Evaluation of Rose Bengal Test, Standard Tube Agglutination Test and Nested PCR for the diagnosis of Human Brucellosis

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Brucellosis affects human populations in many developing countries including the Middle East where it is still endemic. The clinical diagnosis of human brucellosis is difficult so, physicians depend upon laboratory confirmation. Peripheral blood specimens were taken from 40 patients having compatible signs and symptoms that were clinically diagnosed to have brucellosis. They were 23 males and 17 females, and their ages range between 21 and 74. They were tested by Rose Bengal test (RBT), Standard tube agglutination test (STA) and nested PCR using four primers to amplify 677bp fragments. 23 patients (57.5%) were positive by (RBT). 25 patients (62.5%) were positive by (STA). 28 patients (70%) were positive by nested PCR assay. Our results show that the PCR assay is more sensitive than conventional serological methods and this coupled with its speed and reduction in risk to laboratory workers. So, PCR technique is a very useful tool for the diagnosis and follow-up of human brucellosis.

INTRODUCTION

Brucellosis is a zoonosis widely distributed around the world (1). Brucella species namely; Brucella abortus, B. Canis, B. Suis and especially B. melitensis are able to cause human infections (2). The disease has a worldwide distribution and constitutes a major public health problem in the Mediterranean countries, the Middle East, Southeast Asia, and Central and South America (3).

Brucellosis is prevalent in Egypt and in the recent years there is an increase in number of positive human cases. (4). Brucellosis is transmitted by the ingestion of raw or unpasteurized milk and other dairy products, by direct contact with infected animal tissues, or by accidental ingestion, or inhalation of infected materials.

Human brucellosis is manifested by septicemic febrile illness or localized infection of bone, tissue, or organ systems (5). The variable symptoms, the paucity of distinctive physical signs and the occurrence of subclinical and atypical infections in both the acute and the chronic stages make the clinical diagnosis of human brucellosis difficult (6). Clinicians therefore rely substantially on laboratory confirmation, even though they are confident in most of the cases that the clinical picture is highly suggestive of brucellosis.

Diagnosis of human Brucellosis can be established by laboratory methods such as blood cultures and serology. The culture techniques are time-consuming and lack sensitivity for patients with chronic infections. In addition, cultures are not always positive when other tests are positive (7). Many serological tests have been used for the diagnosis of human brucellosis such as agglutination tests, The most commonly used tests are the standard tube agglutination test (STA),the Rose Bengal test (RBT) and the Coombs anti-Brucella test (8). Each test has its own advantages and disadvantages and the presence of antibodies doesn't always mean an active case of brucellosis, since individuals from endemic areas often show weak serological responses. Cross-reactions with other gram negative bacteria occur, giving false-positive results (9). As for other fastidious pathogens, amplification of DNA by PCR offers an alternative way for diagnosis of brucellosis PCR assay shows high specificity and sensitivity, in acute and chronic disease and allows rapid diagnosis (10).

Many reports evaluate and compare blood culture, Rose Bengal test and PCR assay in the diagnosis of Brucellosis (11). Furthermore no much works have been done to explore the laboratory diagnosis of Brucellosis in Egypt, Therefore the present study was designed to compare Rose Bengal
test, standard tube agglutination test and PCR assay in the diagnosis of human Brucellosis.

PATIENTS AND METHODS

Forty clinically suspected brucellosis patients (ages: 21 to 74 years-old, 23 males and 17 females) from Tanta Fever Hospital included in this study. Most patients manifested low-grade fever, malaise, insomnia & arthralgia with prominent back pain. Other causes of fever were excluded clinically & by laboratory tests if possible.

Sample collection

Blood samples were collected aseptically by vein puncture from each patient at the initiation of therapy & serum separated in two sterile eppendorfs, one for serology& other for PCR.

Serological tests:

Slide agglutination test (Rose Bengal test)

It was conducted as described by the manufacturer using B. abortus antigen (Rose Bengal reagent, Spinreact, S.A. Ctra. Santa Coloma, Spain). The presence of agglutination indicates Brucella antibodies concentration equal or greater than 25 IU /ml (Reference value up to 25 IU /ml).

Standard tube agglutination (STA) test

It was conducted as previously described by the manufacturer using killed stained Brucella antigens (Brucella abortus and Brucella melitensis) (ANTEC reagent, U. K.). A titer of 1/160 was considered positive

Polymerase chain reaction (PCR)

Nested PCR technique was used for the detection of Brucella DNA because it is more sensitive, it involves three successive steps; DNA extraction, amplification and detection of the amplified specific DNA product.

DNA extraction

DNA was extracted from sample as described by the manufacturer (Fermentas chemical company). Briefly 200 µl of serum, treated with 400 µl of lysis solution and incubated for 5 minutes at 65°C, 600 µl of chloroform was added and centrifuged at 10,000 rpm for 2 min. DNA in upper aqueous phase was treated by precipitation solution and centrifugation at 10,000 rpm for 2 min. The DNA pellets were dissolved in 1.2 M Na Cl solution. The DNA was precipitated by cold ethanol and centrifugation at 10,000 rpm for 4 min. The DNA pellets were washed once with 70% ethanol and dissolved in sterile deionized water by gentle vortex.

DNA amplification:

The primers used were genus-specific primers to amplify a highly conserved region within the 16S rRNA of the genus Brucella. The size of the amplification products was a 677 base pair fragment of the 16S rRNA genes. Four Primers were used:

External Sense:
5-TCGAGAATTGGAAGAGGTC-3

External Antisense:
5-GCATAATGCCTTTAAGA-3

Internal Sense:
5-AAGAGGTCGGATTTATCCG-3

Internal Antisense:
5-CGAGCATTTGCAGTCGAA-3

The reaction mixture in the 1st round PCR:
25 µl of 2 × master mix (Fermentas),0.5 µl external sense primer(conc. 0.1 µM), 0.5 µl external Antisense primer(conc. 0.1 µM),14 µl sterile deionized water & 10 µl template DNA, with a total volume of 50 µl. The cycling conditions were an initial denaturation at 95°C for 5 min, template denaturation at 94°C for 60s, annealing at 55°C for 60s, and extension at 72°C for 90s for a total of 30 cycles. (12)

The reaction mixture in the 2nd round PCR:
25 µl of 2 × master mix (Fermentas),0.5 µl internal sense primer(conc. 0.1 µM), 0.5 µl internal Antisense primer(conc. 0.1 µM),19 µl sterile deionized water & 5 µl of 1st round PCR amplified product with a total volume of 50 µl. The cycling conditions were template denaturation at 94°C for 60s, annealing at 55°C for 60s, and extension at 72°C for 90s for a total of 30 cycles then post extension at 72°C for 10 min. (12)

Detection of the amplified specific DNA product:
Ten microliters of the PCR product, negative control and positive control and two microliters of 6 X loading dye (Fermentas) and ten microliters of marker(Fermentas) were run by electrophoresis in a 2% agarose gel in 1 ×
TBE buffer and gels were stained with ethidium bromide (2 µg/ml). PCR amplification products were detected by visualization of the bands under UV light at 677 bp. \(^{(13)}\) (Marker gave different bands ranging between 100 - 2000 bp.)

**RESULTS**

Table 1: Epidemiological and clinical results of 40 cases of human brucellosis.

<table>
<thead>
<tr>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients studied</td>
</tr>
<tr>
<td>Male: Female</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
</tr>
<tr>
<td>No. of patients with fever</td>
</tr>
<tr>
<td>No. of patients with muscle pain and anorexia</td>
</tr>
<tr>
<td>No. of patients with adenitis and splenomegally</td>
</tr>
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(Table 1) show male/female ratio, the mean age in years and clinical presentation of the patients. The mode of infection gained by history taking indicated that, out of 40 patients included in this study, 25 (62.5%) had contact with animals (e.g., sheep and goats) either raised nearby where they live or at work (e.g., farmers or butchers). 16 (40%) acquired their infections by consuming unpasteurized milk or homemade dairy products (e.g., soft cheese). The other 5 (8%) patients acquired their infection from unknown sources. The mean duration of symptoms before diagnosis of the brucellosis was 25 days (range 10–60 days).

Table 2: Sensitivity of serological & PCR tests: (no. of patients = 40)

<table>
<thead>
<tr>
<th>Test</th>
<th>Brucella assay result</th>
<th>% Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>-Rose Bengal</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>-Standard tube agglutination</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>-PCR</td>
<td>28</td>
<td>12</td>
</tr>
</tbody>
</table>

(Table 2) show out of 40 patients with clinical suspicion of Brucellosis only 23 patients (57.5%) were positive by Rose Bengal test, 25 patients (62.5%) were positive by standard tube agglutination test with titers ranging from 1/160 – 1/2560 and 28 patients (70%) were positive by PCR.
Figure (1): Agarose gel electrophoreses and ethidium bromide staining of PCR product derived from Brucella DNA in patients with brucellosis. lane 1 marker, lane 2 positive control, lane 3 negative control, lane 4 positive case, lane 5 negative case, lane 6 positive case, lane 7 negative case, lane 8 positive case

677bp

Figure 2: show the percentage of positive and negative patients according to the different studied methods.
DISCUSSION

In the present work three laboratory methods namely; Rose Bengal test, standard tube agglutination test and nested Polymerase chain reactions were evaluated as laboratory tools for the diagnosis of human brucellosis. The classical Rose Bengal test (RBT) is often used as a rapid screening test. RBT is based on the agglutination of serum antibodies with a stained whole cell preparation of killed Brucella (14). Our work showed that Rose Bengal test was positive in 57.5 % of the patients. Rose Bengal test correlated well with STA except for two cases which were positive by STA and negative by Rose Bengal test. In this work, five patients were positive by PCR tested negative in the RB test and two of them positive by STA., however these two cases had a titer 1/160. Furthermore these two negative cases showed symptoms but not signs such as splenomegally or lymphadenitis. The negative results of these two patients may be false negative due to an unsatisfactory antigen preparation. A negative result may be confirmed by testing of a follow-up sample. (15)

The present study indicated that the sensitivity of Rose Bengal test is 57.5 %, while Cernyseva et al. found that the sensitivity of the test was 68.6%, (16). Mesa et al and Altwegg et al put the figure at 93.8 and 100%, respectively (17 & 18). The sensitivity of the RB test depends on the antigenic concentration and commercial antigenic preparation used which offer an explanation for the differences of opinion found in the literature regarding the sensitivity of the RB test. Moreover, the low sensitivity of RBT in our result may be also due to selection of the patients or early infection. The RB test is a very simple test that often is used as a first screening step for patients with clinical suspicion of brucellosis.

The second test used in the present work was the STA which had been evaluated and compared with RBT and PCR. We reported that STA had superior sensitivity (62.5 %) compared to RBT test (57.5 %) but it had inferior sensitivity to PCR (70 %). In the present study the titer cut value of STA was 1/160 because we selected patients from high risk group. Several studies indicated that an optimal break point of 1/160 gave an excellent sensitivity (19). However, in other studies when clinical evidence suggested brucellosis, even values <1/160 may not rule out the diagnosis (20). STA recognizes both IgG and IgM antibodies.

Our work showed that STA was positive for 62.5 % and negative for 37.5 % of the patients. Our work is in basic agreement with work of Wallach et al on Argentine patients (21). On the other hand, the result of STA of the present study differed from the findings of a previous report by Clavijo et al who found that the positive cases were 90.2 % (15). The high figure reported by Clavijo et al may be due selection of patients with illness lasting more than 3 months, while higher percentage of negative cases in our study may be due to selection at early onset of the infection (15). Follow up study by RBT and STA are needed to rule out such argument. In contrast, to our results Al-attas et al reported that STA was positive in all cases studied and the sensitivity was 100 % (1). However, the number of patients (14) that have been involved in the work of Al-attas et al study was too small to reach such conclusion. Recent study was done by El faki et al on twenty Saudi patients with clinically diagnosed brucellosis and confirmed by STA and PCR.16(80 %) patients out of 20 were positive with STA with titer 1/ 80 (22) This titer is not sensitive for diagnosis of Brucellosis in endemic area as Saudi Arabia, therefore if patients with titer 1/ 80 were excluded from EL Faky et al study, the number of positive cases will be 12 (60 %) that was similar to our results. STA must not be used alone for confirmation of clinically diagnosed brucellosis. Two serological methods should be used in the diagnosis of human brucellosis to distinguish both immunoglobulin classes and to determine the stage of the infection (23).

The third test used in the present study is nested PCR assay with four primers to amplify targets sequence of 677 bp of 16S rRNA sequence of brucella. The primers used in the present study were described previously by Nimri (10). The PCR results confirmed the clinical diagnosis in 28 (70 %) patients. Twelve cases were negative by PCR and also negative by serological methods, might be due to the absence of infection. The high sensitivity of this assay is superior to Rose Bengal and
STA provide a valuable tool for the diagnosis of brucellosis. Our work is in basic agreement with work of Queipo-Ortuno et al (23) and Al-Nakkas et al (24). In contrast, to our results Vrioni et al (25) reported that PCR was positive in 61.3 % of cases studied. PCR can reduce the time needed for blood cultures and its limitations caused by the low number of bacteria in the blood especially in chronic and focal type of the disease (26). The PCR assay can be used for the diagnosis of acute, chronic relapse, and focal complications in patients with brucellosis (27&28). Other important advantages of the PCR assay that make it superior in diagnosis of infectious disease specially brucellosis a) PCR is rapid assay, providing results in less than 24 h, which is much less than the time required for culture to rescue a fastidious microorganism such as a Brucella sp (29). b) The risk of infection of laboratory personnel is reduced or minimal (30). c) The sample can be stored at −20°C until processing. The main disadvantages of PCR technique are costly and highly equipped laboratory is required for performing the assay.

Conclusion and recommendations:
We conclude that serological tests are important for screening patients with brucellosis. Two serological tests must be used for screening and preliminary diagnosis and rising titer between two serum samples 15 days apart is essential. Nested PCR method is sensitive and can be used for definite diagnosis but is costly. PCR can use for diagnosis of acute, chronic cases. It is useful technique in diagnosis of focal brucellosis and relapse cases and evaluate the treatment regimen. If serology is negative in patients with strong clinical suspicion, nested PCR assay must be performed. Further study is recommended for seroprevalence people among high risk groups. We recommend performing a similar study with large number of sample before and after treatment. Also we recommend applying nested PCR assay on wide range of specimens to include cerebrospinal fluid and joint fluid

REFERENCES
10- Nimiri.L (2003): Diagnosis of recent and relapsed cases of human brucellosis by PCR assays. BMC Infect Dis, 3: 5-10


لا يمكنني قراءة النص العربي بشكل طبيعي. يرجى تقديم نص يمكنني قراءته بشكل طبيعي.