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Comparison of duplex polymerase chain reaction and Rose Bengal test for diagnosis of brucella abortus and brucella melitensis

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Original Article

Abstract

BACKGROUND: Diagnosis of brucellosis requires a rapid and accurate method such as the polymerase chain reaction (PCR). The purpose of this study was simultaneous detection of Brucella abortus (B. abortus) and Brucella melitensis (B. melitensis) in serum samples using the duplex PCR technique and then comparing the results using the Rose Bengal test (RBT).

METHODS: In this comparative-descriptive study, 100 serum samples were collected from a veterinary station located in Shahriar City, Iran. Moreover, the monoplex-PCR of B. abortus and B. melitensis and duplex-PCR for both agents was optimized. The limit of detection (LOD) and specificity test were also checked. Besides, deoxyribonucleic acids (DNAs) were extracted from the serum samples by the DNA extraction solution (DNG-plus) technique. The PCR product was cloned in pTZ57R plasmid by T/A cloning.

RESULTS: B. abortus (494bp) and B. melitensis (733bp) amplicons were observed in 1.5% gel electrophoresis. The LOD of the monoplex-PCR test for both of the agents was 100 genomes per reaction. Additionally, 40 out of 100 samples were positive for RBT, out of them, 35 samples were positive with duplex-PCR, 31 samples were positive for B. abortus, and 4 for B. melitensis; moreover, 20 samples were positive with duplex PCR from 60 negative RBT. From this number, 17 samples of B. abortus and 3 samples of B.melitensis were detected.

CONCLUSION: The number of positive samples by duplex-PCR was more than the RBT; therefore, we can assert duplex-PCR for confirming the RBT results.

KEYWORDS: Brucellosis; Rose Bengal; Culture; Polymerase Chain Reaction

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Introduction

Brucellosis is a common infectious disease among humans and animals (zoonotic disease), which is caused by the Brucella species of bacilli.[1,2] More than 500000 new human brucellosis cases are reported annually worldwide.[2] In Iran, during the last decade, a total of 173526 cases were reported from different provinces of Iran.[3] Although it is rarely fatal, it can cause some severe complications in the absence of a rapid and appropriate diagnosis.[3,4] In addition, Brucella is injected into the body of its mammalian host through cuts and scratches on the skin surface,

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consuming the contaminated food, direct contact with the infected animal, and inhaling the contaminated particles and is also one of the most important diseases that affect humans and animals in developing countries.[5-7] In humans, it causes fever, sweating, weakness, lethargy, and weight loss. In addition to pathogenicity in humans and health problems, Brucella has many economic disadvantages in the livestock industry because it causes abortion, reduced milk production, infertility, and the cost of veterinary and medical care.[8] Brucellosis has various clinical manifestations causing diagnostic problems for the treatment.[9] The classic discovery of this bacterium is usually performed based on serologic methods and culture.[10,11] These techniques are time-consuming, with relatively low sensitivity, and require facilities and also sufficient experience to interpret the results.[12,13] Overall, serological methods such as tubular agglutination and Rose Bengal test (RBT) are commonly used in clinical laboratories to diagnose brucellosis. Despite false-positive reactions due to cross-reactive microorganisms such as Vibrio cholerae (V. cholerae) and low sensitivity, these tests continue to be the most important paraclinical tests for the diagnosis of brucellosis. Due to the prevalence of brucellosis in Iran, rapid diagnosis by choosing a sensitive and specific laboratory method and timely treatment of patients is very important.[14]

In the last few decades, several molecular methods have been developed, especially for the propagation of specific nucleic acid sequences of pathogens, which made the rapid detection of microorganisms with high sensitivity and specificity compared to the traditional methods.[15,16] Queipo-Ortuño et al. showed that LightCycler-based real-time polymerase chain reaction (LC-PCR) assay had 91.9% sensitivity and 95.4% specificity when tested with 65 negative control samples and 62 serum samples from 60 consecutive patients with active brucellosis.[17] Soleimani et al. reported that real-time quantitative loop-mediated isothermal amplification was highly specific and no amplification products were observed from the non-Brucella organisms.[18] Dal et al. showed that the sensitivity of multiplex real time-polymerase chain reaction (mRT-PCR) in the samples that were positive by immunocapture test (ICT), standard tube agglutination test (STAT), Coombs test, and blood culture was 70.2%, 77.3%, 83%, and 97.2%, respectively. Polymerase chain reaction (PCR) can be considered a useful diagnostic tool in patients who have negative serologic test results, and in the detection of Brucella species.[11] Considering the importance of rapid and accurate detection of Brucella abortus (B. abortus) and Brucella melitensis (B. melitensis) in patients with brucellosis, for timely treatment and prevention of the onset of secondary complications, and due to the time-consuming culture method, the need for a quick, sensitive, and easy method at the same time with a low cost, proprietary, and expert advice is essential.[17,18]

The RBT serologic test is a quick, simple, and sensitive test that is used as one of the screening tests for brucellosis. If the test result is positive, other bacteriological and serological methods will be used for the final confirmation of brucellosis. Molecular methods are more sensitive, and easy, with a low cost,[11,17,18] in identifying pathogenic microbes and the type of microbes. Therefore, comparing molecular methods with serological methods and determining the accuracy level of the response of the two methods will help the therapist and the researcher to use the better method according to the conditions of the samples. This study aimed to improve the duplex-PCR assay for the optimization of the detection of B. abortus and B. melitensis and then to compare its results with the RBT.

Methods

In this comparative-descriptive study, 100 samples of serum from cattle and sheep were

collected randomly, in sterile microtubules in a veterinary laboratory located in Shahriar City, Iran, and were then stored after being transferred to the laboratory of Islamic Azad University, Qom Branch, Iran (in 2020), in a refrigerator for performing further experiments. Sampling was done from animals that were healthy and did not have infectious or non-infectious diseases. Sick animals were excluded from the study. Further, the strains of B. abortus (ATCC23448) and B. melitensis (ATCC23457) were prepared as positive control strains. Moreover, the strains of B. abortus (ATCC23448) and B. melitensis (ATCC23457) and the deoxyribonucleic acid (DNA) of each strain were extracted using the kit (Sinaclon's DNG-Plus, Iran).

For the RBT, 30 µl of each serum was dispensed on a white glossy ceramic tile and then mixed with an equal volume of RBT antigen. Afterward, the tile was rocked for 4 minutes at room temperature, and any visible agglutination was taken as a positive result.[19]

Optimization of the PCR: In this study, the primers IS711gene were used for the detection of B. abortus and B. melitensis. To optimize the PCR technique, the appropriate concentration and volume of the components required for this method were evaluated, and finally, the required values were obtained in 25 µl PCR reaction consisting of 2.5 µl of 10X PCR buffer, 0.5 µl deoxynucleotide triphosphates (dNTPs) (10 mM), 0.75 µl magnesium dichloride (MgCl2) (50 mM), 0.3 µl Taq DNA polymerase enzyme (1.5 U), 0.5 µl of each 10 µM primer (Table 1), 15 µl of distilled water [deuterium depleted water (DDW)], and 5 µl of DNA template. PCR amplification was performed under thermal conditions as follows: initial denaturation for 5 minutes at 94 ℃, followed by 35 cycles each of 30-second denaturation at 94 ℃, 30-second annealing at 61 ℃, 1-minute extension for 72 ℃, and the final extension for 5 minutes at 72 ℃. The duplex-PCR product was then electrophoresed on a 1.5% agarose gel containing SYBR Green in a tris-borate-ethylenediaminetetraacetic acid (TB-EDTA) 0.5X buffer. Notably, the specific band produced for B. abortus and B. melitensis was visible.[20,21]

Determination of the sensitivity and specificity of PCR assay: To determine the sensitivity of the optimized PCR test, for different dilutions of DNA extracts of B. abortus and B. melitensis, a PCR test was performed with a positive control and a negative control. To determine the specificity of the optimized PCR test for B. abortus and B. melitensis, DNA extracts were obtained from Escherichia coli (E. coli), Listeria monocytogenes (L. monocytogenes), Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus), Legionella pneumophila (L. pneumophila), Salmonella typhimurium (S. typhimurium), and Streptococcus pyogenes (S. pyogenes) using DNG-plus method and an optimized PCR.

PCR product cloning: After purification, the PCR product was cloned using the T/A cloning kits (Thermo Fisher Scientific Inc., USA) and vector pTZ57R. The resulting plasmids were then extracted. Afterward, the obtained plasmids were extracted using the alkaline lysis method.

Statistical analysis: Data analysis was performed using Minitab software.

Table 1. Primer sets and expected amplicon sizes specific for Brucella abortus (B. abortus) and Brucella melitensis (B. melitensis)

Target genes	Primer sequences (5/ to 3/)	Size of amplicon base pair (bp)	References
IS711 element of B. abortus	5'-AAATCGCGTCCTTGCTGGTCTGA-3' 5'-TGCCGATCACTTAAGGGCCTTCAT-3'	731	20
IS711 element of *B.* melitensis	5'-GACGAACGGAATTTTTCCAATCCC-3' 5'-TGCCGATCACTTAAGGGCCTTCAT-3'	498	21

B. abortus: Brucella abortus; *B. melitensis:* Brucella melitensis

Considering the selection of 100 samples, our samples represent a normal population, which will prove our hypothesis by testing the mean difference between two normal populations to show the superiority of the results of the duplex PCR test over the RBT. We assume that the information about the two populations is as follows: we want to test the hypothesis that the means of the two populations are the same (Table 2) (thesis code in Islamic Azad University, Qom Branch: 15430507941001).

Table 2. Two studied populations

Community 1	Formula	Community 2	Formula
Average	$x_1 = \frac{1}{n_1}\sum x_i$	Average	$x_2 = \frac{1}{n_1}\sum x_i$
Statistics	1	Statistics	2
Number of samples	n1	Number of samples	n2

If the hypotheses of a study refer to the comparison of the means of an attribute in two populations, we can follow all the assumptions made in the tests related to a population and only concerning the equality or inequality of variance to follow the test steps as before.

Test statistics, depending on whether the variances of the two communities are assumed to be equal or not, are in two general ways:

1- Test index when the variances of two populations are equal:

$$Q_{H_0} = \frac{\bar{x}_1 - \bar{x}_2}{\sigma\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

2- Test index when the variances of two populations are not equal:

$$Q_{H_0} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

In tests related to the difference between the mean of two populations, due to the unknown variance and sample size, the distribution of the test index will eventually lead to one of the distributions of z or t.

If the distribution of the statistical population is normal when the number of samples is less than 30 and the variance of the population is unknown, the statistical index of the distribution test is t. The curve of this distribution is similar to the normal distribution and the characteristic of this distribution is its degree of freedom (df) which is determined by n-1.

$$\begin{cases} H_0 : \mu_1 = \mu_2 \\ H_1 : \mu_1 \neq \mu_2 \end{cases} \qquad |Q_{H0}| > Z_{\frac{\alpha}{2}}$$

$$\begin{cases} H_0 : \mu_1 = \mu_2 \\ H_1 : \mu_1 > \mu_2 \end{cases} \qquad Q_{H0} > Z_{\alpha}$$

$$\begin{cases} H_0 : \mu_1 = \mu_2 \\ H_1 : \mu_1 < \mu_2 \end{cases} \qquad QH0 < -Z_{\alpha}$$

Results

Of the 100 sera from cattle and sheep yielded only 40 were positive for the RBT and 60% were negative for B. abortus and B. melitensis. However, the result of duplex-PCR evaluated 48 B. abortus and 7 B. melitensis.

Hypothesis test results for two parameters (for the average of a population) and the decision rule used in this research are as follows:

$$\begin{cases} H_0 : \mu_1 = \mu_2 \\ H_1 : \mu_1 > \mu_2 \end{cases} \qquad Q_{H0} > Z_{\alpha}$$

1) We formulate test hypotheses. The significance level is set at 0.5. We reject the null hypothesis when the value of the test index is greater than the value of table z:

Reject H_0 if $Z > Z_{1-a}$

If we form the first population with the results of the RBT and the second population with the results of the duplex-PCR test, the results of this method will indicate the superiority of the duplex-PCR test for testing 100 livestock serum samples. Testing this hypothesis will prove our theory as follows.

Statistical calculations of the data from the present study were performed with Minitab software (Table 3).

Table 3. Statistical results

Statistical index	Unit	Results
Average normal distribution 1	μ1	0.40
Average normal distribution 2	μ2	0.55
Variance 1	Var1	0.24
Variance 2	Var2	0.25
SD 1	α1	0.49
SD 2	α2	0.50
Total	Z (0.975)	1.96

SD: Standard deviation

Moreover, using these data (Table 3), the normal distributions of the RBT (Figure 1) and duplex PCR test were drawn (Figure 2).

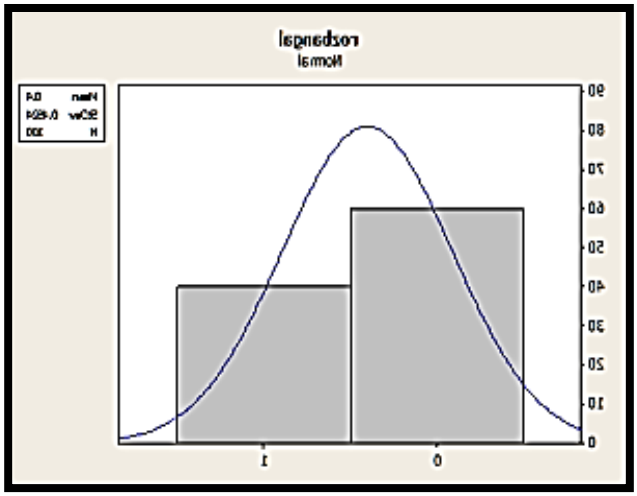

Figure 1. Normal distribution of Rose Bengal plate test

The results of the PCR assay showed that only 100 copies of DNA were replicated, and in the titers of less than 100 copies of the DNA, no band was detected, which indicates the high sensitivity of the test. The PCR assay for E. coli, L. monocytogenes, P.aeruginosa, S. aureus, L. pneumophila, S. typhimurium, and S. pyogenes showed no unwanted product and band, which indicated that the PCR test had a very high profile and the specific band produced for B. abortus and B. melitensis was visible.

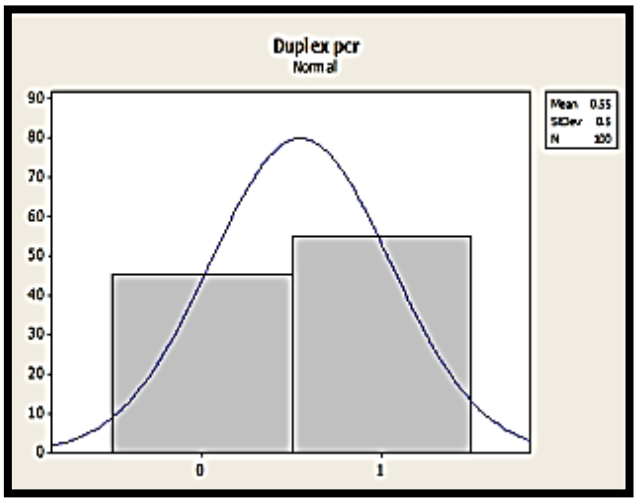

Figure 2. Normal distribution of duplex polymerase chain reaction (PCR) assay

Discussion

Brucellosis is one of the most important zoonotic diseases, which is frequent in most countries such as the Mediterranean and the Middle East regions, including Iran.[22-24] The purpose of this study was simultaneous detection of B. abortus and B. melitensis in serum samples using the duplex PCR technique and then comparing the results using the RBT.

The results of this study indicate that the RBT is one of the most prevalent tests. More commonly, this method can be used for early diagnosis of brucellosis control and eradication programs as a test that can be performed before performing the other serological tests.[17,23] It is recommended that all the serum samples should be tested using the tube method due to the probability of occurrence of prozone phenomenon and observation of a false-negative answer. Furthermore, the bacterial growth phase can produce false-negative reactions. As the antibodies (agglutinin) in the serum of the individual tested may be due to bacterial infections rather than Brucella bacteria, it should be noted that the incidence of agglutination-positive testing may not be due to the Brucella bacteria.[24,25] Saadat et al. described that the specificity and

sensitivity of PCR tests were more than the conventional methods for brucellosis diagnosis.[15] AL-Shemmari compared conventional and molecular tests to detect brucellosis in cattle and buffaloes, and concluded that PCR could be considered the most important and reliable test compared to the other techniques.[13] In other research, Hekmatimoghaddam et al. noted that the PCR assay was the golden test and could be used for the determination of brucellosis.[1] Dal et al. compared real-time PCR (RT-PCR) with the serological and culture methods for detecting human brucellosis and their results indicated that RT-PCR could be considered a suitable method for the detection of Brucella species in false-negative serologic results.[11] Molecular techniques are now standardized, valid, and accepted by international authorities.[1,11,15] Moreover, according to the calculations performed using the Minitab software, the test statistic is greater than Z (0.975), which rejects the assumption of the equality of the two pre-Bengal and duplex PCR tests. Additionally, our theory is that the duplex PCR test for diagnosis of B. abortus and B. melitensis is more appropriate. The samples of this study were taken from animals, and it is better to conduct similar research on human samples. Besides, two tests were compared in this study. For further studies, it is suggested that different molecular tests and different serological tests be compared.

Conclusion

It can be concluded that the duplex PCR method used in this study to detect B. abortus and B. melitensis was safer. Further, the reason for having more positive outcomes compared to the RBT was the use of this technique in the rapid and accurate diagnosis of brucellosis.

Conflict of Interests

Authors have no conflict of interests.

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