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Detection of different species of *Brucella* in stray dogs using serological and molecular techniques: A strategy for controlling *brucellosis* in humans and animals

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Abstract

Brucellosis is one of the most common infections in humans and animals. The dog has a main role in maintaining and transmitting this pathogen between humans and livestock. Close contact between dogs, humans, and animals may lead to the transmission of *Brucella* species, causing shared illnesses in humans and livestock and economic losses from abortion and stillbirth in animals. This research investigated the occurrence of brucellosis in dogs and compared the sensitivity and specificity of the Rose Bengal test with the traditional Wright test for diagnosing *brucellosis* in dogs. Blood was taken from 46 dog collars without an owner. Rose Bengal, Wright, and 2-ME tests were performed on serum and PCR was conducted on whole blood. The serum samples were first screened with the Rose Bengal test. All sera were then tested with Wright's or standard tube test and 2-mercaptoethanol to confirm positive animals. Based on the Rose Bengal test, the prevalence of brucellosis in the studied dogs was 15.21%. The rate of disease prevalence was determined by Wright and 2-ME methods, 10.86%, and by PCR method, 13.04%. In a titer of 1:80, the positive Wright test served as the diagnostic threshold. The sensitivity, specificity, and positive and negative predictive values for the Rose Bengal test were 100%, 95.12%, 71.42%, and 100%, respectively. According to this research, the Rose Bengal test has significant validity in the contamination of dogs with common strains between humans and livestock. It can also be used as a primary screening test with a titer of 1:80. For a definitive diagnosis, other tests and culture can be used if necessary.

Introduction

Dogs can get *brucellosis* through contact or inhalation of infected secretions, aborted fetuses, or infected placentas, semen, or milk contaminated with *Brucella*. Infection can also be transmitted through ingestion of contaminated materials or sexual intercourse (1). The bacteria can also be found in semen weeks or months after infection, and intermittent shedding

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of smaller amounts may occur for years. *Brucella* species are also excreted through urine, and low concentrations of bacteria may be excreted through saliva, nasal and eye secretions, and feces, so dogs play an important role in maintaining *Brucella* species and their possible transmission to other dogs, cattle, and humans (2). Dog infection with *Brucella* species has been frequently reported in various regions of America, Europe, and Asia. In Iran, several indigenous and mixed dog breeds have been tested for *Brucella*-specific antibodies in the provinces of Fars (3), Khuzestan (4), Tehran (5), Alborz (6), East Azerbaijan (7), Razavi Khorasan, and Markazi and West Azerbaijan (7). Nevertheless, none of these researches focused on isolating and identifying the *Brucella* strains present in dogs in Iran.

Diagnosis typically relies on identifying the causative agent or utilizing serological techniques. Even with a strong focus on finding a conclusive diagnostic method for *brucellosis*, the current diagnosis still depends on multiple tests to prevent false negative outcomes. Serological tests include Rose Bengal, Wright, and 2-ME. The Rose Bengal test has a lot of false positives and is more of a qualitative test, but Wright and 2-ME, in addition to determining the positive *brucellosis*, also gives a titer. In these samples, if the titer is higher than the standard or increases at least 4 times after two weeks, it is reported as positive. These two methods do not determine the *Brucella* strain. The gold standard for the diagnosis of *brucellosis* is bacterial culture and PCR. Unfortunately, cultivation takes 3 to 4 weeks (8). Serological studies have been conducted on dogs in different regions of Iran and reported high to moderate serological prevalence from 15.8% to 3.5% in different provinces (4; 3; 7). This difference in the serological prevalence of *Brucella* among dogs can be related to several factors such as the prevalence of *brucellosis* in the country or region, the study sample and the diagnostic test used, as well as the sampling methods (9). In Iran, *B.melitensis*, biovar 1 was isolated for the first time in 1996 from Iranian sheep-dogs (6).

Up to now, data on strains causing *Brucella* infection in dogs in Iran has been extremely scarce. Even though multiple studies in Iran have documented cases of brucellosis in dogs, the current situation regarding the strains circulating in dogs remains uncertain. *Brucellosis* is a global zoonotic disease caused by different species of *Brucella*. Considering that dogs are one of the reservoirs of this bacterium and by excreting this pathogen they can infect humans and other animals, it is a threat to the economy and public health of countries (10). Therefore, in this study, the presence of different species of *Brucella* in stray dogs was determined by the PCR method. The knowledge about the prevalence of brucellosis in dogs may help to design prevention and control strategies to limit the spread of *Brucella* species to other animals and humans.

Materials and methods

Sampling

Blood samples were collected from stray dogs between 2022 and 2023. In this study, dogs were selected regardless of the symptoms of *brucellosis*, with the possibility of contact with guts and viscera, domestic animal excreta, consumption of placentas, and aborted fetuses. A total of 46 blood samples were analyzed. From each dog, two blood samples with and without anticoagulant were taken for serology and PCR, respectively. Serum and blood samples were kept at -20°C until serology and PCR examinations.

Serological examination

At first, sera were tested for anti-*Brucella* antibodies with the Rose Bengal Test (RBT), a standard qualitative test for *brucellosis* in both humans and animals. The *B. abortus* antigen was bought from the Pauster Institute (Tehran, Iran). To conduct the test, a single drop of RBT antigen and three drops of serum were combined on a white ceramic surface, stirred with a sterile tool, gently agitated for 4 minutes, and examined for agglutination. A positive result was achieved

when specific pink granules (agglutination) were formed (1).

For examination of the Wright serum agglutination test (Wright SAT) and 2-ME, a serial dilution of serum in tubes was prepared. Then, specific brucella antigen for Wright and 2-ME (Pauster Institute, Tehran, Iran) was added (4; 11).

Molecular detection

For DNA extraction, a DNA extraction kit (Cinaccolon-Iran) was used, according to the instructions of the manufacturer. For PCR; 12.5 µL Mastermix, 9. 5 µL sterile distilled water, one µL of forward and reverse primers with a concentration of 10 pmol (Tables 1 and 2), and one µL template DNA with a concentration of 50 ng/µL were prepared and mixed. The amplification of 16SrNA was used for the identification of *Brucella* spp infection (12).

To determine the serovars of *Brucella* species, an enzyme digestion examination (Restriction Fragment Length Polymorphism, RFLP) was used in the presence of *PstI* endonuclease. Electrophoresis of PCR products in each of the above steps was performed on 1.5% agarose gel containing DNA-safe stain solution (SinaGene, Iran) in the presence of a 100 bp DNA marker with a constant voltage of 90 volts for about 1 hour. The desired gel was examined with a UV transilluminator device (Uvitec, UK).

Table 1- The Primer sequence for PCR examination

Fragment length (bp)	Primer sequence (5' to3')	Specify	Reference
223	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCTTTCAGGTCTG	*Brucella spp.*	**(12)**
700	GGCTATTCAAAATTCTGGCG ATCGATTCTCACGCTTTCGT	*B. melitensis*	
1100	CCTTCAGCCAAATCAGAATG GGTCAGCATAAAAAGCAAGC	*B. abortus*	
300	CCAGATAGACCTCTCTGGA TGGCCTTTTCTGATCTGTTCTT	*B. canis*	

Statistical analysis

Data were analyzed using SPSS statistical software, version 26. The prevalence of *Brucella* infection in the sampled population was expressed as a percentage. In this study, the presence or absence of a statistically significant relationship between the frequency of infection with different *Brucella* strains together and the frequency of *Brucella* infection using different serological tests were analyzed with Fisher's exact test or Chi-square test, at a confidence level of 95 percentage.

In this section, using the crosstab tables, thecharacteristics of the Rose Bengal test were evaluated using the results of Wright's serology method and 2-ME to identify *brucellosis*. At first, using the crosstab table, true positive, false positive, true negative, and false negative values were obtained and then used to calculate the sensitivity, specificity, and predictive value of the positive and negative test.

All procedures were performed according to the ethical guidelines set forth by the Faculty of Veterinary Medicine and were approved by the faculty's ethics committee (No.: IR.IAU.SHK.REC.1402.023).

Results

Out of the 6 samples that were positive in PCR (16, 22, 34, 35, 42, and 46), two samples amplified 300 bp fragments related to *B. canis* (35 and 42), and four samples amplified 700 bp fragments related to *B. millitensis* (16, 22, 34, and 46) (Table 5). In the RFLP, 4 positive samples of *B. millitensis* induced 110-280-400-500 bp fragments related to *B. millitensis*biovar 1.

Table 2- Temperature program for PCR reaction (Celsius)

PCR volume (50 µL)	PCR program	Gene
5 µL PCR buffer 10X 2 mM $Mgcl_2$ 200 µM dNTP (Fermentas) 1 µM of each primer F & R 1 U Taq DNA polymerase (Fermentas) 2.5 µL DNA template	1 cycle: 93 ^{0C} ---------- 5 min. 40 cycles: 90 ^{0C} ---------- 60 s 60 ^{0C} ---------- 60 s 72 ^{0C} ---------- 60 s 1 cycle: 72 ^{0C} ---------- 10 min	Detection of *Brucella* spp.
5 µL PCR buffer 10X 2.5 mM $Mgcl_2$ 300 µM dNTP (Fermentas) 0.4 µM of each primer F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template	1 cycle: 94 ^{0C} ---------- 5 min. 30 cycles: 94 ^{0C} ---------- 60 s 50 ^{0C} ---------- 120 s 72 ^{0C} ---------- 180 s 1 cycle: 72 ^{0C} ---------- 7 min	*B. melitensis*
5 µL PCR buffer 10X 2.5 mM $Mgcl_2$ 300 µM dNTP (Fermentas) 0.6 µM of each primer F & R 1.5 U Taq DNA polymerase (Fermentas) 3 µL DNA template 5 µL DMSO	1 cycle: 95 ^{0C} ---------- 5 min. 30 cycles: 94 ^{0C} ---------- 45 s 58 ^{0C} ---------- 60 s 72 ^{0C} ---------- 60 s 1 cycle: 72 ^{0C} ---------- 10 min	*B. abortus*

Table 3- The Results of *brucellosis* diagnosis in 46 studied dogs

Animal code	Rose Bengal	Wright	2-ME	PCR	Animal code	Rose Bengal	Wright	2-ME	PCR
1	-	-	-	-	24	-	-	-	-
2	-	-	-	-	25	-	-	-	-
3	+	-	-	-	26	-	-	-	-
4	-	-	-	-	27	+	+	+	-
5	-	-	-	-	28	-	-	-	-
6	-	-	-	-	29	-	-	-	-
7	-	-	-	-	30	+	+	+	-
8	-	-	-	-	31	-	-	-	-
9	-	-	-	-	32	-	-	-	-
10	-	-	-	-	33	-	-	-	-
11	+	-	-	-	34	-	-	-	+
12	-	-	-	-	35	-	-	-	+
13	-	-	-	-	36	+	+	+	-
14	-	-	-	-	37	-	-	-	-
15	-	-	-	-	38	-	-	-	-
16	-	-	-	+	39	-	-	-	-
17	-	-	-	-	40	-	-	-	-
18	-	-	-	-	41	+	+	+	-
19	-	-	-	-	42	-	-	-	+
20	-	-	-	-	43	-	-	-	-
21	-	-	-	-	44	-	-	-	-
22	-	-	-	+	45	-	-	-	-
23	+	+	+	-	46	-	-	-	+

Table 4- Frequency (number and percentage) of *brucellosis* infection in different diagnostic methods in 46 studied dogs

Diagnostic Method	Number	Percent
Rose Bengal	7	15.21
Wright	5	10.86
2-ME	5	10.86
PCR	6	13.04

Table 5- Frequency of *brucellosis* with different *Brucella* strains in the PCR method in 46 studied dogs

***Brucella* strain**	**Number**	**Percent**
B. mellitensis	4	8.69
B. canis	2	4.34

Characteristics of the Rose Bengal in comparison to standard serological tests

Out of 7 positive samples in the Rose Bengal, 5 true and 2 false positive samples were identified in the Wright and 2-ME at the cut point of 1:80and 1:40, respectively. Also, out of 39 negative samples in Rose Bengal, all of them were found to be negative in the Wright (true negative). At this cut-off point, 95.12% (39 out of 41 samples) that were identified in Wright's as negative, were also reported as negative in the Rose Bengal. Table 6 shows the frequency distribution of Rose Bengal and Wright results at the cut point of 1:80 in dogs with *brucellosis* separately. The Rose Bengal had a sensitivity of 100, a specificity of 95.12, a positive predictive value of 71.42, a negative predictive value of 100, and an accuracy of 95.6% at the 1:80 titer cut point of Wright. In Table 7, the sensitivity, specificity, positive and negative predictive value, and accuracy of the Rose Bengal according to the result of the Write test at the cut-off point of 1:80 have been determined separately. In this study, the presence or absence of a statistically significant relationship between the frequency of infection with *B. canis* and *B. millitensis* was statistically evaluated. In data analysis with Fisher's exact test or K2 test, at the 95% confidence level, there is a statistically significant difference between the frequency of *Brucella* infection in PCR and antibody detection using 2-ME or Rose Bengal examination (P=0.036). In addition, there is a statistically significant difference between the frequency of infection with *B. millitensis* and other *Brucella* species in the PCR (P=0.041).

Table 6- The results of Wright and Rose Bengal at the 1:80 cut point of 46 studied dogs

		2-ME / Wright		
		Pos.	Neg.	Total
Rose Bengal	Pos.	5	2	7
	Neg.	0	39	39
	Total	5	41	46

Table 7- Characteristics of the Rose Bengal test in comparison with Wright/ 2-ME for diagnosis of *brucellosis*

Actual Positive	**5**
False Positive	2
Actual Negative	39
False Negative	0
Accuracy (%)[1]	95.6
Sensitivity (%)[2]	100
Specify (%)[3]	95.12
Positive Predictive Value (%)[4]	71.42
Negative Predictive Value (%)[4]	100

[1]Accuracy: Total actual positive and negative samples to total samples
[2]Sensitivity: Actual positive samples to actual positive and false negative samples
[3]Specify: Actual negative samples to actual negative and false positive samples
[4]Positive predictive value: Actual positive to Actual positive and false positive
[5]Negative predictive value: Actual negative to Actual negative and false negative

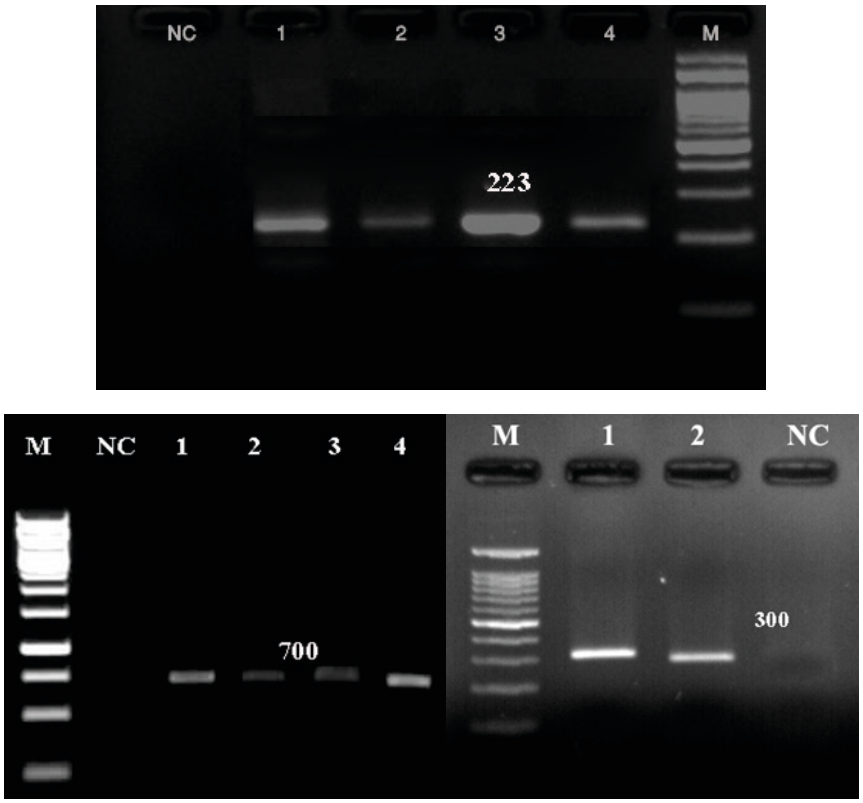

Fig. 1. The gel electrophoresis of the PCR products related to the detection of the*Brucella* genus and species. Column M = 100 bp DNA ladder, Column NC = negative control. **A.** *Brucella* genus,Columns 1-4 = positive samples; **B.***B.mellitensis,*Columns 1-4 = positive samples; **C.***B. canis,*Columns 1-2 = positive samples

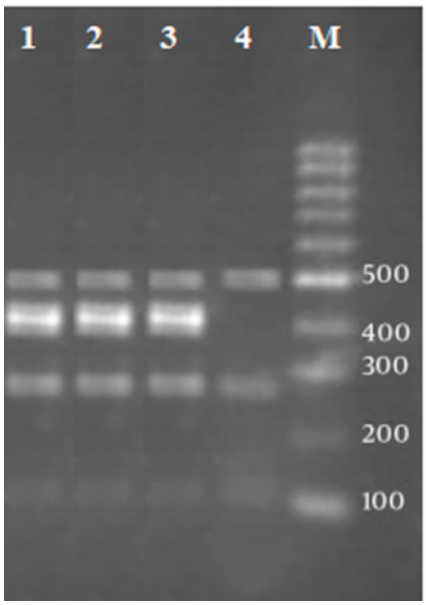

Fig. 2. The gel electrophoresis of the RFLP product related to the detection of *B. mellitensis*serovars

Column M = 100 bp DNA ladder, Columns 1-4 = positive samples (110, 280, 400, and 500 bp fragments).

Discussion

In this study, the sera of 46 dogs were examined for *brucellosis* using Rose Bengal tests, Wright SAT, 2-ME, and PCR. The serum samples of the dogs were first screened with the Rose Bengal test. 7 dogs (15.21%) were positive in the Rose Bengal screening test. Then all sera were tested with Wright's test and 2-ME to confirm positive samples. A titer of 1:80 or higher in Wright's test and 2-ME assays for *Brucella*-specific antibodies was considered positive according to the standard methods of the World Health Organization (WHO). 5 dogs (10.86 %) showed a positive reaction with 1:80 ratio in Wright's test and 2-ME, and the prevalence of *brucellosis* by PCR method was 13.04 % (6 out of 46 dogs).

In this study, the Rose Bengal test had a sensitivity of 100, a specificity of 95.12, a positive predictive value of 71.42, a negative predictive value of 100, and an accuracy of 95.6% at the titer cutoff point of 1:80 of Wright's test. Among the serological methods in the diagnosis of *brucellosis*, Wright's serum agglutination test is the most widely used. There are also false negatives in the Write test for various reasons. This may be due to test performance at an early stage of infection or the presence of blocking antibodies (prozone phenomenon) (13). In this study, all the dogs that were positive in the Rose Bengal, Wright, and 2-ME tests, were negative in the PCR method. In the study by Morshedi et al. (2010) evaluating the PCR test for diagnosing bovine brucellosis, similarly, it was found that the PCR test failed to detect several positive sampleswhen compared to the standard serological method (14). Serology tests detect the presence of antibodies in the blood, while PCR shows the presence of the genome of bacteria in the blood. In all these samples, the bacteria were probably not in the blood at the time of blood collection and were localized in the tissue, so the result of the PCR test in the blood was negative. Generally, PCR on a blood sample is effective for detecting *brucellosis* in the acute phase. The bacteremia decreases over time and decreases the probability of being positive in infected dogs. In a study by Mahzounieh et al. (2015), it was found that the seropositive animals did not test positive for*Brucella*using PCR, which could be due to the occurrence of cross-reactions in serological tests or the activation of the immune system and elimination of bacteria from the internal organs (15).

In this study, positive PCR samples were found-alongside negative serology tests. This may indicate thatthese samples are from the early stages of the disease, where immune response against the bacteria is not yet strong enough to be detected by serological methods. Tadjebakhche and Gatel (1972) were the first to examine dog blood serum for brucellosis in Iran (5). After that, numerous serological investigations have been carried out in various areas employing various diagnostic methods. In the current study, the occurrence of serum and molecular *brucellosis* ranged from 10-15% across various methods. In prior Iranian reports, the specified ranges vary greatly, either lower or higher at times. Climatic variations are responsible for the disparity in the occurrence of canine *brucellosis* between Isfahan city and other areas of Iran. Furthermore, there has been a substantial increase in farmers' understanding of *brucellosis* in recent years. This has reduced the risk of stray dogs being in contact with livestock and their miscarried fetuses. There is uncertainty regarding the impact of dogs on human *brucellosis* in Iran because of the absence of a thorough report on the subject. Nonetheless, the presence of antibodies in dogs infected with zoonotic *Brucella* species suggests the potential for these bacteria to spread from dogs to both humans and farm animals in the area.

In the study conducted by Gharekhani and Sazmand (2019) in Hamedan, *brucellosis* in dogs was detected by Rose Bengal and Wright methods. These researchers reported a prevalence rate of 3.3% (7). However, these two methods do not test for *B. canis*-specific antibodies. In Ahvaz city, 102 blood samples from companion dogs were analyzed using a commercial kit, and revealed an infection rate of 4.9% (16). Furthermore, in a study conducted in Fars province

using the same kit found that 10.6% of the examined dogs tested positive (3). Also, in Kerman province, using the immunofluorescence antibody kit (IFA), 15.8% of the samples tested positive for *B. canis* (4). This rate was 20.9% in São Paulo, Brazil (using blood culture method), 4.9% in Mississippi, USA (using rapid serology method) and 4.4%in South Africa (using tube agglutination test (2-mercapto-ethanol) (1, 17, 18). Since rapid diagnostic and IFA kits for B. canis are not often brought into Iran, it is recommended that Iranian researchers concentrate on making these diagnostic kits domestically. In the sole PCR study conducted in Iran, it was found that 14 out of 94 blood samples (14.9%) taken from companion dogs in Isfahan and Shahrekord tested positive (19). In our study, the prevalence of *B. canis*infection was determined to be 4.34% by PCR method, which was similar to the results of Moslinejad et al. (16).

Considering the challenges in performing molecular and ELISA methods (longer processing times and higher costs) in most regions, serological methods remain the most widely used method of diagnosis in many regions. These methods are cheaper and more accessible method with relatively acceptable sensitivity and specificity. Among the serological tests, we can mention Rose Bengal, Wright, 2-ME test. In one part of the study, the performance and characteristics of the Rose Bengal screening test were compared to those of the standard, more accurate serological tests (Wright and 2-ME). According to the final results, the sensitivity and specificity ofthe Rose Bengal test was reported as 100% and 95.12%, respectively. Matović et al., (2008), determined the sensitivity of Rose Bengal, Wright, and ELISA serological tests to be 86.6%, 100%, and 100%, respectively (20). Farazi and Hosseini (2012), examined 297 serum samples from a flock of sheep that had a history of abortion by serological tests. In this study, the sensitivity and specificity of the Rose Bengal were reported as 81.5% and 94%, respectively. The positive predictive value of tests for Rose Bengal was 93 and that of 2-ME was 94.8, whereas the negative predictive values for Rose Bengal and 2-ME were 84% and 80.1%, respectively (21). The differences in reported data may be due to the difference in the statistical population studied because, in the above study, the dogs of the region were randomly sampled without considering the clinical symptoms suspected of *brucellosis*, but they were present in the region. In several studies, only dogs suspected of *brucellosis* were examined. Also, the slight discrepancy can be due to the sample size, the use of the negative control group, and the difference in the kit used.

In the present study, in the RFLP test, all 4 positive samples of *B. melitenensis* were related to biovare 1. Identification of biovars is very important in the epidemiology of *brucellosis*. In the biotyping results of Sadeghi et al. (2022), all three samples isolated from goat blood culture were also detected as *B. meliensis* biovar 1 (22).

In Iran, *B. melitensis* was initially found in a sheep in Isfahan in 1950, and later its biovar 1 hasbeen occasionally found in other regions of the country, affecting sheep, goats, cattle, camels, sheepdogs, and humans. Biovar 1 of *B. melitensis* is particularly prevalent in Isfahan, Khorasan, Gilan, Khuzestan, Yazd, and Kermanshah regions, while biovars 1, 2, and 3 are commonly found in Tehran and Azerbaijan (23). According to this study conducted in Isfahan province reveals the detection of biovar 1 in dogs, indicating an outbreak of this biovar in the area.

Conclusions

Considering the relatively high seroprevalence of *Brucella* species observed in this study, designing screening programs to control infection across various regions of Iran is highly recommended. The results of the current research indicated that the Rose Bengal test is considered positive at a cutoff point of 1.80 Wright. This test displays satisfactory sensitivity, and specificity, as well as positive and negative predictive values for diagnosing Brucellosis.Therefore, the diagnostic power and accuracy of the Rose Bengal test are very similar to those of the Wright

test. Thus, it seems that the use of the Rose Bengal test in areas at risk forbrucellosi*s* in stray dogs is highly effective for detecting infections caused by common *Brucella* species.

Acknowledgments

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Ethical approval

The study was approved by the ethical committee of the Veterinary Medicine Faculty, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran (Ethical no. IR. IAU. SHK. REC. 1402.023).

Conflict of interests

There is no conflict of interest between the authors.

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