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Detection of *Brucella canis* infection in Pit Bull breed dogs in Turkey

Detección de infección por *Brucella canis* en perros de raza Pit Bull en Turquía

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ABSTRACT

Brucella canis infection is an often neglected but important zoonotic disease. This study aims to determine its seroprevalence in Pit Bull dogs from the Western Region of the Turkish Anatolian Peninsula. In the Province of Manisa, 2 mL blood samples were taken from the antebrachial region of 35 Pit Bull dogs using sterile K_2EDTA (3.6 mg) blood tubes, and the samples were analyzed using both the mercaptoethanol (ME) microagglutination test and *B. canis*–specific PCR techniques. Of the 35 dogs tested by 2–ME RSAT, 13 (37.14%) tested positive and 22 (63%) tested negative. Of the 13 dogs that tested positive for 2–ME RSAT, 8 (22.85%) were female, and 5 (14.28%) were male. Subsequent PCR analysis of all samples revealed that 7 (20%; 7/35) of the samples that tested positive for 2–ME RSAT were actually *B. canis*–specific PCR positive. These findings suggest that *B. canis* is present in Pit Bull dogs, although they provide a general idea of the disease's prevalence of the disease in the region. Multicentre studies with larger numbers of cases in different groups of Pit Bulls, such as healthy, patient and risk groups, are needed to provide comprehensive evidence.

Key words: *Brucella canis;* Pit Bull brucellosis; 2–ME RSAT; PCR

RESUMEN

La infección por *Brucella canis* es una enfermedad zoonótica a menudo desatendida pero importante. Este estudio pretende determinar su seroprevalencia en perros Pit Bull de la región occidental de la península turca de Anatolia. En la provincia de Manisa, se tomaron muestras de sangre de 2 mL de la región antebraquial, de 35 perros Pit Bull utilizando tubos de sangre estériles con K_2EDTA (3,6 mg), y las muestras se analizaron utilizando, tanto la prueba de microaglutinación con mercaptoetanol como técnicas de PCR específicas de *B. canis*. De los 35 perros analizados mediante 2–ME RSAT, 13 (37,14%) dieron positivo y 22 (63%) negativo. De los 13 perros que dieron positivo por 2–ME RSAT, 8 (22,85%) eran hembras y 5 (14,28%) machos. El posterior análisis por PCR de todas las muestras reveló que 7 (20%; 7/35) de las muestras que dieron positivo a 2–ME RSAT eran en realidad positivas a la PCR específica de *B. canis*. Estos hallazgos sugieren que *B. canis* está presente en los perros Pit Bull, aunque proporcionan una idea general de la prevalencia de la enfermedad en la región. Se necesitan estudios multicéntricos con un mayor número de casos en diferentes grupos de Pit Bulls, como sanos, pacientes y grupos de riesgo, para proporcionar evidencia completa.

Palabras clave: *Brucella canis;* Pit Bull brucelosis; 2–ME RSAT; PCR

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INTRODUCTION

Brucellosis is a zoonotic disease caused by a group of Gram-negative, aerobic (which may require additional CO_2), coccobacillus, facultative and intracellular bacteria belonging to the *Brucella* genus, which can cause serious Public Health problems as well as significant economic losses on a global scale due to its potential to infect animals. There are twelve species of *Brucella* genus that are accepted, and dogs (*Canis familiaris)* can be infected with four of the six species of *Brucella* spp. (including *B. canis*, *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, and *B. neotomae*) [1, 2, 3, 4]. Contact with contaminated fluids from infected dogs is also an important but rare source of infection in humans. It is estimated that only 1% of diagnosed human brucellosis cases are due to *B. canis* infection [5]. The pathogen has the ability to breach the human body's defences through a variety of entry points, including cracks in the skin, mucous membranes, and the conjunctiva, as well as infiltrating the respiratory, cardiovascular, and gastrointestinal systems. This invasion often results in a systemic infection that can manifest in two distinct phases: acute and chronic. The clinical presentation of this disease may encompass a range of symptoms, including fever, chills, joint pain or inflammation, enlargement of the liver or spleen, and the swelling of lymph nodes [6, 7].

Puppies can be infected through intrauterine vertical transmission or oronasal transmission, which can occur via contaminated milk after birth, contact with placental membranes, or vaginal discharge after abortion. Surviving infected puppies may become permanent carriers of *B. canis*. However, spayed dogs may still many develop complications [5, 8]. Dogs can also be infected in many ways (FIG.1) [9]. Symptoms of infection in dogs are sometimes not obvious. Males may experience problems like epididymitis, prostatitis, and orchitis, affecting testicular size, sperm absence, and fertility [10]. Female dogs may abort between 45–59 days (d) of pregnancy, with a discharge lasting 1–6 weeks (wk). After abortion, around 100 billion microorganisms per mL can spread from infected uterine discharges to the environment in 4–6 wk [11, 12]. In addition, endometritis and placentitis can be seen in female dogs [13].

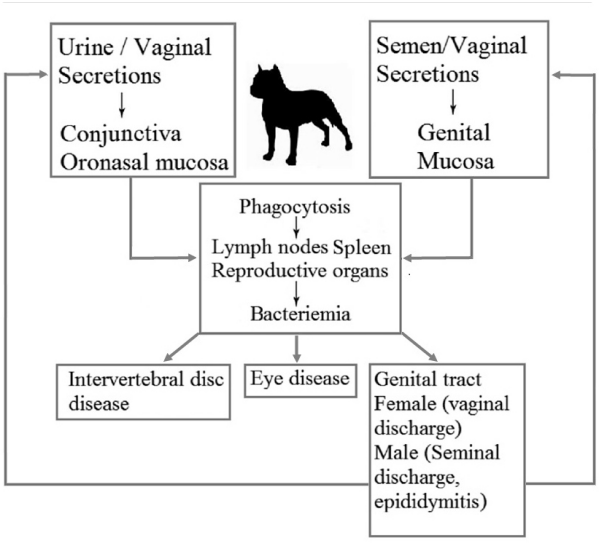

FIGURE 1. The summarized general sequence of the transmission and subsequent clinical events of *Brucella canis* infection in dogs

B. canis enters via genitals, nose, or eyes, taken up by immune cells, settles in lymph nodes, spleen, and genitals through blood. Bacteremia lasts 1–4 wk, can extend to 6 months [11]. Canine brucellosis spreads venereally, orally, via secretions, placenta, and semen. The pathogen remains unaffected by the procedures conducted during semen freezing, retaining its vitality [14]. Bacteria can infect the reticuloendothelial cells such as osteoblasts, osteoclasts, and fibroblasts, particularly macrophages [15], and placenta [16]. It is noteworthy that these microorganisms are classified as hidden pathogens, as they avoid producing conventional virulence markers such as *Brucella* spp. toxins or adhesins within host macrophages [17]. In pregnant dogs with brucellosis, symptoms may appear between 45–55 d of pregnancy. Weak puppies or stillbirths that may die a few days after birth or abortion can be seen on the day of birth [5]. The disease also has zoonotic significance. *B. canis* has the potential to cause severe illness in humans [5]. Some cases of human infection have been linked to non-clinical domestic dogs that have had close contact with individuals diagnosed with brucellosis. However, identifying subclinically infected pet dogs, which lack visible clinical symptoms, can be challenging for both owners and veterinarians [18]. Even when infected domestic dogs exhibit no symptoms, transmission of *B. canis* to humans has been observed [19].

This study aimed to investigate the presence of canine brucellosis in the Western part of Turkey using a 2-mercaptoethanol rapid slide agglutination test and species-specific PCR method in blood samples obtained from Pit Bulls housed at a shelter.

MATERIALS AND METHODS

Sample collection

Between March and April 2023, a total of 35 Pit Bull breed dogs located in the Temporary Animal Care Center of Manisa Metropolitan Municipality had 2 mL blood samples taken from their vena cephalica antebrachii using sterile ethylenediaminetetraacetic acid (K_2EDTA) (3.6 mg) blood tubes (BD, Plymouth, United Kingdom). The blood samples were transported in ice boxes (Igloo, Playmate, Texas, USA) to the Microbiology Department of Aydin Adnan Menderes University Faculty of Veterinary Medicine Research laboratory. The samples were kept at room temperature for 30 min and centrifuged for 15 min at 2,770×G (Hettich, Tuttlingen, Germany). The obtained sera were transferred into 1.5 mL Eppendorf tubes and stored in a -4°C freezer (Bosch, Series 4, Germany) for serological analysis. Ethical approval for this study was obtained from the Dokuz Eylul University Animal Experiments Local Ethics Committee (08/02/2023, and protocol no:08/2023).

Serological tests

The serological test antigen 2-ME (2-mercaptoethanol) rapid slide agglutination test (RSAT) was prepared as previously described [20]. Each serum sample was mixed with 25 µL of 0.2 M 2-ME solution (Thermo Fisher Scientific, Massachusetts, USA) for at least 45 s. Then, 25 µL of *B. canis* antigen was added, and after 2 min of orbital shaking, agglutination was observed. Agglutination formation was considered a positive result [21].

Polymerase chain reaction (PCR), serotyping and DNA extraction

The BcSS primers (Sentebiolab, Ankara, Turkey), specific to the *B. canis* species, used in our study were as follows: F: 5'-CCAGATAGACCTCTCTGGA-3', R: 5'-TGGCCTTTTCTGATCTGTTCTT-3'

[22]. The *B. canis* RM-666 ATCC 23365 standard strain was used as a positive control. The standard serotype was provided by the Department of Microbiology at Selcuk University Faculty of Veterinary Medicine (Konya).

Genomic deoxyribonucleic acid (gDNA) was isolated from blood samples and standard strains using a Genomic DNA Purification Kit® (Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's protocol. The isolated genomic DNA was utilized as a template for PCR amplification. The DNA samples were stored at -20°C in a freezer (Bosch, Series 4, Germany) until they were ready for use in PCR.

B. canis species-specific PCR

B. canis-specific PCR procedures were performed according to the protocol reported by Kang *et al.* [23]. Genomic DNA extracted from each blood sample was amplified in a PCR reaction mixture (25 µL) containing KCl containing 10X reaction buffer (Fermentas, Vilnius, Lithuania), 1.75 mM $MgCl_2$ (Fermentas, Vilnius, Lithuania), 0.2 mM each dNTP (Fermentas, Vilnius, Lithuania), 1 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 1 µL forward primer *B. canis* (10 pmol), 1 µL reverse primer *B. canis* (10 pmol) (Sentebiolab, Ankara, Turkey), and 2 µL template DNA (10 pg–1 µg). PCR amplification was performed using a thermal cycler (Mastercycler Personal; Eppendorf, Netheler, Hinz GmbH, Hamburg, Germany). The PCR cycling parameters were as follows: initial denaturation at 94°C for 7 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 59°C for 40 s, extension at 72°C for 35 s, and final extension at 72°C for 5 min [23].

Agarose gel electrophoresis

PCR products were analyzed through 1.5% agarose gel electrophoresis (Agarose-ME, Classic Type; Nacalai Tesque, Kyoto, Japan) stained with ethidium bromide (AppliChem GmbH, Darmstadt, Germany). The DNA bands were visualized using a gel documentation system (Infinity VX2, Strasbourg, France). Positive DNA samples were scanned at 300 bp.

RESULTS AND DISCUSSION

Serologic identification

In this study, 35 Pit Bull dogs were analyzed for 2-ME RSAT and PCR testing. Of the 35 blood serum samples analyzed, 13 (37.14%) were found to be positive for 2-ME RSAT, while 22 (63%) were found to be negative. Further analysis showed that 22.85% (8/35) of the 2-ME RSAT positive dogs were female, while 14.28% (5/35) were male.

Molecular identification

To confirm the presence of *B. canis* infection, PCR testing was applied to all samples, and 7 (20%) of the 2-ME RSAT positive samples were found to be *B. canis* specific PCR positive. Agarose gel electrophoresis micrograph were given in FIG. 2.

Of the 7 positive samples, 11.42% (4/35) belonged to female animals and 8.58% (3/35) to male animals. Molecular and serological results are shown in TABLE I.

Interestingly, 6 samples (4 females and 2 males) were found positive (17.14%) by serological testing, but PCR testing did not confirm the positivity. This was considered a result of potential cross-reactions in serological tests.

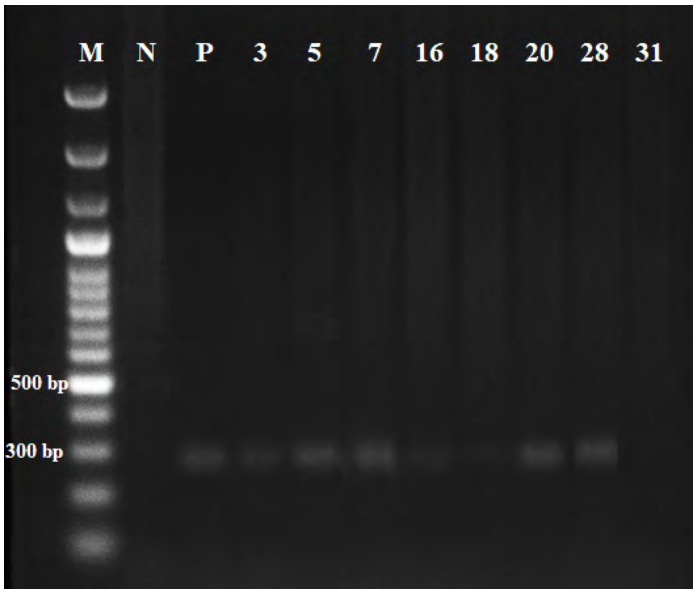

FIGURE 2. *B. canis* specific PCR results of Pit Bull dogs. M: 100 bp ladder (Hibrigen), N: Negative control; P: *B. canis* RM666 ATCC 23365 positive control; 3,5,7,16,18,20,28: *B. canis* positive samples; 31: *B. canis* negative samples

TABLE I
The serological 2-ME RSAT and PCR test results of the Pit Bull blood samples. 2-ME RSAT, 2-mercaptoethanol rapid slide agglutination test; PCR, polymerase chain reaction

Pit Bull blood samples (n=35)	Positive		Serological positivity (%)	PCR positivity (%)
	2-ME RSAT	PCR		
Female	8	4	22.85	11.42
Male	5	3	14.28	8.58
TOTAL	13	7	37.14	20

B. canis infection in humans is usually acquired through direct contact with infected dogs or their reproductive or blood products. Culture is the primary method for diagnosing *B. canis* in humans, but it's complicated by low and intermittent bacteremia. Commercial serological tests for smooth *Brucella* species may not detect *B. canis* antibodies. Although canine serological tests have been adapted for use in humans, their results should be interpreted with caution [24]. While dogs may initially test results positive, their serology values can gradually decrease over time until they become negative [25]. In the present study, Pit Bull dogs were identified as subclinically infected with *B. canis*, even though they displayed no clinical signs. The presence of the pathogen was verified through serological and molecular genetic screening. The incidence of *B. canis* infections has risen in Turkey and other Countries, recently.

Studies have shown that 5.26-31.57% of dogs have tested positive for *B. canis* using tube agglutination (TAT), while ELISA has shown a prevalence of 2.12-15.78%. In a different study, *B. canis* antibodies were discovered in 12 (0.8%) out of 1559 dog sera and 13 (5.8%) out of 225 human sera [26]. Symptoms of brucellosis from *B. canis* in humans are similar to other *Brucella* species, with a risk of death due to endocarditis or meningitis complications at a rate of 2-5%. It is important to take precautions against contamination

and transmission, as the potential for venereal contamination can continue for at least two more years in dogs known to be healthy [26].

A higher prevalence of *B. canis* has been reported in stray dogs in rural settings and on the streets. However, the prevalence of *B. canis* infection in shelter dogs was reported as 2.3%, with a seroprevalence of 17.8% [27]. The prevalence of *B. canis* seropositivity varies from 2 to 30% in different countries [28]. Studies in Brazil have used serological surveys to identify cases of *B. canis* infection in dogs. Reports show that seropositivity in dogs can range from 0 to 54.8% [29]. A study in the United States found a seroprevalence of *B. canis* infection in dogs of 6.8%, with age, breed and breeding history being risk factors associated with the disease [30]. In Mississippi, a recent study found a 2.3% prevalence of *B. canis* infection in shelter dogs [27]. Dogs in Colombia had a seroprevalence of 1.96% for *B. canis* [31]. In Egypt, research showed an apparent prevalence of 3.8% and an actual prevalence of 13.2%, with stray dogs having a higher estimated true prevalence of 15% compared to owned dogs at 12.5% [32]. Positive *B. canis* antibodies were also found in studies conducted in Italy, where 25 out of 2328 sera were positive (1.1%), and Brazil, where 72 out of 280 sera (25.7%) were positive results.

The prevalence of *B. canis* antibodies in dogs varies across different countries. In Canada, out of 33 sera tested, 60.6% were positive, with a range of 0.8 to 44.5%. In Argentina, 14.7% of 224 sera were positive, while in Japan and Korea, 2.5 and 39.1% of 485 and 463 sera, respectively, were positive [33]. In Turkey, studies have shown seroprevalence in dogs ranging from 5.4% and 7.7% [34]. Positive *B. canis* antibodies were found in 7.7% of 362 sera by Oncel *et al.* [28], in 5.4% of 111 sera by Yılmaz and Gümüşsoy [33], in 6.3% of 222 samples by Diker *et al.* [35], and in 6.7% of 134 sera by IstanBulluoğlu and Diker [36]. A study found that dogs fed with leftovers and poor-quality food had the highest prevalence of canine brucellosis (25%), while dogs fed with commercial and formulated quality dog food had lower prevalence values (0.19%) [37].

An epidemiological study of brucellosis among 415 domestic dogs in Beijing, China, between 2006 and 2007 reported a seroprevalence of 0.24%. Another study of domestic dogs in 2012–2013 reported an incidence rate of 47% [22]. Pit Bull dogs have been found to have a significantly higher rate of seropositivity. In fact, it has been suggested that males are more likely to be seropositive for *B. canis* than females, while females may be more susceptible to seropositivity than males [30]. However, in the current study, all seropositive dogs were either neutered or spayed and the gender distribution was almost equal. Therefore, gender was not considered to be an important factor in the disaggregation of data. Due to transmission associated with reproduction, it is normal for *B. canis* seroprevalence to be reduced in neutered or spayed dogs. Also, when the results of the current study were compared with those of previous studies, a lower proportion of samples were found to be positive for *B. canis* antibodies. It was thought that the change in the number of positive samples might be due to the difference in the strains used to prepare the antigen.

The serological methods most commonly used to screen for *B. canis* infection are the rapid slide agglutination test, the 2-mercaptoethanol rapid slide agglutination test (2-ME RSAT), agar gel immunodiffusion and ELISA [24]. The 2-ME RSAT can detect antibodies to *B. canis* in serum samples from dogs [38]. However, this test has restrictions such as low specificity and sensitivity [5, 24]. The limited humoral response observed in dogs infected with *B. canis* may account for this reduced sensitivity of serological tests. This may be due to the intracellular nature of *Brucella* bacteria [39]. Also, treatment of serum with 2-mercaptoethanol increases the specificity of the test by destroying IgM pentamers, which can interfere with the evaluation of IgG, but does not completely eliminate false positives due to heterologous cross-reactions [24].

Molecular techniques are also often used to diagnose canine brucellosis [5, 39]. PCR is a rapid sensitive, and specific test that can be used on blood samples, semen samples from male dogs and vaginal fluid samples from female dogs. PCR can detect inactive bacteria and is unaffected by other bacterial contaminants [40, 41]. However, several factors, including the presence of inhibitors, the use of antibiotics and blood collection techniques involving heparin, can reduce the sensitivity of PCR results [29]. The present study demonstrated that the detection rates of *B. canis* antibodies in pit Bull blood samples ranged from 22.85 to 20% when assessed by 2-ME RSAT and PCR, respectively. Notably, six sera (17.14%) that were initially positive by 2-ME RSAT were negative by PCR. In general, the use of the 2-ME RSAT test for the diagnosis of brucellosis-positive dogs increases the specificity of the test but may result in reduced sensitivity and an increased number of negative results that may still be present in the population [27]. The present diagnostic sensitivity of 2ME-RSAT (37.14%) was similar to that reported by Keid *et al.* [39] (31.76%) and Hensel *et al.* [24] (31.76–70%). When PCR was compared with the 2ME-RSAT serological test, Keid *et al.* [41] stated that PCR diagnostic sensitivity and specificity for the detection of *B. canis* DNA in dog blood was 100%. Sensitivity and specificity results for PCR and 2ME-RSAT are low due to the small number of dogs in our study. However, our research has shown that the combination of 2ME-RSAT and PCR as complementary diagnostic tools for canine brucellosis can significantly increase diagnostic accuracy. This finding also highlights the potential to increase diagnostic accuracy through the synergistic use of these tests. The RSAT test has a high sensitivity, resulting in minimal false negative results. However, its lack of specificity is known to contribute to frequent false-positive results. The reduced humoral response observed in dogs infected with *B. canis* may offer an explanation for the reduced sensitivity of serological tests, given that *Brucella* are facultative intracellular organisms [39, 42].

Therefore, complementing the analysis with PCR testing is essential to achieve accurate results. Several factors could contribute to the discrepancy in test results, including possible infections at different stages in the animals, the presence of different immunoglobulins in the blood serum, or the occurrence of cross-reactions. Human infection with *B. canis* is rare and self-limiting, with only an estimated 1% of diagnosed cases of human brucellosis attributed to this agent [5]. Despite the relatively low prevalence of brucellosis, dog breeders and veterinarians must remain vigilant because of the associated public health risk.

CONCLUSIONS

This study concludes that the combination of the 2-ME RSAT test with PCR is recommended to achieve accurate results and avoid false-positive results in the serological diagnosis of *B. canis* infection in dogs. Although 2-ME RSAT is a widely used diagnostic method for canine brucellosis, PCR-based assays offer higher sensitivity and specificity for the detection of *B. canis*. In addition, PCR-based assays have demonstrated good diagnostic performance for various sample types, making them a valuable tool for the early and accurate diagnosis of canine brucellosis. Further studies are needed to understand the prevalence and risk factors associated with *B. canis* infection in Pit Bull dogs.

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Conflict of interest

The authors have no declaration of competing interests

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