

The Optimization of Molecular Detection of Clinical Isolates of *Brucella* in Blood Cultures by *eryD* Transcriptase Gene for Confirmation of Culture-Negative Samples

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Abstract

Background: Brucellosis is a zoonosis disease which is widespread across the world.

Objectives: The aim of the present study is the evaluation of culture-negative blood samples.

Materials and Methods: A total of 100 patients with suspected brucellosis were included in this experimental study and given positive serological tests. Diagnosis was performed on patients with clinical symptoms of the disease, followed by the detection of a titer that was equal to or more than 1:160 (in endemic areas) by the standard tube agglutination method. Blood samples were cultured by a BACTEC 9050 system, and subsequently by *Brucella* agar. At the same time, DNA from all blood samples was extracted by Qiagen Kit Company (Qia Amp Mini Kit). A molecular assay of blood samples was carried out by detection of *eryD* transcriptase and *bcsP 31* genes in specific double PCR reactions. The specificity of the primers was evaluated by DNA from pure and approved *Brucella* colonies found in the blood samples, by DNA from other bacteria, and by ordinary PCR. DNA extraction from the pure colonies was carried out by both Qiagen Kit and Chelex 100 methods; the two were compared.

Results: 39 cases (39%) had positive results when tested by the BACTEC system, and 61 cases (61%) became negative. 23 culture-positive blood samples were randomly selected for PCR reactions; all showed 491 bp for the *eryD* gene and 223 bp for the *bcsP 31* gene. Interestingly, out of 14 culture-negative blood samples, 13 cases showed positive bonds in PCR. The specificity of the PCR method was equal to 100%. DNA extraction from pure cultures was done by both Chelex 100 and Qiagen Kit; these showed the same results for all samples.

Conclusions: The results prove that the presented double PCR method could be used to detect positive cases from culture-negative blood samples. The Chelex 100 method is simpler and safer than the use of Qiagen Kit for DNA extraction.

Keywords: Detection, Chelex 100, *eryD* Gene, *Brucella*

1. Background

Brucellosis is a zoonose disease that is found worldwide; it can affect humans as well as many domestic and wild animals. Transmitted infection to humans might be the result of direct contact with animals, or drinking non-pasteurized milk (1). Brucellosis is one of the biggest global health problems, especially in developing countries. Brucellosis in Iran is caused mainly by *Brucella abortus* and *Brucella melitensis* (2). Brucellosis is endemic in some parts of Iran. The outbreak rate of this disease varies from 0.5 to 10.9% in different provinces (3). Each year, half a million people are affected by this disease. In some countries, there are 10 cases of the disease annually among every 100,000 people, especially in the Mediterranean region (southern

Europe, north and east of Africa), the Middle East, India, and Middle Asia (4). The laboratorial diagnosis of human brucellosis is done by separating bacteria from blood culture, or by observing special antibodies. The blood culture procedure is the standard method. Based on the stage of the disease, the type of brucellosis, the culture medium, and the amount of bacteria in the blood, the accuracy of this method is between 53 and 90%. Serological tests are another key method when the bacteria cannot be diagnosed by culture. These tests are frequently simple and quick, but it is difficult to interpret their results, especially in the early stages of the disease (5). Due to low sensitivity, serological cross-reactivity, and the inability to distinguish between active and inactive infections, antibody tests are not efficient in a diagnosis in the early stages (6). In contrast to

methods such as nucleic-acid probe, sequential polymeric reactions, and other microbial-molecular detection techniques, the blood culture method remains as a reliable and feasible method for the diagnosis of blood infections (7). In endemic areas, blood culture is necessary for a conclusive diagnosis of the disease, as the symptoms of brucellosis are non-specific (8). *Brucella* species often grow slowly in the initial culture and subculture environments; accordingly, modern systems for blood culture have been developed which have the ability to detect bacteria in the blood in less than seven days. However, such systems are expensive and not used routinely (9). The main problem is that *Brucella* culture is time-consuming. To address these issues, various techniques are used for DNA polymorphism detection and *Brucella* molecular typing (10). In order to detect *Brucella* via the PCR method, based on different zone sequences of various bacterial isolates, a number of primers have been designed and several PCR protocols proposed (11). Because of its high accuracy and sensitivity, the PCR method makes a valuable tool for diagnosing brucellosis. This method has several advantages, including repeatability at different times and in various centers, simplicity and quickness, lack of cross-reactivity, ability to detect the infecting element in diverse samples, low level of risk for laboratory personnel, and minimum amount of the required sample (12). Different genes are used in the molecular diagnosis of the bacteria, including *Bcsp 31* and *omp 31*, which produce 31-kDa outer membrane protein (13, 14). In addition, *eryD* is a conserve gene which exists in all isolates of *Brucella*. Until now, this gene has not been used in diagnosis (15, 16).

The microbial acid nucleic tracing for the detection of infection and diagnosis of the disease is dependent on the success of acid nucleic extraction from the sample. In order to increase access to DNA, commercial kits are used in laboratorial detections. Considering the important role of the DNA extraction method in the results obtained by PCR, the current study was conducted in order to evaluate the efficiency of the two different methods of DNA extraction.

2. Objectives

The aims of the present study are the evaluation of existent genes, the use of the *eryD* gene for molecular detection of *Brucella*, and the comparison of two different methods of DNA extraction.

3. Materials and Methods

3.1. Sample Collection

In this experimental study, during a ten-month period covering 2013 - 2014, we tested patients with brucellosis

symptoms at Farshchian hospital in Hamadan, Iran. According to our previous studies, in which the confidence level specified was 95% (0.95 probability), the required sample size for this study was:

$$n = \frac{(Z_{\frac{\alpha}{2}})^2 \times pq}{E^2} \quad (1)$$

$$= 100 \text{ patients}$$

Patients were selected who had clinical symptoms of brucellosis, including fever, overnight perspiration, and pain in joints, backbone and muscles. The code and date of ethical approval were 92-144-1, 2/2/1392, from Arak University of Medical Sciences. Serological diagnosis in the endemic area was done by an anti-*brucella* antibody titer of $\geq 1/160$ by the standard tube agglutination method; this was accomplished by one person in an approved governmental laboratory.

3.2. Microbiological Methods

Blood cultures were carried out with BACTEC 9050 (Becton Dickinson, USA), by inoculating 5 - 7 mL of each patient's blood into standard aerobic BACTEC bottles; these were incubated for one week at 37°C. The blood contents of the positive BACTEC bottles were cultured in *brucella* agar culture media (Merck, German), and incubated in a 5% CO₂-enriched atmosphere for 48 - 72 hours at 37°C.

At the end of the first week, the negative bottles were incubated for an additional three weeks; they were also sub-cultured weekly in *brucella* agar medium (Merck, German). Identification of grown cultures was carried out by gram ordinary tests for staining, oxidase, catalase, urease, and the production of H₂S in colonies.

3.3. DNA Extraction from Peripheral Blood Samples

Isolation of DNA from peripheral blood samples was carried out by a commercial DNA extraction kit (Qia Amp Mini Kit), as recommended by the manufacturer.

3.4. Gene Selection and Primer Design

It has been proved that housekeeping genes are very important in the process of detection of *Brucella* Mutation does not occur in regulatory gene groups and transcription. Housekeeping genes have more stability in different isolates of *Brucella*, and perform better in the process of detection. In this study, the gene bank was examined; an involved gene in transcription, which lacked mutation, was selected. The *eryD* gene is an erythritol transcriptional regulator. This gene has a Locus tag (BruAb2-0365). Its place on the second chromosome of *Brucella* is NC-006933.1 363458.364408, complement.

Blast was used to confirm that the gene and genus specificity could be used in the process of detection. By examining this gene in the gene bank, it was revealed that this bacterium has the capability to detect all types of *Brucella*, and that there is no uniform equivalent of it in non-*Brucella* pathogenic species. The Blast results showed that these gene uniformities are observed only in different species of *Brucella* with MAX ID.

In this study, we designed the necessary primers for transcription genes (*eryD*), whose encoded site contains 461 base pairs. The Mega 4, Oligo 6, IDT, and Blast programs were used in the design. The specificity primers, which were designed by Integrated DNA Technology, were evaluated with regard to TM, GC content, and the creation of Hairpin secondary structures (Table 1).

In addition, two pairs of primers were used to amplify a target sequence of 223-bp within a genetic code to produce a 31-kDa outer membrane protein that was specific to *B. abortus*, which is conserved in all *Brucella* species (*bcs*p31) and has a target sequence of 350 pairs of outer membrane proteins (*omp*p31) (Table 2).

3.5. Specific Double PCR Methods

DNA amplification was carried out by two different PCR sets of primers: B4/B5 (223 bp) and *eryDF/eryDR* (461bp).

Primers B4 (5'-TGG CTC GGT TGC CAA TAT CAA-3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG -3') were used to amplify a target sequence of 223 bp that was present in a gene encoding a 31 kDa *Brucella* antigen. PCR was performed using a 25 μ L volume reaction mixture, DNA template of 5 μ L, master mix of 12.5 μ L, primer R of 2 μ L, primer F of 2 μ L, and H₂O at 3.5 μ L. The reaction included an initial five minute incubation step at 94°C, followed by 32 cycles with denaturation at 94°C, annealing at 60°C, extension at 72°C (each for one minute), and final extension at 72°C for 10 minutes.

Primers of *eryDF* (5'-ATG ACT CAG TCT GCC GTT GCC -3') and *eryDR* (5'-GGG CAC CGG CAT CAC ATA AGC G -3') were used to amplify a target sequence of 461 bp. Isolated DNA was amplified as described above. The PCR products were evaluated by 1% electrophoresis in agarose gel, and stained with safe stain solution. The intended bands were observed in a trans-illuminator.

3.6. Specificity of the Primers

To confirm the data, specificity of the primers was evaluated by accomplishing the PCR reaction for extracted DNA from pure cultures of *Brucella* that were isolated from blood samples; the other bacteria included *E. coli*, *Kelebsiella pneumonia*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.

3.7. DNA Extraction From Pure Colonies

Two methods were used for extraction of DNA from the bacterial colony. The first method was carried out using a commercial DNA extraction kit (Qia Amp Mini Kit). The second was an extraction technique using the Chelex 100 method, as follows: 0.07 grams of Chelex100 particles were added to 270 microliters of TAE 1X buffer; three or four new colonies of bacteria from the *bucella* agar culture medium were then added. These were kept at 95°C for 45 minutes. They were then centrifuged three times at 14000 rpm for 10 minutes each time; after each cycle, the supernatant was transferred to a 1.5 μ L tube and the sediments were removed. Finally, the obtained supernatants, containing the *Brucella* genome, were kept at -20°C until analysis.

4. Results

4.1. Blood Samples

100 patients were included in the study; a total of 100 bottles of blood culture were used, which contained the samples collected from the suspected patients.

4.2. Bacterial Isolation

After six days of incubation, 39 (39%) samples were positive according to the BACTEC system, and 61 (61%) were negative. Conversely, identification by ordinary detection tests proved that all the isolated bacteria in the *Brucella* agar media were categorized under the *Brucella* genus. The grown colonies were used for molecular examination.

4.3. DNA Extraction

The two DNA extraction methods applied were compared in order to evaluate them. The bands obtained by the Chelex 100 method were weaker than those obtained by the Kit method. It was concluded that the genome yield in the Chelex 100 method was lower than that of the Kit method, but the Chelex 100 method was simple and safe. By doubling the DNA concentration in the PCR components, we achieved the same bands at identical quality for both methods.

4.4. PCR Amplification

23 culture blood samples were positive, and all showed 491 bp for the *eryD* gene and 223 bp for the *bcs*p 31 gene (Table 3). Interestingly, out of 14 culture-negative blood samples, 13 showed positive bands in PCR (92%).

Table 1. Profile Primers Designed for Gene *eryD*

| Primers for <i>eryD</i> Gene | GC Content, % | TM | Length | ΔG (Probability of Hairpin Formation) |
|------------------------------|---------------|--------|--------|---------------------------------------|
| <i>eryDF</i> | 57.1 | 59.9°C | 21 | -0.4 |
| <i>eryDR</i> | 61.9 | 61.3°C | 22 | -0.39 |

Table 2. Primers Used in PCR Reactions

| Genes | Primers' Sequences | Length | Reference |
|---------------|---|--------|-----------|
| <i>Bcsp31</i> | 5'-TGGCTCGGTGGCCAATATCAA-3'; 5'-CGCGCTT-GCCTTTCAGGCTG-3' | 223 bp | (15) |
| <i>eryD</i> | <i>eryDF</i> : 5-ATG ACT CAG TCT GCC GTT GCC-3; <i>eryDR</i> :5-GGG CAC CGG CAT CAC ATA AGC G-3 | 461 bp | This work |

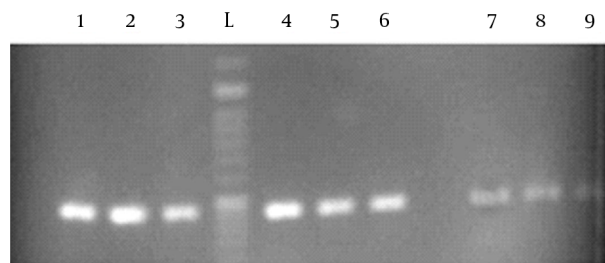
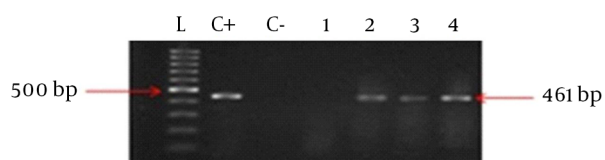


Figure 1. L: Ladder number (1, 2, 3), PCR product with 1 microliter of DNA template extracted with Qia Amp Mini kit; Number (4, 5, 6), PCR product with 2 microliters of DNA template extracted with Chelex method; Number (7, 8, 9), PCR product with 1 microliter of DNA template extracted with Chelex method.

Figure 2. Agarose Gel Electrophoresis Analysis for Clinical Samples Using *eryD* Gene



From left to right: L, molecular weight DNA ladder (100 bp); C+, positive control; C-, negative control lanes; 1, negative PCR; 2, 3, 4, positive PCR.

4.5. Specificity of the Primers

Specificity of the primers was confirmed by PCR of DNA from pure cultures of *Brucella* that were isolated from blood samples; the other bacteria included *E. coli*, *Kelebsiella pneumonia*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Besides the DNA from *Brucella*, all other tested bacteria had a negative reaction by the specific primers (specificity 100%).

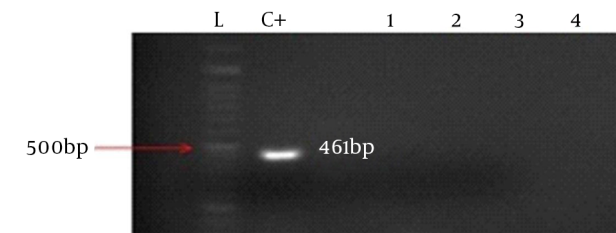


Figure 3. Specificity of the primer *eryD* with *Brucella* (C+) and *E. coli*, *Kelebsiella pneumonia*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (1- 4)

5. Discussion

Laboratory diagnosis of brucellosis is based on the immunological and culturing methods. Culturing is a time-consuming method with a low sensitivity and a risk of contamination (17). The suitable specificity and sensitivity of the PCR method makes it a preferable tool for diagnosing brucellosis (18).

In this study, we compared the culturing, serological, and PCR methods for the diagnosis of brucellosis. All patients showed high titer anti-*Brucella* antibodies.

In the last decade, automatic blood culture systems such as BACTEC have been developed for clinical practice. Recent studies have indicated that such techniques enable most clinical microbiology laboratories to detect *Brucella* within the seven day incubation (9). Maleknejad et al. recovered 41 (41%) of 102 isolates with the BACTEC 9120 system during the seven day incubation (19). Ruiz et al. (9) isolated 16 (94%) of 17 samples with the same system and timeframe. In this study, the sensitivity of the BACTEC system (39%) was in accordance with these previous works (20).

The specificity and sensitivity of the PCR method varies, based on the laboratory characteristics. Up to now, no standard method has been determined for providing samples, target genes, and diagnostic methods (21).

Our data, obtained by PCR, indicated that the whole blood PCR assay is rapid, safe, highly sensitive, and specific. Our specific double PCR reaction showed 100% sensitivity in patients with positive blood cultures, and 92% specificity in patients with negative cultures.

The double PCR detection method by the *eryD* gene yielded good results, and can be used in routine clinical di-

Table 3. Comparison of the PCR Evaluated with Microbiological Tests for the Diagnosis of Brucellosis

| | Positive Whole Blood | Negative Whole Blood | eryD | Bcsp 31 |
|------------------------|----------------------|----------------------|------|---------|
| Positive Blood Culture | 23 | 0 | 23 | 23 |
| Negative Blood Culture | 13 | 1 | 13 | 13 |
| Total | 36 | 1 | 36 | 36 |

agnosis of brucellosis by a suitable specificity.

The specificity and sensitivity of any PCR method is dependent on many factors, including technical errors, unsuitability of chemical materials, and the quantity and quality of DNA. In all cases, the PCR method may produce false negative results. Another effective factor in the PCR method is DNA extraction from the sample (22). There are various methods of DNA extraction, such as the use of commercial kits and manual methods (18). In this study, we investigated the efficiency of two methods (Qiagen Company (Qia Amp Mini Kit) and Chelex 100), as well as the PCR method for tracing the DNA of *Brucella*.

It was concluded that the Chelex 100 method is the most cost-efficient, safe, and simple. Heating of the sample at 95°C in the first step could kill the bacteria and reduce health risks for operators.

Another advantage of this method is that after extraction, further DNA is obtained which can survive for over five years. One disadvantage is the low level of DNA concentration. By increasing the amount of DNA in the PCR reaction, this deficiency has been removed in the present study. The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

In this study, for the first time, the *eryD* gene was introduced to diagnose human brucellosis. The sensitivity of PCR by the use of *eryD* like BCSP 31 was 100%.

5.1. Conclusion

We conclude that the introduced double PCR reaction could be successfully used in the detection of brucellosis. We detected 13 cases from 14 negative cultures by this method. The *eryD* gene was used for the first time for this purpose. DNA extraction by Chelex 100 is simple, safe, and economic for routine work.

Footnote

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