 16S rRNA.

database using the BLAST algorithm (Basic Local Alignment Tool). Computer analysis was performed using the DNASIS computer program (Hitachi Genetic Systems).

RESULTS

The 16S rRNA partial fragment of *B. canis* strains was successfully amplified and the size of the PCR products was 905 bps, as expected. The sequence of the 16S rRNA partial fragment had 98% homology to *B. canis* when compared to the GenBank database using the BLAST algorithm. For infected and non-infected groups, the results of the PCR assay showed 100% similarity with 2ME-MPAT and blood culture tests (Table 2). These results showed that the PCR assay revealed 100% sensitivity and specificity (Table 3). For suspected groups, the PCR assay showed one positive out of 16 samples (Table 2).

DISCUSSION

The present study showed the potential use of a PCR assay using primers specific for 16S rRNA as a rapid confirmatory test for canine brucellosis diagnosis. The PCR assay showed 100% sensitivity and specificity (n=6) in accordance with the positive result of 2ME-MPAT and blood culture (Keid *et al.,* 2007a, b, c). In the infected group, the PCR assay had positive results in 16 out of 16 cases (100%) when compared to 2ME-MPAT and blood culture tests. The 2ME-MPAT positive results were highly specific for detecting antibodies against *B. canis* (Keid *et al.,* 2007a, b, c). The 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) MPAT may be a good serological screening test for canine brucellosis because it can avoid the agglutinating activity of IgM and IgA. Blood culture is a gold standard for the definitive diagnosis of canine brucellosis. However, blood culture is a time-consuming procedure and often produces a negative result,

Table 2 PCR results for the detection of *Brucella canis* using 16S rRNA compared to 2ME-MPAT and blood culture.

PCR condition	Dog health status			Total
	Infected[1]	Non-infected[2]	Suspected[3]	
	Group 1	Group 2	Group 3	
Positive	16[a]	0[b]	1[b]	17
Negative	0[c]	16[d]	15[d]	31
Total	16	16	16	48

[1]dogs positive by 2ME-MPAT and blood culture, [2]dogs negative by 2ME-MPAT and blood culture, [3]dogs positive by 2ME-MPAT but negative by blood culture, [a]true positive [b]false positive [c]false negative [d]true negative.

Table 3 The sensitivity, specificity, positive and negative predictive value and accuracy of PCR in blood samples for *Brucella canis* diagnosis in dogs.

PCR	%
Sensitivity	100
Specificity	100
Positive predictive value (PPV)	100
Negative predictive value (NPV)	100
Accuracy	100

especially in long-standing disease (Corbel, 2006; Keid *et al.,* 2007a, b, c). The PCR assay using primers F4-R2 is the most sensitive and most specific test for *Brucella* diagnosis (Romero *et al.*, 1995). Recently, Noosud *et al.* (2008) reported that the detection limit of the PCR assay using primers F4-R2 for the detection of *B. canis* was 1 fg/μl using purified plasmid containing 16S rRNA gene of *B. canis* and 2.65 pg/μl using *Brucella* spiked with a blood sample.

For the non-infected group, the PCR assay had negative results in 16 out of 16 cases (100%) as did the 2ME-MPAT and blood culture tests as shown by previous reports (Keid *et al.,* 2007a, b, c). Thus, the specificity of the PCR assay was 100%. For the suspected group, 15 of 16 dogs were negative and one dog was positive by PCR. However, these 16 dogs were negative from a blood culture test and were positive when the 2ME-MPAT assay was used. Thus, the PCR assay produced results more similar to blood culture testing than the 2ME-MPAT assay. The specificity of the PCR assay decreased to 93.75% (Keid *et al.,* 2007 c). Although 2ME-MPAT was highly specific for detection antibodies against *B. canis*, false negative results were reported in a previous study (Keid *et al.,* 2007c). The false-positive reactions by the 2ME-MPAT assay are common because lipopolysaccharide antigens in certain other bacterial species can cross-react with *B. canis* antigens and lead to false-positive results (Keid *et al.,* 2007a, b, c). The limitation of blood cultures may have been due to the low numbers of *B. canis* in blood samples especially in the chronic phase of infection, as the bacteraemia may be absent, intermittent or present in low numbers in the chronic stage (Wanke, 2004; Corbel, 2006; Greene and Carmichael, 2006; Keid *et al.,* 2007a, b, c). Romero *et al.* (1995) have published that *Ochrobactrum anthropi,* as the closest known relative to *Brucella*, can cross-react in this PCR assay, but that *O. anthropi* has rarely been found to be pathogenic (Romero *et al.*, 1995; Bricker,

2002). However, the PCR assay using F4-R2 primers had high sensitivity and specificity for the detection of *Brucella* spp. in blood specimens (Romero *et al.*, 1995; Bricker, 2002; Corbel, 2006). There were several reports that the results of dogs testing positive by PCR but negative by blood culture could have been a consequence of the presence of the low number of circulating organisms or the presence of non-viable brucella in the clinical samples (Greene and Carmichael, 2006; Keid *et al.,* 2007a, b, c).

According to these results, the PCR assay using the genus-specific primers could provide a rapid, sensitive and specific testing alternative to serology and the microbiological culture method for canine brucellosis diagnosis. Moreover, the PCR assay can minimize the human health risk of infection in laboratory workers and provides a practical approach for a rapid diagnosis of canine brucellosis from blood samples (Romero *et al.*, 1995; Nimri, 2003; Wanke, 2004; Keid *et al.,* 2007a, b, c).

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