Brucellosis is difficult to diagnose based on clinical symptoms of the disease, which are nonspecific and often atypical signs. Therefore, the diagnosis mostly relies on the results of laboratory testing. Culture of the organism is the diagnostic method of choice; however, cultures involve risk of infection and require special precautions in the laboratory. An infectious dose for Brucella in humans is 10 to 100 organisms; consequently, diagnostic laboratory personnel who cultivate these organisms are at significant risk of accidental exposure. Brucellosis is one of the most commonly reported laboratory-acquired infections. Current methods of testing cultures for Brucellae are time-consuming and lack sensitivity, particularly in chronic infections.

Most laboratories apply serological tests that do not provide suitable sensitivity and specificity for this organism. Enzyme-linked immunosorbent assay (ELISA) methods that detect immunoglobulin G (IgG) are sensitive but have low specificity. Measurement of specific immunoglobulin M (IgM) levels has lower sensitivity than IgG but is more specific.

Molecular diagnostic assays minimize the risks associated with handling potentially infectious specimens and increase the sensitivity, specificity, and speed of testing, although some studies have reported only moderate sensitivity (50%) using these methods. However, few laboratories diagnose brucellosis using culture methods because cultures have limited sensitivity, are time-consuming, and require specially biosafety equipment. Therefore, data on the frequency of Brucella infections are often unreliable. Recently, multiplex polymerase chain reaction (PCR) protocols that overcome these problems have been described. In this study, we used a multiplex extraction method had been used. Specific primers targeting IS 711 were used for Brucella melitensis, B abortus, and B suis.

Brucellosis was confirmed in 110 patients (58.2% male and 41.8% female) based on applied diagnostic methods and clinical features. Results of ELISA, the SAT, and PCR were positive in 92, 42, and 51 patients, respectively. B abortus and B melitensis were detected in 16 and 35 patients, respectively. B suis was not detected in any of the specimens.

All methods tested in our study need to be used to ensure accurate diagnosis of brucellosis, although ELISA displayed the highest level of efficiency. Also, B melitensis showed a higher frequency rate than B abortus in our cohort.

**Keywords:** B abortus, B melitensis, B suis, ELISA, Multiplex PCR, SAT

**ABSTRACT**

**Objective:** To determine the sensitivities of various testing methods for diagnosing brucellosis.

**Methods:** The 267 patients in our cohort were suspected to be infected with Brucella and had been referred to a hospital in Mianeh City, Iran. The mean (SD) age was 37.0 (11.3) years for female patients and 37.1 (13.7) years for male patients. All serum specimens from these patients were examined by the standard agglutination test (SAT), Coombs Wright test, 2-mercaptoethanol (2ME) test, enzyme-linked immunosorbent assay (ELISA) for determining levels of immunoglobulin G (IgG) and immunoglobulin M (IgM), and multiplex polymerase chain reaction (PCR). DNA was extracted. Extracted DNA was checked with human PCR–targeting CRP gene–encoding 485 base pair (bp) as an internal control to ensure that the proper extraction method had been used. Specific primers targeting IS 711 were used for Brucella melitensis, B abortus, and B suis.

**Results:** Brucellosis was confirmed in 110 patients (58.2% male and 41.8% female) based on applied diagnostic methods and clinical features. Results of ELISA, the SAT, and PCR were positive in 92, 42, and 51 patients, respectively. B abortus and B melitensis were detected in 16 and 35 patients, respectively. B suis was not detected in any of the specimens.

**Conclusions:** All methods tested in our study need to be used to ensure accurate diagnosis of brucellosis, although ELISA displayed the highest level of efficiency. Also, B melitensis showed a higher frequency rate than B abortus in our cohort.

**Keywords:** B abortus, B melitensis, B suis, ELISA, Multiplex PCR, SAT

**Abbreviations**

ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; STA, standard tube agglutination; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; SAT, standard agglutination test; 2ME, 2-Mercaptoethanol; Tris, Tris(Hydroxymethyl)aminomethane; TBE, Tris/borate/EDTA; dNTP, deoxyribonucleotide triphosphate

**Department of Microbiology, Health Reference Laboratories, Ministry of Health and Medical Education; and Pediatrics Infectious Research Center (PIRC), Shahid Beheshti University of Medical Sciences**

*To whom correspondence should be addressed. E-mail: lari@iums.ac.ir

DOI: 10.1309/LM4J9MWOBIPA6RBN

**Comparison of Methods for Diagnosing Brucellosis**

Massoud Hajia, PhD, Fatemeh Fallah, PhD, Goli Angoti, MSc, Abdollah Karimi, MD, Mohamad Rahbar, PhD, Latif Gachkar, MD, Bahram Mokhtari, MD, Anahita Sanaei, MD, Abdolaziz Rastegar Lari, PhD*
PCR method to simultaneously differentiate and quantify the type and frequency of *Brucella* infections.

**Materials and Methods**

In this study, the 267 patients in our cohort had been admitted to the hospital, suspected of brucellosis based on clinical assessment. The subjects were entered into the study from August 1, 2009, through July 31, 2010. They had been referred to Imam Khomeini Hospital in Mianeh (East Azarbaijan province), located in northwest Iran.

**Inclusion Criteria for Patients Suspected of Having Brucellosis**

All patients suspected to have brucellosis based on symptoms and who had positive results on their serological tests via standard tube agglutination (STA), ELISA, or PCR were diagnosed as having brucellosis. According to the recommendation of the Laboratory Affairs Office of Iran, a titer of 1:80 and higher, rather than 1:160 and higher, might be considered as a positive result for *Brucella* by tube agglutination testing because of the confirmation of the former ratio in several Iranian brucellosis reports. Tube agglutination testing was carried out using the Pasteur-protocol kit (Iranian Institute for Health Sciences Research Co, Tehran). ELISAs for measuring IgG and IgM antibodies used a 10 U/mL cut-off according to the manufacturer’s protocol (Immuno Biological Laboratories Co Ltd, Fujioka, Japan; the unit was defined therein for each enzyme). Cultures were not included in the study because most of the patients were being treated at the time that samples were collected.

**Sampling**

*Brucella* species are highly infectious. Therefore, all specimen manipulations were performed in a protective hood that provided containment level 3 or higher. Blood specimens (5 mL) were collected by venipuncture in an ethylenediaminetetraacetic acid (EDTA) tube. One mL of blood from the sample was used for PCR and the rest was used for tube agglutination and ELISA testing.

**Tube Agglutination Methods and ELISA**

Specimens were analyzed by the standard agglutination test (SAT), 2-mercaptoethanol (2ME), and the Coombs Wright test. The ELISA kit was provided by Immuno Biological Laboratories Co Ltd. Positive sera and normal saline were used as a quality control for the tests before use with patient specimens.

**PCR**

DNA was extracted using the High Pure PCR Template preparation kit (F. Hoffman-La Roche Ltd, Basel, Switzerland). The PCR protocol was based on IS 711 in the *Brucella* chromosome using the primers reported by Redkar et al (Table 1). We used the conventional multiplex PCR approach, which detects *B abortus* and *B melitensis* by electrophoresis instead of using the real-time procedure. The PCR protocol was set up and optimized by *B abortus* strain 544 and *B melitensis* serotype 1 (strain 16M); unfortunately, we did not have access to the *B suis* strain. Therefore, *B suis* reverse primer was added to the mixture after optimizing the test for *B abortus* and *B melitensis*.

PCR reactions proceeded in 25 μL of reaction mixture, composed of 10 mM Tris(Hydroxymethyl)aminomethane (Tris)/HCl (pH, 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of deoxyribonucleotide triphosphate (dNTP), 10 pmol of each primer, and 0.8 U of Taq DNA polymerase. PCR products were separated by agarose gel electrophoresis in Tris/borate/EDTA (TBE) buffer (89 mM boric acid, 89 mM Tris pH 8.0, and 10 mM EDTA); DNA was stained by ethidium bromide. All extracted DNA were checked with human PCR–targeting CRP gene–encoding 485 bp as an internal control to ensure adequate extraction.

**Results**

Optimization tests confirmed previously reported concentrations of reaction mixture (Image 1). We changed amplification time in each segment of the PCR cycles and increased the annealing temperature from 58°C to

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward, for all 3 species</td>
<td>5’CATGGGCTATGTCGTTTCA-3’</td>
</tr>
<tr>
<td>Reverse, for <em>Brucella abortus</em></td>
<td>5’GGTCTTCCTACGGTATTC-3’</td>
</tr>
<tr>
<td>Reverse, for <em>B melitensis</em></td>
<td>5’AGTGTTCGCTAGATATG-3’</td>
</tr>
<tr>
<td>Reverse, for <em>B suis</em></td>
<td>5’ACCCGACATGCAAATG-3’</td>
</tr>
</tbody>
</table>

*NA*, not available.
59°C because we used conventional PCR detection by electrophoresis rather than the real-time method. Our results were most sensitive when we used the following amplification sequence: 2 minutes at 95°C, followed by 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 59°C, and 30 seconds at 72°C. Finally, the mixture was maintained at 72°C for 5 additional minutes. We did not observe significant improvement in the detection limit of B abortus by increasing the cycles to more than 35. The sensitivity of the multiplex format was the same as that of single tests for B abortus and B suis. We were not able to determine the sensitivity of multiplex PCR for detection of B suis because we did not have access to a control strain for this species.

Brucellosis was confirmed in 110 patients in our cohort based on laboratory results and the clinical symptoms exhibited by these patients (64 [58.2%] females and 46 [41.8%] males). The frequency of brucellosis in different age groups is shown in Figure 1. The mean (SD) age of patients with brucellosis was 37.0 (11.3) for females and 37.1 (13.7) for males. Analysis of the results revealed positive ELISA results in 92 cases (83.6%) and positive results for IgG and IgM in 64 (58.2%) and 32 (29.1%) cases, respectively.

The SAT method yielded positive results in 42 cases (38.2%), all of which yielded positive results on Coombs Wright testing as well. 2ME results were negative in 7 SAT-positive cases; overall, 14 patients had an SAT titer at least 4-fold higher than the 2ME titer. 50 ELISA-positive specimens displayed no reaction via SAT, 2ME, or Coombs Wright.

PCR results were positive in 51 cases; among them, 34 were positive by ELISA. Among the remaining 17 ELISA-negative samples, 1 was positive by SAT. Among the 59 specimens that were negative by PCR, 58 were positive by ELISA; the single ELISA-negative specimen in this group was positive by SAT (Table 2).

PCR testing confirmed B abortus infection in 16 cases and B melitensis in 35 cases. One patient was infected...
with *B. abortus* and *B. melitensis*. PCR results failed to detect *B. suis* in any of the patients.

### Discussion

In this study, brucellosis was confirmed in 110 patients based on antibodies to *Brucellae* and the results of PCR testing. Among the applied methods we used for detecting *Brucellae*, the SAT displayed the lowest positivity rate. The low reliability of the SAT method has been previously reported. The ELISA test, used to detect IgG and IgM, had the highest efficiency in diagnosis of brucellosis (83.6%) among all the methods we used, although it yielded negative results in 17 cases; in these cases, PCR and SAT results were positive in all of the SAT-negative cases and the ELISA results were positive in 16 of the 17 SAT-negative cases. These cases of PCR positivity with concurrent seronegative results via antibody methods need to be more thoroughly investigated. Culture-positive seronegative brucellosis has been previously reported.

Overall, PCR detected *Brucellae* in 46.4% of the study subjects. The lower sensitivity of the PCR method compared with ELISA is consistent with the findings of other studies. One reason we observed lower sensitivity of PCR might be that we applied conventional multiplex PCR instead of the real-time method. Also, the causative agent in some of the brucellosis cases might be *B. suis*; we had no positive control strain of this species to check the sensitivity of the test. In future work, genus-specific multiplex PCR should be applied and the results evaluated. Another potential reason for the lower sensitivity of the PCR method may be that brucellosis was wrongly diagnosed in some of our subjects at presentation.

Some of us previously reported ELISA, tube agglutination, and PCR testing results in patients admitted to Imam Khomeini Hospital. The conclusion was that the lower sensitivity observed for PCR (48.9%) resulted because all of the patients had been admitted with a documented medical history of incorrect antibiotic treatment. The seropositive specimens yielded negative results via PCR soon after the patients began appropriate treatment. In the present report, all of the subjects were outpatients but some may have had previous treatment with an incorrectly prescribed antibiotic before their arrival at the outpatient facility and enrollment in our study. Therefore, PCR may not be highly sensitive for chronic brucellosis or in patients who had previously been treated with incorrectly prescribed antibiotics. ELISA testing cannot confirm brucellosis because it yielded positive results in only 83.6% of the patients with brucellosis in our cohort.

Two studies have reported differentiation of *Brucella* species in Iran. In a report by Khosravi et al, none of the examined specimens tested positive for *B. abortus*. However, in 2011, Doosti et al collected specimens from the provinces of Isfahan and Chaharmahal Va Bakhtiari and reported that, among 76 PCR-positive cases, 41 had tested positive for *B. abortus* and 6 had tested positive for *B. melitensis*. They reported that the frequency of *B. abortus* was higher than *B. melitensis* in Chaharmahal Va Bakhtiari, compared with Isfahan. Hence, the frequency of *Brucella* species can vary by region in Iran.

Brucellosis is an uncommon infectious disease. The isolation rate for *Brucella* is high in countries in which it is endemic; serologic testing for the condition can often confirm the diagnosis in suspected patients. However, none of the available methods for diagnosis of brucellosis is highly sensitive. Diagnostic laboratory methods based on serology are used in most countries because culture methods have low sensitivity and require special biosafety accommodations. Use of multiplex PCR that enables simultaneous detection of all species may be a strong alternative for confirming brucellosis.

### Conclusion

ELISA yielded the highest sensitivity among the applied tests in our study, although it yielded negative results in several cases of brucellosis. By contrast, multiplex PCR can be a favorable method for diagnosis of the disease and identification of *Brucella* species. This method is especially helpful epidemiologically in patients who have
brucellosis signs and symptoms but for whom serologic testing does not provide suitable results. LM

Acknowledgments

This study was funded by Tehran University of Medical Sciences (grant no. M.T/444).

References