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Polymerase chain reaction (PCR) diagnosis of human brucellosis (L7/L12 and 16srRNA genes) compared with immunocapture-agglutination test (brucellacapt) and common serological tests

Pakzad I.1,4*, Hosseinzadegan H.2, Ghafouryan S.1 and Abtahi H.3

1Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.
2Department of Microbiology, Faculty of Nursing and Midwifery, Maragah, Tabriz University of Medical Sciences, Tabriz, Iran.
3Department of Microbiology, Faculty of Medicine and Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran.
4Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran.

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Diagnosis of the human brucellosis mainly depends on blood culture and serological tests. The most commonly used tests are the serum agglutination test (SAT), Coombs anti-Brucella, and Rose Bengal tests. New diagnostic tests such as immunocapture-agglutination test (Brucellacapt) and polymerase chain reaction (PCR) have been used. This study aims to compare PCR method by using L7/L12 and 16srRNA genes with Brucellacapt and serological tests in diagnosis of Brucellosis. A total of 754 different suspected brucellosis were tested during the period from March, 2008 to February, 2009. They were assayed by Rose Bengal test, Brucellacapt, Coombs tests, SAT and PCR. Our results had shown that out of 754 sera, 125 were positive by Rose Bengal test. Thus, frequency of brucellosis by Rose Bengal test was 16.5%. In PCR, all of samples were differentiated. Forty nine (6.5%) samples in Coombs test and 47 (6.2%) samples in SAT were positive. The results in 1:40 and 1:80 were equal for Brucellacapt and Coombs tests and different for SAT. The results from the present study show a higher sensitivity and specificity of PCR for the diagnosis of human brucellosis than serological tests. Sensitivity of the PCR by L7/L12 gene and 16srRNA were similar and could be used for diagnosis of human brucellosis. Sensitivity of Brucellacapt test was higher than Coombs tests and SAT, although the sensitivity of PCR assay was higher than all of them.

Key words: Brucellacapt, Rose Bengal, L7/L12, ilam.

INTRODUCTION

Brucellosis is one of the world’s major zoonotic disease, and responsible for enormous economic losses, as well as considerable human disease in endemic areas (Queipo-Ortuno et al., 2005). The disease is endemic in the Mediterranean basin, Middle East, India, and Central and South America. Furthermore, the constant growth by migration and tourism, together with the variable incubation period of the disease, explains the appearance of cases of human brucellosis in countries where the disease in animals has been eradicated. (Queipo-Ortuno et al., 2005). The importance of brucellosis is not known precisely, but it can have a considerable impact on human and animal health, as well as socioeconomic impacts, especially in rural areas in which income relies largely on livestock breeding and dairy products (Araj,
2010; Lulu et al., 1988; Pakzad et al., 2009; Young, 1991). Human brucellosis is caused by exposure to livestock and their products. Infection can result from direct contact with infected animals and can be transmitted to consumers through raw milk and milk products. In humans, the symptoms of disease are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly and night sweats (Pakzad et al., 2009). Brucellosis is an common health problem in some Middle Eastern, Mediterranean countries and Iran (Karimi et al., 2003). Brucellosis is an infectious disease caused by different species of the genus Brucella spp. This disease is the cause of significant economic losses in livestock production due to reproductive disorders and reduced production of affected animals (Pakzad et al., 2010). The prevalence rate of brucellosis in different parts of Iran varied from 1.5 up to 107.5 per 100,000 of population. The highest levels of infection reported from Hamedan, Kurdistan, West Azerbaijan and Zanjan with 107.5, 83.5, 71.4 and 71.1 per 100,000 people respectively (Zamani et al., 2011). Thus, its prevention, control and eradication are a major challenge for public health program.

Brucella is a Gram negative coccobacillus and diagnosis of brucellosis is based on isolation of the microorganism from the blood culture that ensures diagnosis of the disease, but sensitivity of this method is depends 30 to 90% on the stage of the disease (Surucuoglu et al., 2009). When the culture is found negative, investigation of classic serologic tests and antibodies are important tests in diagnosis of brucellosis. Antibodies begin to form 2 weeks after the beginning of disease. Immunoglobulin (Ig) M type antibodies appear in one week and reach a peak in three months. IgG antibodies, on the other hand, appear in three weeks and reaching a peak in six to eight weeks. Coombs test have been using to investigate blocking antibodies. Dilutions of the patient sera need to be performed in very high ratios in order to overcome the occurrence of prozone (Gomez et al., 1999). In recent years, the immunocapture agglutination test, which is based on sandwich Enzyme-linked immunosorbent assay (ELISA) system, has been introduced. This method is brucella agglutination test that occurs in microwell and performed with Coombs antisera and determines the three classes of antibodies (IgG, IgM and IgA), that form against brucella (Maleki et al., 2011). The purpose of this study was to compare the diagnostic values of Immunocapture agglutination and PCR methods, which are used for the diagnosis of brucellosis with reference to Coombs test.

MATERIALS AND METHODS

Experimental design

A total of 754 different suspected to brucellosis were tested during the period from March, 2008 to February, 2009. The diagnosis was based on clinical findings compatible with brucellosis (arthralgia, fever, sweating, malaise, hepatomegaly, splenomegaly), supported by detection of specific antibodies at significant titers. Antibody titers were determined by standard agglutination tube (SAT), the corresponding titers considered positive were ≥ 1/160.

Bacteriological method

Conventional culture method was done for isolation and identification from blood samples in diphasic medium (Castañeda, Roche) (Gemechu et al., 2011; Pakzad et al., 2010).

Serological methods

About 5 to 7 ml of blood was collected by using a sterile disposable syringe and needle. For serology, blood samples were centrifuged (3000 x g for 10 min) and the serum divided into aliquots and stored at -20°C until needed. All sera were evaluated using the Rose Bengal, serum agglutination, Coombs’ PCR and brucella capt tests. Brucellosis was diagnosed on the basis of clinical evidence, a SAT titer of > 1/160, or a fourfold rise in SAT or Coombs test titers between two samples collected within 15 to 30 days of each other (Lulu et al., 1988).

Brucellacapt test

The Brucellacapt Test (Vircell SL) was performed as specified by the manufacturer. Briefly, 0.050 ml of serum dilutions were added to wells of a U-bottom microtiter plate coated with anti-total human immunoglobulin. Then 0.050 ml of an antigen suspension was added to all the wells. The plates were sealed with adhesive tape and incubated at 37°C for 24 h in a dark humid chamber. Positive reactions show agglutination on the bottom of the well. Negative reactions are indicated by a pellet at the center of the bottom of the well (Casanova et al., 2009).

Serum agglutination test (SAT)

The assay was performed as described by Briefly, 0.5 ml of Brucella SAT antigen was added to 0.5 ml of each serum sample, diluted serially from 1:5 to 1:640 in physiological saline solution and mixed thoroughly. SAT reactions were read after 24 h incubation at 37°C. The agglutinations ++ and stronger, observed in sera at dilution 1:20 and higher, was considered to be positive (Jain and Tilak, 2008).

Rose Bengal Test (RBT)

Briefly 30 µl of serum was mixed with equal volume of antigen on a white enamel plate circled approximately 2 cm in diameter with manicure. The mixture was rocked gently for 4 min at room temperature and then observed. Any sign of agglutination was considered positive (Diaz et al., 2011).

Coombs test

The Coombs test was carried out with the SAT tubes by washing three times with phosphate-buffered saline (pH 7.2) by centrifugation at 30,003 g for 20 min. After the last wash, the bacteria were suspended in 1 ml of phosphate-buffered saline and 0.05 ml of previously standardized anti-total human immunoglobulin (Sanofi Pasteur) was added to each tube. The tube contents was mixed and incubated at 37°C for 24 h (Casanova et al., 2009).
Table 1. Specific primers were used for PCR of 16S rRNA and L7-L12 genes.

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>Gene</th>
<th>Primer</th>
<th>Annealing TM</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>905</td>
<td>16S rRNA</td>
<td>TCG AGGGCCCGCAAGGGG AACCATAGTGCTCCACTAA</td>
<td>44°C</td>
<td>F4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R2</td>
</tr>
<tr>
<td>375</td>
<td>L7-L12</td>
<td>CCGTCTAGAAAATGGCTGTACTCTCGCAAAG</td>
<td>56°C</td>
<td>FL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGTCTAGATTACTTGAGTCAACCTGGG</td>
<td></td>
<td>RL</td>
</tr>
</tbody>
</table>

Table 2. Frequency of clinical characteristics in patients with brucellosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clinical characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fever</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Patients 745 [n(%)]</td>
<td>393(52.7)</td>
</tr>
<tr>
<td>Rose Bengal positive 125/745 (%)</td>
<td>79</td>
</tr>
<tr>
<td>SAT 1/160 positive 113/745 (%)</td>
<td>92</td>
</tr>
</tbody>
</table>

Table 3. Comparison between Capt, SAT and Coombs test.

<table>
<thead>
<tr>
<th>Test</th>
<th>1/40 [n (%)]</th>
<th>1/80 [n (%)]</th>
<th>1/160 [n (%)]</th>
<th>1/320 [n (%)]</th>
<th>1/640 [n (%)]</th>
<th>1/1280 [n (%)]</th>
<th>Total [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT</td>
<td>2(1.6)</td>
<td>20.0(16.0)</td>
<td>35.0(28.0)</td>
<td>43.0(34.4)</td>
<td>19.0(15.2)</td>
<td>6.0(4.8)</td>
<td>125(100)</td>
</tr>
<tr>
<td>Coombs test</td>
<td>0(0)</td>
<td>13.0(10.4)</td>
<td>39.0(31.2)</td>
<td>35.0(27.0)</td>
<td>27.0(21.6)</td>
<td>11.0(8.8)</td>
<td>125(100)</td>
</tr>
<tr>
<td>Brucellacapt test</td>
<td>0(0)</td>
<td>12.0(9.6)</td>
<td>34.0(27)</td>
<td>41.0(32.8)</td>
<td>24.0(19.2)</td>
<td>14.0(11.2)</td>
<td>125(100)</td>
</tr>
</tbody>
</table>

DNA extraction and PCR detection of 16S rRNA and L7/L12 genes

Sera samples were taken at the time blood culture and preserved in -20°C until processing. DNA from 200 µl serum was prepared by QIAamp DNA mini kit (Qiagen Hilden, Germany) according to manufacture procedure. The samples were centrifuged at 15,000 × g for 15 min. The supernatant was removed, and the pellet was resuspended in deionized water and centrifuged at 15,000 × g for 10 min. The supernatant was removed, and the pellet was resuspended in 40 µl of deionized water, subjected to boiling at 100°C in a water bath for 10 min, cooled on ice, and centrifuged at 15,000 × g for 10 s before it was stored at −20°C until processing. As positive controls for PCR, DNA samples extracted from reference strains B. abortus S19. This PCR assay amplifies a 905-bp sequence of the gene 16S rRNA and 375-bp sequence of the gene L7/L12, which is conserved in all Brucella species. Specific primers in Table 1 were used.

RESULTS

There were 567 (75.1%) males and 187 (24.9%) females enrolled in this study. Age range of this study population was 17-69 years with mean and SD of 38.63 ± 11.58 years. Information on clinical characteristics of the cases and seropositivity are shown in Table 2. Our results had shown of 754 sera isolates, 125 were positive by rose Bengal test. Thus frequency of brucellosis by Rose Bengal test was 16.5%. All the initial sera from the 125 patients gave titers of >1:40 in the Bruccellacapt, Coombs tests and SAT. All the initial sera from the 125 patients gave titers higher than 1:80 for Bruccellacapt while only 123 (99%) were positive in the SAT at the same titer. In 1:160 dilution, 91, 90 and 80% of sera were positive by Bruccellacapt, Coombs tests and SAT, respectively. Blood culture of 76 (60%) patient was positive from seropositive cases. The result revealed 60% accuracy for sensitivity and specificity of 88.1% using blood culture as gold standard. Results had shown in Tables 3 to 5 in detail. PCR Bruccellacapt, Coombs tests and SAT had 64, 55 and 48% in titer of 1:320, respectively. In titer of 1:640 percentage of positive sera were as an equal for Bruccellacapt and Coombs tests (31%) while this was 20% for SAT but in titer of 1:1280 results were different and were 12, 9 and 4.8% for Bruccellacapt and Coombs tests and SAT, respectively.

The PCR amplified a 905 and 375-bp sequence from positive controls, DNA samples extracted from reference strains B. abortus S19, and Rose Bengal positive sera. (Figure 1) Two PCR assays were carried out at admittance, and the results were positive in the cases the sera of the 125 (100%) patients (Table 4). In 50 results were negative by Rose Bengal test, as well as the results were negative by two PCR assays (100%).

DISCUSSION

Gender distribution in this study was 75.1% males and 24.9% females. In India Gemechu et al studied a sample...
Table 4. Results of Capt, SAT and Coombs test in different titer.

<table>
<thead>
<tr>
<th>Test</th>
<th>1/40 (%)</th>
<th>1/80 (%)</th>
<th>1/160 (%)</th>
<th>1/320 (%)</th>
<th>1/640 (%)</th>
<th>1/1280 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capt</td>
<td>125 (100)</td>
<td>125 (100)</td>
<td>113 (91)</td>
<td>79 (64)</td>
<td>38 (31)</td>
<td>14 (12)</td>
</tr>
<tr>
<td>SAT</td>
<td>125 (100)</td>
<td>123 (99)</td>
<td>103 (83)</td>
<td>68 (48)</td>
<td>25 (20)</td>
<td>6 (4.8)</td>
</tr>
<tr>
<td>Coombs test</td>
<td>125 (100)</td>
<td>125 (100)</td>
<td>112 (90)</td>
<td>73 (55)</td>
<td>38 (31)</td>
<td>11 (9)</td>
</tr>
</tbody>
</table>

Table 5. Comparison between Rose Bengal, PCR of l7/l12, PCR 16srRNA, Capt, SAT and Coombs test.

<table>
<thead>
<tr>
<th>Rose Bengal</th>
<th>PCR of 16srRNA</th>
<th>PCR of l7/l12</th>
<th>Brucella Capt test</th>
<th>SAT</th>
<th>Coombs test</th>
</tr>
</thead>
<tbody>
<tr>
<td>125/754 (100%)</td>
<td>125(100%)</td>
<td>125(100%)</td>
<td>113(91%)</td>
<td>103(83%)</td>
<td>112(90%)</td>
</tr>
<tr>
<td>50 Negative sera</td>
<td>Negative</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 1. A: Electrophoresis of PCR product of 16srRNA gene for sera samples on 1% agarose gel, L (Ladder 100 bp), 16srRNA = 905 bp (lane 1 to 7), lane 8, negative sample by rose Bengal test, lane 9 to 10: positive controls, DNA samples extracted from reference strains B. abortus S19. B: Electrophoresis of PCR product of l7/l12 gene for sera samples on 1% agarose gel, L (Ladder 100 bp), L7/L12 = 375 bp (lane 2-5), lane 1, negative sample by Rose Bengal test, lane 6: DNA samples extracted from reference strains B. abortus S19.

that males were 97.4% and reported median age of 38 years (range 19 to 64). In study of Ali et al in Egypt males were 72% and reported median age of 32 years (range 13 to 55) whereas in Spain Queipo-Ortuno et al. reported mean age of 37.9 years (range 14 to 91) (Queipo-Ortuno et al., 1997, Gemechu et al., 2011). Eight percent of the patients had no clinical suspicion of brucellosis but diagnosed as seropositive. Mantur et al. also made diagnosis in 88.7% cases only by routine serology. Although many serological tests and new automated blood culture techniques have been developed to diagnose brucellosis, there are still significant problems in the diagnosis of the disease. Blood cultures are time-consuming, also handling the organism cause a high risk of contagion for laboratory personnel. The sensitivity of blood cultures is from 53 to 90%. (Yagupsky, 1997c), (Mitka et al., 2007; Yagupsky et al., 1997b). Isolating Brucella from BACTEC bottles need 2.5 to 5 days, with 94 to 97% of Brucella strains growing within this time period (Yagupsky, 1997a). Serological methods lack specificity, and titers often remain positive for a protracted period after therapy even in cases of complete recovery. Although the SAT test, which is cost-effective, easy-to-use, and appropriate test, especially in regions endemic for brucellosis, but, it
has been shown that false negatives could be appropriate to a decrease in the agglutination ability of antibodies at the low pH of the test medium. While the RBT yields results within minutes, a minimum of 24 h is needed for the SAT test. This increases up to 48 h when Coombs’ test needs to be used to clarify negative results (Pabuccuoglu et al., 2011). Most important antigenic structure which has been used in the diagnosis of brucellosis in agglutination tests is the smooth lipopolysaccharide. One of the reasons for Negative results in agglutination tests is blocking antibodies (Ozdemir et al., 2011). Coombs and Brucellacapt test have been using to overcome the blocking antibodies phenomenon. On the other hand, PCR offers an alternative choice over the conventionally available methods for an accurate diagnosis of brucellosis. Many studies indicated PCR assays are simple, highly sensitive, specific, and relatively inexpensive, allowing the use of a PCR assay as a routine test in clinical laboratory practice (Yu and Nielsen, 2010). In addition few studies using Brucellacapt test have shown very promising sensitivity and specificity (Casao et al., 2004). In this study the aims was to compare PCR with Brucellacapt test reference to Coombs test and blood culture. The results from the present study show a high sensitivity and specificity of Brucellacapt for the diagnosis of human brucellosis. All the initial sera from patients with brucellosis included in the study had Brucellacapt and Coombs titers of >1:80, while only 99% of them were SAT positive. However, problems in the interpretation arose with the use of a 1:80 diagnostic titer, in particular in areas of endemicity, where the prevalence of anti- Brucella antibodies is high due to previous episodes of brucellosis or exposure to infected animals in a high proportion of the population (Holt et al., 2011; Jama’ayah et al., 2011). The definition of a diagnostic titer, indicative of an active infection, has not been possible in human brucellosis, even in tests such as the Coombs test and SAT, which have been in use for a long time (Diaz et al., 2006). Mainly researchers consider a SAT titer of >1:160 as an indicator of active brucellosis. However, active brucellosis cannot be excluded in patients with lower SAT titers, especially during the first stages of the infection, in chronic brucellosis and in relapses (Casanova et al., 2009; Orduna et al., 2000). In the present study, nearly 48% of the initial sera from infected patients showed SAT titers of 1:160. This implies a serious limitation for disease diagnosis, especially since prompt treatment is very important for a good prognosis (Orduna et al., 2000).

Many studies were reported that the use of conventional PCR techniques, the PCR-ELISA and Light Cycler Real-Time PCR assay with whole blood and serum samples provides better results than conventional microbiological techniques for the diagnosis of both primary infection and relapses of brucellosis (Morata et al., 1999; Queipo-Ortuno et al., 2005). For diagnosis of human brucellosis by PCR assay we used three different fragments including: (i) a gene encoding a 31-kDa Brucella abortus antigen, (ii) a sequence 16S rRNA of B. abortus, and (iii) a gene encoding an outer membrane protein (omp-2). The sequence of B. abortus 16S rRNA using primers F4/R2 is still the most sensitive target for PCR detection (Navarro et al., 2002). We compared PCR assay using primers F4/R2 with Brucellacapt test, as well the sequence I7/I12 (ribosomal protein) of B. abortus using primers FL/RL.

Although, there is no standardization among studies in this technique such as extraction method, primers, and target sequences, storage conditions of samples or experimental setup. For that reason different results have been reported in the researches (Surucuoglu et al., 2009). A study showed that the 16S rRNA gene (F4/R2 primers) used for detection of bovine blood samples was insensitive (Mukherjee et al., 2007). However in another study, in comparison of three PCR methods for detection of human Brucellosis, F4/R2 was the most sensitive primer (Navarro et al., 2002). Previously it was reported, where PCR using primers B4-B5 (bcsp31) and p1/p2 (omp2) were the most sensitive for detection of Brucella spp. (Elfaki et al., 2005; Mitka et al., 2007). It is controversial which one of these primers is better, on the other hand the I7/I12 is a ribosomal protein gene, essential for ribosomal function and protein synthesis present in all of Brucella spp. and showing nearly the identity 98 to 100% (Bachrach et al., 1994; Oliveira et al., 1994). In fact we used I7/I12 primers. Results of PCR assay differentiated all of the samples and using 16S rRNA and I7/I12 gene showed excellent specificity and good sensitivity.

Our study shows a higher sensitivity and specificity of PCR for the diagnosis of human brucellosis than serological tests. Sensitivity of PCR by I7/I12 gene similar with 16S rRNA, so could suggest its use for diagnosis of human brucellosis. Sensitivity of Brucellacapt test was higher than Coombs tests and SAT, although the sensitivity of PCR assay was more than all of them.

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