Enzyme Linked Immunosorbent Assay Versus Polymerase Chain Reaction for Diagnosis of Brucellosis

M. Hasibi, A. Amirzargar, S. Jafari, A. Soudbaksh, M. Hajiabdolbaghi, A. Rashidi and M. Gharouni

The aim of study was to determine the diagnostic value of Polymerase Chain Reaction (PCR) and Enzyme Linked Immunosorbent Assay (ELISA) in 37 confirmed cases of brucellosis and 78 healthy controls. The diagnosis of brucellosis was based on a compatible clinical picture plus a positive blood culture and/or an antibody titer of ≥ 1:160 at the serum agglutination test. Controls were asymptomatic individuals with negative blood cultures and no previous history of brucellosis. PCR and ELISA were performed on all samples. PCR was positive in 15 (40.5%) patients and no controls. Mean±SD (range) ELISA IgG levels in patients and controls were 198.9±63.1 (97.7-231.9) IU mL⁻¹ and 14.6±27.2 (0-145.0) IU mL⁻¹, respectively (p<0.001). The area under the receiver operating characteristic curve for distinguishing between cases and controls was 0.977. The cutoff point for ELISA results yielding maximal sensitivity plus specificity was 167.35 IU mL⁻¹. ELISA proved to be a more appropriate diagnostic method than PCR in the series, with sensitivity, specificity, positive predictive value and negative predictive value of 89.2, 100, 100 and 95.1%, respectively.

Key words: Brucellosis, diagnosis, PCR, ELISA

1Amir-Alam Hospital, Tehran University of Medical Sciences, Tehran, Iran
2Department of Immunogenetic, Tehran University of Medical Sciences, Tehran, Iran
3Imam Hospital, Tehran University of Medical Sciences, Tehran, Iran
INTRODUCTION

Brucellosis is an infectious zoonotic disease that is associated with chronic debilitating infections in humans (Pappas et al., 2005). It is an important public health problem in Iran, with a seroprevalence of 1-2% in the general population (Karimi et al., 2003). The clinical manifestations of the disease are nonspecific and may simulate several other febrile diseases; thus the clinical diagnosis must usually be supported by the results of bacteriologic and/or serologic tests. Isolation of Brucella in cultures is definitive, but attempts at isolation are frequently unsuccessful (Queipo-Ortuño et al., 2005).

The Serum Agglutination Test (SAT) is the most popular diagnostic tool for brucellosis, though sometimes yielding misleading results. The lack of seroconversion can be attributable to the performance of the test early in the course of infection, the presence of blocking antibodies, or the inhibition of agglutination at low dilution due to an excess of antibodies (Young, 1991). The enzyme linked immunosorbent assay (ELISA) detects Brucella specific antibodies and has been reported to have higher sensitivity and specificity. However, the results obtained by different studies have not been consistently supporting (Fadell et al., 2006; Gad El-Rab and Kambal, 1998). Recently, the development of Polymerase Chain Reaction (PCR) has offered a new dimension in the rapid diagnosis of brucellosis (Mitka et al., 2007; Zerva et al., 2001). The PCR assay is rapid and it may be considered a useful tool for diagnosis of human brucellosis. In the present study, the diagnostic value of ELISA was investigated and compared with peripheral blood PCR assay. Furthermore, the optimal diagnostic cutoff point for ELISA results was determined.

MATERIALS AND METHODS

A total of 37 consecutive patients with brucellosis diagnosed in the Infectious Diseases Department of Imam Hospital, Tehran University of Medical Sciences between Oct., 2005 and Nov., 2007 were evaluated. The study protocol was approved by the ethics committee of our university and all patients and controls gave informed consent prior to inclusion in the study. The diagnosis of brucellosis was based on a compatible clinical picture plus a positive blood culture and/or significant antibody titers at SAT defined as ≥1:160. Blood cultures were processed by standard bacteriologic techniques with a BACTEC 9240 system (Becton Dickinson Diagnostic Instrument Systems, Towson, Md.). For those patients in which the system failed to detect any growth, the incubation was maintained for 30 days, with blind subcultures performed after 10, 20 and 30 days. Brucella species (B. melitensis in all cases in present series) were identified as reported by Hausler et al. (1984). Patients with a earlier history of brucellosis or anti-brucellosis therapy were not included. Blood samples from 78 healthy controls (asymptomatic individuals with negative blood cultures and no earlier history of brucellosis) were included in the study.

The sera obtained from all patients and controls were tested for Brucella antibodies using Brucella ELISA IgG test (Immuno Biological Laboratories Company, Germany). All sera were routinely diluted from 1:80 to 1:20, 480 to overcome prozone phenomenon. Each batch of tests included a positive 1:1, 280 control and a negative saline control. A definite agglutination of the suspension was read as a positive reaction. If prozone phenomenon was encountered, the higher dilution agglutination was recorded.

Details of the PCR method can be found in Elfaki et al. (2005a). Five hundred microliter of blood was collected in sodium citrate and stored at -20°C, firstly treated with lysis buffer (10 mM Tris/HCl [pH 7.5], 0.3 M saccharose solution, 1% Triton X-100, 5 mM Mg(II)). The mixture was centrifuged at 14000 x g for 1 min. The PCR target sequence of 223-bp present on a gene encoding a 31-kDa Brucella abortus antigen was selected for amplification. The primers of B4 and B5 (Pharmacia LKB, Barcelona, Spain) were used to amplification as reported by Baily et al. (1992). PCR reactions were performed in a total volume of 25 μL in 10 mMTris/HCl (pH 8.3), 50 mM KCl, 1 mM MgCl2, 0.2 mM of each dNTP, 0.7 μM of each primer and 2.5 U of Taq polymerase (Boehringer). Reaction mixture was amplified for 5 min at 95°C in first cycle and sequentially 30 sec at 90°C, 30 sec at 61°C and 30 sec at 72°C. Then mixture was kept at 72°C for 5 min additionally. This amplification program was repeated 35 cycles. PCR products were analyzed by agarose gel electrophoresis in TBE buffer (8 mM boric acid, 89 mM Tris [pH 8.0], 10 mM EDTA) and DNA visualized by ethidium bromide. PCR test was optimized with known strains of Brucella abortus for which the lowest detection limit was 1 pg. All PCR's were performed in duplicate with appropriate inclusion of negative and positive controls. Strict precautions including working in separate rooms for master mix preparation, sample extraction and PCR analysis were taken to prevent contamination.

Age, sex and ELISA IgG levels were compared between the groups by the Student's t-test, the Chi-squared test and the Mann-Whitney U-test, respectively. To determine the optimal cutoff point for ELISA results, the Receiver Operating Characteristic
(ROC) curve was drawn and the IgG level yielding maximal sensitivity plus specificity was selected. Data were analyzed with the SPSS statistical program (SPSS Inc., SPSS/PC+, Chicago, Illinois, USA), all tests were two-sided and p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The groups were matched in age (44.8±14.7 years in the case versus 43.6±15.6 years in the control group; p = 0.678). The proportion of females was higher in the control than in the case group (43 versus 9, respectively; p = 0.002). Blood culture was positive in 21 (56.8%) patients, all except two of whom had antibody titers ≥1:160 at SAT. All culture negative patients had antibody titers ≥1:160 at SAT. Therefore, laboratory diagnoses of patients was based on positive results in both blood culture and SAT in 19 (51.4%) patients, positive results only in SAT in 16 (43.2%) patients and positive results only in blood culture in 2 (5.4%) patients. Diagnosis in all patients was heavily supported by a compatible clinical picture and appropriate response to therapy. PCR was positive in 15 (40.5%) patients and no controls.

Mean±SD (range) ELISA IgG levels in patients and controls were 198.9±63.1 (9.7-231.9) IU mL⁻¹ and 14.6±27.2 (0.145.0) IU mL⁻¹, respectively (p<0.001). Controlling for the potentially confounding effect of sex did not reduce the significance of this difference (data not shown). The area under the ROC curve for distinguishing between cases and controls was 0.977 (Fig. 1), significantly different from 0.5 (p<0.001). The cutoff point for ELISA results yielding maximal sensitivity plus specificity was 167.35 IU mL⁻¹ (sensitivity: 89.2%; specificity: 100%). Table 1 shows a comparison between PCR and ELISA using four measures (sensitivity, specificity, positive predictive value, negative predictive value).

![Fig. 1: ROC curve for distinguishing between cases and controls using ELISA IgG](image)

Table 1: Comparison between PCR and ELISA IgG

<table>
<thead>
<tr>
<th>Diagnostic tool</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>40.5</td>
<td>100</td>
<td>100</td>
<td>78.0</td>
</tr>
<tr>
<td>ELISA IgG</td>
<td>89.2</td>
<td>100</td>
<td>95.1</td>
<td></td>
</tr>
</tbody>
</table>

PPV: Positive Predictive Value; NPV: Negative Predictive Value

Table 2: Available reports on sensitivity and specificity of PCR

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of patients</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Queipo-Ortuño et al. (1997) Spain</td>
<td>50</td>
<td>98.8</td>
<td>100.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Zerva et al. (2001) Greece</td>
<td>31</td>
<td>100.0</td>
<td>98.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Cirk and Hizel (2002) Turkey</td>
<td>22</td>
<td>100.0</td>
<td>98.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Nimir (2003) Jordan</td>
<td>165</td>
<td>100.0</td>
<td>98.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Elfaki et al. (2005) Saudi Arabia</td>
<td>20</td>
<td>100.0</td>
<td>98.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Hashi et al. [present study] Iran</td>
<td>37</td>
<td>100.0</td>
<td>98.5</td>
<td>50.0</td>
</tr>
</tbody>
</table>

50 and 100% (Elfaki et al., 2005, Cirk and Hizel, 2002, Nimir, 2003; Zerva et al., 2001; Queipo-Ortuño et al., 1997) (Table 2). The sensitivity of 40.5% obtained for PCR in the present study is rather low. It could be due to the higher detection limit of PCR in this study (1 pg) compared to other reports (e.g. 10 fg) (Queipo-Ortuño et al., 1997). This may limit the detection of the pathogen when very low DNA levels are present. Moreover, serum is the optimal specimen for the diagnosis of brucellosis by PCR and has higher sensitivity compared to whole blood (94 vs. 61%, respectively) (Navarro et al., 2004). Application of whole blood samples instead of serum in this study could be another reason for the low sensitivity.

There is general agreement that ELISA is a more sensitive method than traditional techniques used in the diagnosis of brucellosis (Gad El-Rab and Kambal, 1998; Osoba et al., 2001). Furthermore, SAT does not discriminate between the immunoglobulin classes.
The detection of specific immunoglobulins by a single, simple and rapid test is a major advantage with ELISA. The main problem with widespread use of ELISA in our country has been the lack of a definite cutoff value. We determined, for the first time, the appropriate cutoff value for ELISA in Iran. Using a cutoff of 167.35 IU mL\(^{-1}\) in the series resulted in 100% specificity and 89.2% sensitivity. These results are more promising than those obtained in an earlier study on 68 patients and 70 controls in Saudi Arabia. In that study, ELISA had 45.6% sensitivity and 97.1% specificity (Memish et al., 2002). Present results, among other factors (e.g., higher cost of PCR, availability in only a few laboratories across the country) favor using ELISA rather than PCR, at least in our country.

In summary, the value of PCR and ELISA methods in aiding the diagnosis of Brucellosis in 37 patients and 78 healthy controls was compared. ELISA proved to be a more appropriate diagnostic method, with sensitivity, specificity, positive predictive value and negative predictive value of 89.2, 100, 100 and 95.1%, respectively. If future studies with larger sample sizes confirm the results, PCR will be expected to be soon replaced by ELISA for the diagnosis of brucellosis in our country.

REFERENCES


