

A Novel Polymerase Chain Reaction to Detect *Brucella canis* in Dogs

Zeki ARAS ¹  Mehmet TAŞPINAR ² İbrahim AYDIN ³

¹ Department of Microbiology, Faculty of Veterinary Medicine, Aksaray University, TR-68100, Campus, Aksaray - TURKEY

² Department of Medical Biology, Faculty of Medicine, Yuzuncu Yil University, TR-65080 Campus, Van - TURKEY

³ Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Selçuk University, TR-42075 Campus Konya - TURKEY

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Abstract

In this study, the specific polymerase chain reaction has been standardized and evaluated for the direct diagnosis of *Brucella canis* in vaginal swab samples from dogs. The specific primer sets are directed to the 16S-23S rRNA inter-space region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis*. A total of 21 references and field strains and 35 vaginal swab samples were used for the evaluation of the polymerase chain reaction. It found that polymerase chain reaction is positive for *B. canis* DNA indicated by only amplification of 214 bp product. It detected at least 2.7×10^1 CFU/g of bacteria diluted in vaginal swab samples indicates that the polymerase chain reaction can be used as a practical alternative for bacterial isolation. The novel polymerase chain reaction provides a simple and rapid for the detection of *B. canis* in clinical and field samples in one step and in short time about 24 h.

Keywords: Specific PCR, *Brucella canis*, Vaginal swab samples

Köpeklerde *Brucella canis*'in Teşhisi Amacıyla Yeni Polimeraz Zincir Reaksiyonu Metodunun Geliştirilmesi

Özet

Bu çalışmada, köpeklerin vajinal sıvay örneklerinden *Brucella canis* (*B. canis*)'in direk teşhisi amacıyla spesifik polimeraz zincir reaksiyonu metodu standardize edildi ve değerlendirildi. *Brucella* spp.'in 16S-23S inter-space bölgesine ve *B. canis*'in BMEI1426-BMEI1427 gen bölgesinde ki 351 bp'lik delesyon alanına özgü primer çiftleri seçildi. polimeraz zincir reaksiyonu yönteminin değerlendirilmesinde toplam 21 referans ve saha suşu ile 35 vajinal sıvay örneği kullanıldı. Sadece 214 bp'lik amplifiye polimeraz zincir reaksiyonu ürününün görüntülenmesi *B. canis* için pozitif olarak kabul edildi. Vajinal sıvay örneklerinden en az DNA tespit limiti yeni metot için 2.7×10^1 CFU/g bakteri olarak bulundu. Bu yöntem bakteriyel izolasyon metoduna pratik bir alternatif olabilir. Yeni polimeraz zincir reaksiyonu testi *B. canis*'in klinik veya saha örneklerinden 24 saat sürede ve tek adımda hızlı ve basit tespitini sağlamaktadır.

Anahtar sözcükler: Spesifik PZR, *Brucella canis*, Vajinal sıvay örneği

INTRODUCTION

Brucella (*B.*) *canis* is main etiologic agent of canine brucellosis and induces various reproductive failures in dogs and in human. It was first isolated from dogs in 1966 by Leland Carmichael. Canine brucellosis is an important disease because it causes the great economic losses in commercial breeding kennels and threats public health. The other smooth *Brucella* species (*B. abortus* and *B. melitensis*) have occasionally been isolated from dogs in various regions of the world ^[1]. Canine brucellosis is found most of the world but Australia and New Zealand appear

to be free ^[1] and the global prevalence of the disease is unknown ^[2]. Normally, *B. canis* infections in humans have been seen through either laboratory accidents or contact with positive dogs ^[1,3,4].

The main clinical findings of canine brucellosis are reproductive failure such as late abortion, birth of weak offspring, epididymitis, orchitis, and testicular atrophy. Lymphadenitis is a common finding in both sexes, affecting most lymph nodes in the body ^[1,5]. The only definitive method for diagnosis of canine brucellosis is based on the isolation of *B. canis* from various clinical samples ^[3,6,7].



İletişim (Correspondence)



+90 382 2882941



zekiaras@hotmail.com

However, microbiological culture method has some disadvantages such as it is time consuming, includes complex tests, requires skilled personnel and is hazardous for laboratory workers [1,6,7]. Generally, serology can be used for diagnosis of infection but cross-reactions between *B. canis* and other bacteria can occur in some serological tests. Nonspecific agglutination reactions also cause false-positive results in some dogs [4,8,9].

In fact, the polymerase chain reaction (PCR) is a rapid, very specific, highly sensitive, and inexpensive technique for *Brucella* DNA detection. Hence, it is an alternative to bacteriological isolation for direct diagnosis of canine brucellosis [10,11]. Several studies were carried out in recent years in order to standardize PCR assays for the detection of *B. canis* DNA in various clinical samples including canine blood, semen, vaginal swab, blood serum, lymphoid tissue [11-16]. However, the PCR assays were used *Brucella* genus-specific primer pairs that were directed to the 16S-23S rRNA interspace and/or the *virB2* gene regions of *Brucella* spp. The Bruce-ladder multiplex PCR assay has been developed as a rapid and one-step molecular test for identification, typing of *Brucella* species and enhancing to distinguish between *B. suis* and *B. canis* [17,18]. However, this method is rarely used for direct detection of *Brucella* species DNA in clinical samples, because it was designed for bacterial isolates [19]. It is recently reported that, Bruce-ladder multiplex assay does not yield ideal results when DNA is extracted directly from clinical samples because of uneven amplification pattern [19].

With the above consideration in mind, the aim of this study is to use the novel PCR assay to detect *B. canis* in pure bacterial culture and vaginal swab samples, for the first time. The primers are directed to the 16S-23S rRNA interspace region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis* [15,20]. It proved that the PCR assay discriminates *B. canis* from other *Brucella* species and also detects the other *Brucella* species as *Brucella* spp.

MATERIAL and METHODS

Bacterial Strains

Strains examined in this study are listed in Table 1. *Brucella* isolates were classified according to standard microbiological procedures, as described by Alton et al. [3].

Dogs

Vaginal swab samples were collected from 35 bitches from the city's pound of Konya Province of west-central Turkey in the years 2012. Breeds included Boxer (2), Golden Retriever (1), Kangal (3), and Mongrel (29). The ages of the dogs were in the range of 1 to 10 years. Clinical signs that suggested canine brucellosis were investigated in these dogs by questionnaire forms that were obtained from the Veterinarian of the city pound. These data stated that

Table 1. Strains examined in this study and PCR pattern

Tablo 1. Bu çalışmada kullanılan suşlar ve elde edilen PZR bantları

Strain	Source	Biovar	PCR Result (bp)
<i>Brucella canis</i>	NCTC 10854	-	214
<i>Brucella canis</i>	field isolate 1	-	214
<i>Brucella canis</i>	Field isolate 2	-	214
<i>Brucella canis</i>	field isolate 3	-	214
<i>Brucella canis</i>	field isolate 4	-	214
<i>Brucella abortus</i>	S99	biovar 1	214. 774
<i>Brucella abortus</i>	field isolate	biovar 1	214. 774
<i>Brucella abortus</i>	field isolate	biovar 3	214. 774
<i>Brucella abortus</i>	field isolate	atypical	214. 774
<i>Brucella melitensis</i>	16M ATCC 10094	biovar 1	214. 774
<i>Brucella melitensis</i>	field isolate	biovar 1	214. 774
<i>Brucella melitensis</i>	field isolate	biovar 3	214. 774
<i>Brucella melitensis</i>	field isolate	atypical	214. 774
<i>Brucella suis</i>	Unknown	biovar 1	214. 774
<i>Brucella ovis</i>	ATCC 25840	-	214. 774
<i>Brucella neotomae</i>	ATCC 23459	-	214. 774
<i>Rhizobium tropici</i>	CIAT 899	-	-
<i>Escherichia coli</i>	field isolates	-	-
<i>Pasteurella haemolytica</i>	field isolates	-	-
<i>Staphylococcus aureus</i>	field isolates	-	-
<i>Listeria monocytogenes</i>	field isolated	-	-

generalized lymphadenitis, metritis, vaginal discharge, still-birth, osteomyelitis, uveitis, lethargy, decreased appetite, weight loss, and hyperthermia were seen and some of the dogs were reported to have received antibiotics.

Vaginal swab samples were collected in duplicate from the 35 bitches by sterile swabs. One of the swab samples was placed in a tube containing 2 mL of Brucella Broth (Sigma, B3051, MO, USA) for bacterial isolation. The other sample was put into a tube containing 2 mL of TE buffer (10mM Tris-HCl pH 8.0, 1mM disodium EDTA pH 8.0) and was kept at -20°C until used for the PCR assay.

Vaginal swab samples from three dogs were contaminated with *B. canis* NCTC 10854 (supplied by Refik Saydam Hifzissihha Institute, Ankara, Turkey) to determine the detection limit of the PCR assay. The dogs were previously diagnosed as negative to canine brucellosis by clinical, serological and culture examinations. Tested samples were transported to the laboratory under cool condition. The study protocol was approved by Selcuk University Veterinary Faculty Ethical Committee (2007/24).

Bacteriological Examinations

Samples were processed using the method described by Alton et al. [3]. Vaginal swab sample was immediately cultured onto Blood Agar Base (Oxoid, CM0271, Hampshire,

UK) plates containing 5% defibrinated sheep blood with an antibiotic mixture. The cultures were incubated at 37°C under aerobic conditions for 7 days. *B. canis* were identified by morphological and biochemical characteristics.

DNA Extraction

First, reference and field strains were grown in Brain Heart Infusion Broth (Oxoid, CM225, Hampshire, UK). Bacteria were killed by addition of 0.5% formaldehyde. After that DNA was extracted using the protocol provided in Promega Wizard Genomic DNA purification Kit (Promega, A1120, WI, USA). DNA concentration was determined spectrophotometrically (Eppendorf, Model 6131, Germany) by absorbance readings in the range of 260 to 280 nm.

B. canis DNA from vaginal swab samples was extracted using the protocol reported by Leal-Klevezas et al.^[21]. Two mL of Tris EDTA (TE) buffer containing swab samples was used for this purpose. The DNA samples were kept at -20°C until used as templates for amplification.

PCR Assay

The two primer sets of the 16S-23S rRNA inter-space region of *Brucella* spp.^[15] and the deletion of 351 bp in BME11426-BME11427 in *B. canis* ^[20] used in this study are recorded in Table 2.

The amplification reaction mixture was prepared in a total volume of 50 µL containing 5 µL of 10 x PCR buffer, 250 µM each of the four dNTPs (Fermentas, Vilnius, Lithuania), 1.5 mM MgCl₂, 1.5 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 thermal cycler (Ependorf, Mastercycler gradient, Germany) with the following steps: 1 x 7 min at 95°C, 30 x 45 s at 95°C, 45 s at 63°C, 120 s at 72°C, and a final extension at 72°C for 5 min. DNA extracted from *B. canis* NCTC 10854 and nuclease free water was served as positive and negative controls, respectively. The PCR product (10 µL) was further analyzed by electrophoresis on 2% agarose gel, and the gel was stained with ethidium bromide (1.5 µg/mL) and photographed. Reactions were considered positive for *B. canis* when they yielded unique PCR product of 214 bp but products of 214 and 774 bp were accepted positive for the other *Brucella* species.

Table 2. Primers used in this study

Tablo 2. Bu çalışmada kullanılan primerler

Primers	DNA Sequence (5'-3')	Length (bp)	Source
ITS66	ACATAGATCGCAGGCCAGTCA	214	Keid et al. ^[15]
ITS279	AGATACCGACGCAAACGCTAC		
BME11426	TCGTCGGTGGACTGGATGAC	774	Zygmunt et al. ^[20]
BME11427	ATGGTCCGCAAGGTGCTTTT		

Determination of Detection Limit of the PCR

Detection limit of the PCR assay was evaluated 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) for determine of colony-forming unit (CFU) and concentration of undiluted *B. canis* culture calculated as 2.1 x 10⁷ CFU/mL by microbiological culture method. To determine the sensitivity of the assay, decreasing numbers of *B. canis* culture (methanol killed) were added to the 1 mL of the vaginal swab samples (obtained by three number of non-infected bitches). The final concentrations of *B. canis* in mixture were 2.1 x 10⁷, 2.1 x 10⁶, 2.1 x 10⁵, 2.1 x 10⁴, 2.1 x 10³, 2.1 x 10² and 2.1 x 10¹ CFU/g. DNA extraction was performed with all dilutions of mixture, as described previously ^[21]. Then DNA samples processed by PCR assay as described above.

RESULTS

The PCR assay was evaluated with 21 reference and field isolates (Table 1). All *B. canis* strains exhibited unique PCR band of 214 bp. However, other *Brucella* species such as *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis* and *B. neotomae* showed 2 bands of 214 and 774 bp. No PCR products were amplified with DNAs from bacteria genetically related to *Brucella* such as *Rhizobium tropici* and other non-*Brucella* organism commonly associated with animals (Table 1).

To determine the analytical sensitivity of the assay, decreasing numbers of *B. canis* were added to the vaginal swab samples. A positive PCR product for *B. canis* always achieved with different amounts containing at least 2.7 x 10¹ CFU/g of vaginal swab samples. The limit of PCR detection of *B. canis* was determined to be 2.7 x 10¹ CFU/g at least.

A total of 35 vaginal swab DNA samples were tested by the novel PCR assay. Namely, *B. canis* DNA was not detected from any vaginal swab samples in addition to that the samples were bacteriological negative.

DISCUSSION

Microbiological culture method and serological tests are widely used for diagnosis of canine brucellosis. The isolation of agent from different tissues of dogs is still considered as the gold standard for the definitive diagnosis of infection ^[3,7,22,23]. These methods have some disadvantages, while the PCR assay is fast, simple, highly sensitive and specific for detection of *B. canis* ^[11,15]. A very specific, highly sensitive and reliable diagnostic PCR assay for *B. canis* is very important for controlling the spread of infection in animal population and public health. In the present study, a species-specific PCR assay was designed and evaluated for detection and differentiation of *B. canis* in vaginal swab samples.

The primers that used in the novel PCR assay were directed to the 16S-23S rRNA inter-space region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis*^[15,20]. This PCR assay discriminated *B. canis* from other *Brucella* species in a single reaction. The specificity of the species-specific PCR assay was explored with bacteria related to *Brucella* including *Rhizobium tropici*, *Escherichia coli*, *Pasteurella haemolytica*, *Staphylococcus aureus* and *Listeria monocytogenes* (Table 1) and it demonstrated a remarkable good specificity. However, *B. canis* is main etiological agent of canine brucellosis but the other smooth *Brucella* species (*B. abortus* and *B. melitensis*) have occasionally been isolated from dogs^[1]. A major advantage of the present assay is that it can directly identify *B. canis* at species level and also detected all other *Brucella* species as *Brucella* spp. in clinical samples.

In our study, decreasing numbers of *B. canis* were added to the vaginal swab samples to determine the analytical sensitivity of the assay. The novel PCR assay detected at least 2.7×10^1 CFU/g of bacteria diluted in vaginal swab sample. This good analytical sensitivity agreed with the analytical sensitivity of *Brucella* genus-specific PCR assay from vaginal swab samples as previously described^[14].

The degree of sensitivity of PCR assay is a key issue for its effective use in detection of brucellosis^[24]. In our study, to detect the sensitivity and specificity of PCR assay, a total of 35 vaginal swab samples were tested with the PCR and culture methods. All samples were found negative for *B. canis* by two methods and in a full consistence with data between the PCR assay and culture method, the gold standard method for direct diagnosis of *B. canis*.

Finally, we concluded that, the novel species-specific PCR assay has been developed for the direct diagnosis of *B. canis* in vaginal swab samples from dogs. It was proved that the PCR is highly specific and sensitive for detection of *B. canis*. This technique open a new gate to detect alternative for bacterial isolation. We strongly believe that the PCR assay provides a simple and rapid tool for detection of *B. canis* in one step and one day. It is suitable for routine diagnosis of this disease and can be used for confirmation of *B. canis* cultures.

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