Serological and Molecular Diagnosis of Human Brucellosis in Najran, Southwestern Saudi Arabia

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ABSTRACT

This work aims to investigate the prevalence of human brucellosis in Najran, southwestern Saudi Arabia and to assess the performance of ELISA and PCR as diagnostic tools of brucellosis in comparison to the conventional methods. The study included 340 patients with clinical characteristics of brucellosis. Blood samples from cases were subjected to culture, standard tube agglutination test (SAT), ELISA for IgM and IgG, and brucella PCR. The diagnosis of brucellosis was confirmed in 54 (15.9%) of 340 provisionally diagnosed patients. Blood culture identified only 14 (25.9%) cases. SAT was positive in 50 (92.6%) cases, and ELISA IgM and IgG were found positive in 46 (85.2%) and 52 (96.3%) cases, whereas PCR was positive in 38 (70.4%) cases. The sensitivities of ELISA IgM and IgG were 85.2% and 96.3% respectively and the specificity was 100% for each. For PCR, the sensitivity and specificity were 70.4% and 100% respectively. In conclusion, ELISA IgM and IgG tests seem to be valuable tools for definitive diagnosis of brucellosis in endemic areas. The PCR can be particularly important in patients with clinical picture and negative serological results, allowing early and rapid confirmation of the disease.

INTRODUCTION

Brucellosis is a systemic disease caused by bacteria of the genus Brucella that affects humans and numerous animal species. Transmission to humans occurs by ingestion of raw or unpasteurized milk and other dairy products, by direct contact with infected animal tissues, or by accidental ingestion, inhalation, or injection of the Brucella culture(1).

According to the World Health Organization, half a million of new human cases are reported each year, but these numbers greatly underestimate the true incidence of human disease(2). Although the incidence of the disease has decreased markedly in industrialized countries, it remains a major public health problem in many developing countries. In Saudi Arabia, Brucellosis is hyper-endemic with an incidence of 5.4 per 1,000 per year. Its prevalence has been variable in different regions of the country with reported values of 8.8 to 38%(3). According to Memish et al.(4), more than 8,000 cases are reported per year to public health authorities.

In humans, brucellosis behaves as a systemic infection with a very heterogeneous clinical spectrum(5). The infection is characterized by protean manifestations and prolonged recurrent febrile episodes. The features of acute disease are varied and may be insidious, whereas the features of chronic disease, which may persist or recur for years, are often vague(6). The disease, therefore, cannot be diagnosed on clinical grounds alone, and microbiological confirmation is required through the isolation of Brucella spp. from blood cultures or the detection of specific antibodies through the use of serological tests. However, the established methods for laboratory diagnosis are often unreliable in several respects. Culture is a time-consuming procedure. In addition, failure to detect the pathogen is a frequent occurrence and Brucella spp. are class III pathogen posing considerable risk to laboratory personnel(7). Common serological tests are the Rose Bengal plate agglutination test (RBPT), standard tube agglutination test (SAT), Coombs test and ELISA. Conventional serological methods have important limitations. Their sensitivity is poor in the early stage of the disease, and their specificity is reduced in areas where the disease is highly endemic and in the frequent relapses of the disease(8). The development of specific PCR assays is a recent advance in diagnosis of human brucellosis, but information concerning the use of this diagnostic tool is scarce.

This work aims to investigate the prevalence of human brucellosis in Najran, southwestern Saudi Arabia and to assess the performance of ELISA and PCR as diagnostic tools of brucellosis in comparison to the conventional methods.

MATERIALS AND METHODS

From April 2010 to September 2011, a total of 340 patients attending the Infectious Diseases Clinic of King Khalid Hospital in Najran and presenting with clinical characteristics of
brucellosis were included in this study. According to the duration of symptoms, the patients were classified into 3 groups: acute group with presentation for < 2 months (N = 180), sub-acute group with the symptoms for 2 to 12 months (N = 110) and chronic group with symptoms for > one year (N = 50). One hundred healthy personnel who were blood donors at the hospital’s blood bank were enrolled as controls. Eight ml blood (5 ml for culture and serology and 3 ml mixed with EDTA for PCR assay) were taken from all patients and controls. The diagnosis of brucellosis was established according to one of the following criteria: (i) the isolation of *Brucella* spp. in blood culture or (ii) the presence of compatible clinical picture together with positivity for one or both of SAT and ELISA.

**Bacteriological and serological techniques**

Blood cultures were processed with biphasic blood culture medium (Biomerieux, France), incubated at 37°C in an atmosphere of 5% to 10% carbon dioxide for 30 days and sub-cultured weekly. Suspected colonies were identified according to the standard techniques.

For serology, All sera from patients and controls were tested by SAT and ELISA for IgM and IgG against *Brucella* species. In the SAT, equal volumes of serial dilutions of the serum (from 1:10 to 1:1280) and *B. abortus* and *B. melitensis* antigens (Omega Diagnostic Ltd, UK) were mixed in test tubes and incubated at 37°C for 24 hours. Known negative and positive control sera were used. A titer of ≥1/160 was considered positive. ELISA testing for IgM and IgG against *Brucella* spp. was performed using commercial reagents (Genzyme Virotech, Germany). The absorbance values found were converted into Virotech units (VE) using the following formula according to the manufacturer’s instructions: patient sample (mean) absorbance X 10/mean absorbance value of cut off controls (>11 VE was considered positive). Borderline results were re-tested and confirmed either positive or negative.

**Brucella PCR**

The detection of a target sequence of 223 bp within the gene coding for the production of a 31-kDa membrane protein specific to the genus *Brucella* was performed by PCR, using specific primers (Qiagen, USA) as previously reported. The sequences of these primers were: forward 5'-TGCTCGGGTCTATATATGACAAACGTCATCTG-3' and reverse 5'-CGCGCTGCTTTTCAGGTCTG-3'.

Serum samples from patients and controls were extracted for the isolation of *Brucella* DNA using E.Z.N.A commercial kit (Omega Biotech, USA), according to the manufacturer’s instructions.

The PCR amplification mixture consisted of pure Taq ready-to-go PCR bead (Amersham Bioscience, UK), 10 pmol/µL of each primer and 50 pg of *Brucella* DNA extract in a total volume of 50 µL. The amplification was performed in a thermal cycler (Cyclogene Techn, UK). The reaction mixtures were heated to 90°C for 5 minutes, followed by 40 amplification cycles, each consisting of 60 seconds at 90°C, 30 seconds at 60°C and 60 seconds at 72°C. A final extension cycle of 72°C for 7 minutes was included. The amplified products were electrophoresed in 1.5% agarose gel. The gels were stained with ethidium bromide and visualized under ultraviolet trans-illuminator (Cole-Parmer, USA). The presence of a clear-cut band of 223 bp was considered as a positive result.

**Statistical methods**

Data were entered and analyzed using SPSS 10 for windows statistical package (SPSS Inc, USA). Sensitivity, specificity, and positive and negative predictive values were calculated.

**RESULTS**

We studied a total of 340 patients having presumptive diagnosis of brucellosis. The patients were between 19 and 82 years of age, with the mean age of the patients being 32.18 years with a standard deviation of ± 11.73 years. Two hundred and sixty (76.5%) patients were males and 80 (23.5%) were females; the male to female ratio was 3.3:1. There was no seasonal variation in the cases studied. The noticeable symptoms were with patients having fever, joint pain, low backache, headache, and vomiting. Consumption of raw milk (205 patients) and direct contact with domestic animals (138 patients) were recognized as major risk factors for transmission of brucellosis in our study.

In this work, the diagnosis of brucellosis was confirmed in 54 (15.9%) of the 340 patients. Blood culture identified 14 (25.9%) cases. SAT was positive in 50 (92.6%) cases, and ELISA IgM and IgG were positive in 46 (85.2%) and 52 (96.3%) cases, whereas PCR was positive in 38 (70.4%) cases. The distribution of laboratory tests results among the brucellosis groups is listed in table (1). Control samples were all negative by culture, SAT and PCR.
Table (1): Distribution of laboratory tests results according to the type of brucellosis

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Brucellosis groups</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute No. =180 (%)</td>
<td>Subacute No. =110 (%)</td>
<td>Chronic No. =50 (%)</td>
<td>Total No. =340 (%)</td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>13 (7.2)</td>
<td>1 (0.9)</td>
<td>0 (0)</td>
<td>14 (4.1)</td>
<td></td>
</tr>
<tr>
<td>SAT</td>
<td>28 (15.6)</td>
<td>6 (5.5)</td>
<td>16 (32)</td>
<td>50 (14.7)</td>
<td></td>
</tr>
<tr>
<td>ELISA IgM</td>
<td>32 (17.8)</td>
<td>4 (3.6)</td>
<td>10 (20)</td>
<td>46 (13.1)</td>
<td></td>
</tr>
<tr>
<td>ELISA IgG</td>
<td>28 (15.6)</td>
<td>6 (5.5)</td>
<td>18 (36)</td>
<td>52 (15.3)</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>32 (17.8)</td>
<td>4 (3.6)</td>
<td>2 (4)</td>
<td>38 (11.2)</td>
<td></td>
</tr>
</tbody>
</table>

Twenty one patients with SAT titer of 1/160 and 18 patients with SAT titer of 1/320 yielded positive results by ELISA IgM and IgG. ELISA IgM and IgG were positive in 4 patients where SAT titer was 1/80. PCR test was positive in 38 cases where SAT titers were 1/160 (in 11 cases), 1/320 (17 cases), 1/640 (6 cases) and 1/1280 (4 cases) as presented in table (2). The sensitivity, specificity, and positive and negative predictive values of ELISA-IgM, ELISA-IgG and PCR are presented in table (3).

Table (2): Distribution of ELISA and PCR results according to SAT titers

<table>
<thead>
<tr>
<th>SAT titer</th>
<th>No of cases</th>
<th>ELISA IgM</th>
<th></th>
<th>ELISA IgG</th>
<th></th>
<th>PCR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1/40</td>
<td>96</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>1/80</td>
<td>194</td>
<td>4</td>
<td>190</td>
<td>4</td>
<td>190</td>
<td>0</td>
<td>194</td>
</tr>
<tr>
<td>1/160</td>
<td>18</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>1/320</td>
<td>21</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>1/640</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>1/1280</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table (3): Diagnostic yield of ELISA IgM, ELISA IgG and PCR

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA IgM</td>
<td>85.2%</td>
<td>100%</td>
<td>100%</td>
<td>97.3%</td>
</tr>
<tr>
<td>ELISA IgG</td>
<td>96.3%</td>
<td>100%</td>
<td>100%</td>
<td>99.3%</td>
</tr>
<tr>
<td>PCR</td>
<td>70.4%</td>
<td>100%</td>
<td>100%</td>
<td>94.7%</td>
</tr>
</tbody>
</table>

DISCUSSION

Brucellosis is an important public problem in many developing countries, including the Mediterranean countries and the Arabian Peninsula. The overall prevalence of human brucellosis in this study was 15.9%. In an earlier report, the seroprevalence rate was 19% in the Southern region. In another large-scale study investigating the seroprevalence of brucellosis on 24000 subjects in different Saudi regions, the highest prevalence of brucellosis was in Northern and Southern regions accounting for 20% and 18.3% respectively, while it was 14.6%, 14% and 11.6% in Central, Eastern and Western regions respectively. Many previous Saudi studies showed that the area of residence (northern or southern regions) has a significant effect on seroprevalence. Border locations where there are uncontrolled movements of animals may have a high prevalence rate, especially in villages where Bedouins live in close contact with animals. However, in another recent study in Najran region, the prevalence was 7.3% among 540 healthy subjects. In that study, ELISA IgG was used as the only diagnostic technique and the authors might underestimate the true prevalence of the disease. It had been reported that none of the serological techniques used in the diagnosis of brucellosis are 100% sensitive and specific. Data from Middle East countries reported seroprevalence rates ranging from 8% in Jordan to 12% in Kuwait.

Despite the important advances made in the diagnostics of human brucellosis following the introduction of automated blood culture techniques, diagnosis of this disease is still based mostly on serological and molecular
methods. Among the newer serologic tests, ELISA appears to be the most sensitive. In this work, overall concordant results between ELISA IgM and IgG titers, and SAT titers were found among 88.5% of patients within the three groups. Six patients yielded discrepant results: 4 acute brucellosis patients with negative SAT titers were positive by ELISA for IgM and IgG and 2 chronic brucellosis patients tested positive only in ELISA IgG. This serological picture is very similar to that reported elsewhere in the world. Mantur et al. reported positive ELISA IgM and IgG in 16 of 28 acute brucellosis patients with negative SAT results and positive ELISA IgG in 21 of 29 chronic brucellosis patients with negative ELISA IgM and SAT titers. Irmak et al. found that 9% of 26 acute brucellosis patients tested positive in the IgM assay and negative in the IgG assay, 56% tested positive in both tests, 26% tested positive only in the IgG assay, and 9% tested negative in both tests. It is known that IgM may be detected after the first week following the entry of the organism. The peak level is reached 4 weeks later. IgG has a delayed appearance, although it is found mixed with IgM 4 weeks after the initial antigenic stimulus; the IgM level always exceeds the IgG level in the acute stage of the disease. Our findings agree with the principle that the IgM test is more indicative of acute infection, while IgG is more useful for the diagnosis of the sub-acute and chronic infection.

Different studies showed different results for ELISA sensitivity and specificity. The sensitivity and specificity of ELISA in this work are in agreement with that reported in other studies. Mantur et al. reported sensitivities of 100% and 91% for ELISA IgM and IgG respectively and specificity of 100% for both. Mantur et al. reported ELISA sensitivity of 71.3% and specificity of 100%. Memish et al. found that sensitivity and specificity of ELISA IgM were 79.1% and 100%, whereas they were 45.6% and 97.1% for ELISA IgG. Combining ELISA IgM and IgG positivity in their study increased the sensitivity to 94.1% and the specificity to 97.1%.

The PCR based assays are promising alternatives for the diagnosis of brucellosis. In this work, PCR correctly diagnosed the 32 acute brucellosis patients. In accordance, Mitka et al. found that rate of PCR positive results in 200 acute brucellosis patients was 99%, while it was 91.2% (31 of 36 patients) in the study of Surucuoglu et al. On the other hand, our PCR test results were only positive in 11.1% of patients with chronic disease. This lower rate might be due to low organism load in the blood of patients with chronic brucellosis. One of the main characteristics of the brucellosis is its ability to establish the diagnosis of acute brucellosis.

Although the PCR specificity in this work was 100%, its sensitivity was 70.4%. In previous studies, PCR sensitivities varied from 66% to 94%. This can be related to lack of uniformity and standardization among studies in PCR protocols such as optimal clinical specimen, sample volume, extraction method, primers and target sequences, storage conditions of samples or experimental setup.

**CONCLUSION**

The results of this study showed that ELISA is a valuable test in the diagnosis of brucellosis in endemic areas. Its ability to measure 2 specific antibodies makes this an effective diagnostic tool of brucellosis. This is especially important, since it may be possible to use this test to confirm the clinical stage of the disease. The PCR test results can be particularly important in patients with clinical signs and symptoms, and negative serological results, allowing early and rapid confirmation of the disease.

**Acknowledgment**

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**REFERENCES**

التشخيص المناعي والجزيئي لمرض الحمى المالطية البشرية في منطقة نجران، جنوب غرب المملكة العربية السعودية

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الهدف من هذا البحث هو تحديد معدل انتشار الحمى المالطية البشرية في منطقة نجران، جنوب غرب المملكة العربية السعودية، وتحقيق أداء اختبار الإليزا وتفاعل سلسلة إنزيم البلمرة في تشخيص الحمى المالطية مقارنة بالطرق التقليدية. استخلص البحث على 340 مريض لديه الخصائص السريرية للحمى المالطية. تم تجميع عينات الدم من كل المرضى لإجراء اختبار الإليزا وتفاعل سلسلة إنزيم البلمرة. تم التشخيص المعلي في 54% (151/54) مريض. كان اختبار الإليزا للجسم المضاد أم و/إيجابيا في 46 و 53 مريض بينما تفاعل سلسلة إنزيم البلمرة إيجابيا في 28 مريض. كانت حساسية اختبار الإليزا للجسم المضاد أم و/ إيجابيا 0.9% و 2.3% و 1.3% وكانت خصوصية الاختبار 100%، أما حساسية وخصوصية تفاعل سلسلة إنزيم البلمرة في 4.7% و 0.7% على التوالي.

من هذا البحث تم استنتاج أن اختبار الإليزا أداة ذات قيمة في التشخيص النهائي للحمى المطية في المناطق المستوطنة، اختبار تفاعل سلسلة إنزيم البلمرة يعتبر هاماً في التشخيص المبكر والسريع في المرضى المشكوك في إصابتها بالمرض ويساهم في نتائج سلبية بطرق التشخيص التقليدية.