In Term of Molecular Technique, Taxonomic and Diagnostic Aspects of Chronic Human Brucellosis in Ramadi City
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Abstract

Background: The diagnosis of chronic brucellosis is frequently difficult to establish. The disease may clinically mimic any infectious and noninfectious disease. This study has been laid down to evaluate PCR technique in the diagnosis of chronic brucellosis in comparison to conventional techniques.

Patients and methods: One Hundred Forty Four peripheral blood samples obtained from two group: one hundred twenty four samples from patients with highly suspicion brucellosis and twenty samples from healthy volunteers. The samples were tested by serology using Rose Bengal test (RBT), serum agglutination test (SAT) and 2-Mercapto-ethanol. Blood culture using monophasic blood culture technique, Castaneda biphasic blood culture technique and lysis centrifugation blood culture method. Also, the samples submitted to polymerase chain reaction using primer sets (B4 and B5) to amplify a 223-bp region coding for 31-kDa Brucella antigen was achieved. Furthermore all positive PCR samples were submitted to PCR cocktail to differentiate Brucella species.

Results: Out of 124 (86.1%) blood samples from patients with chronic brucellosis, 36 (29.03%) showed strong positive for RBT. On the other hand, 61 (49.2%) cases were positive when SAT titer ≥1/320, 104 (83.9%) cases revealed positive results when the SAT titer ≥1/160 and 118 (95.2%) represented positive cases when SAT titer ≥1/80. Also all blood samples submitted to mono and biphasic blood culture technique were 50 (40.3%) and 64 (51.6%) represented positive results respectively, while only 40 blood samples were submitted to lysis centrifugation blood culture technique, 35 (87.5%) revealed positive results. Also, 103 (83.1%) showed positive results for PCR. Out of these cases, 77 (74.8%) represented positive results (B. melitensis), while 26 (25.2%) showed negative cases. Finally, among the twenty (13.9%) controls, serological test, blood culture and PCR were negative.

Conclusions: The study suggested that combination of Rose Bengal test and serum agglutination test ensured the diagnosis of brucellosis. Also Castaneda biphasic blood culture method had improved the rate of isolation and reduce the period of incubation. Further, lysis centrifugation blood culture technique showed increase in the rate of isolation especially in chronic stage. On the other hand, the current study suggested that PCR has several advantages over the conventional methods for the diagnosis of human brucellosis such as speed, safety, high sensitivity and specificity. Furthermore, PCR is very specific and highly sensitive technique that can be used not only for detection of Brucella antigen in any stage of the disease but also in differentiating Brucella species by using PCR cocktail which used different sets of primers.

Key words: chronic brucellosis, serology, blood culture, PCR, PCR cocktail.

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Introduction

Brucellosis is a zoonotic infection of domesticated and wild animals caused by organisms of the genus *Brucella*. The disease is endemic especially in countries of the Mediterranean basin, the Arabian Gulf, the Indian subcontinent and parts of Mexico and Central and South America. Human brucellosis is found to have significant presence in rural/nomadic communities where people live in close association with animals.

Brucellosis is caused by bacteria from genus *Brucella*. It is Gram-negative bacteria, stained red using modified Ziehl-Neelsen technique and appearing as coccoid or short rod shaped cells from 0.5-0.7x0.6-1.5 microns in size. They are aerobic, non-motile, non-spore forming, non-toxigenic, non-fermenting, facultative, intracellular parasite. *Brucella* spp. are facultative intracellular bacteria that have the ability to avoid the killing mechanism and proliferate within the macrophages, similar to other intracellular pathogens.

The onset of human brucellosis may be acute or insidious. The disease is generalized and may involve any organ or system of the body. Clinically, human brucellosis divided into subclinical illness, acute or subacute disease, localized disease and complications, relapsing infection, and chronic disease. The diagnosis of human brucellosis cannot be made solely on clinical grounds due to the wide variety of clinical manifestations of this disease.

The diagnosis of patient with possible brucellosis is often missed despite modern and suitable technologies especially in chronic stage of the disease which may be misleading where the conventional diagnostic techniques have serious limitations in chronic stage of this disease. Diagnosis of brucellosis requires the combination of several approaches, including medical history, clinical examination, and should always be validated by serological and bacteriological tests and confirmed by molecular methods. The diagnosis of brucellosis traditionally relies on serological test such as Rose Bengal test and serum agglutination test. In general, Rose Bengal test is used as a screening test and the positive results are confirmed by serum agglutination test.

The Definitive diagnosis of human brucellosis requires the isolation of the etiologic agent. The bacterial isolation rates are variable depending on the stage of disease, previous use of antibiotics, the clinical specimen, total volume of the sample and last but not least, the culture methods.

Molecular methodology offers an alternative way of diagnosing brucellosis. The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus, species and even biovars levels has becoming extended to improve diagnostic tests and a diversity of methods have been developed. Molecular biology as a diagnostic tool is advancing and will soon be at the point of replacing actual bacterial isolation.
Patients and Methods

A total of 144 peripheral blood samples were obtained from 124 patients with highly suspicion brucellosis and twenty samples from healthy volunteers over a five months period. Out of 124 patients, 52 were males and 72 were females, aged between 14 and 80 years with mean 36.3 ± 13.57 years.

All the patients were submitted to serological test (RBT and STA), blood culture techniques (monophasic and biphasic Castaneda blood culture technique) and molecular method (PCR and PCR cocktail). Selected patients 40 (32.3%) were submitted to lysis centrifugation blood culture technique.

Serological test

For serology, blood samples were centrifuged (3000xg for 10 min) and serum stored at −20°C. All sera were evaluated using Rose Bengal test (RBT) and serum agglutination test (SAT). The RBT was done according to the method of 15. Regarding to SAT, heat killed Brucella abortus strain 99 was used as a Brucella antigen. the test was performed according to the technique mentioned by Alton and associates, (1988) 16. In order to confirm the stage of the disease, 2-Mercaptoethanol test (2ME) destroys disulfide bonds of IgM antibodies so only IgG remains as agglutinable antibodies (if present). This test was performed according to the methods mentioned by Young, (2006) 17.

Bacteriological test

Three blood culture techniques were used, monophasic, biphasic Castaneda blood culture technique, and lyses centrifugation blood culture method were used under aseptic condition as mentioned by Brooks and associates, (2004) 18. A loop full was taken from monophasic blood culture bottle and the deposited yield of lysis centrifugation tubes and cultured in to ordinary culture media (Brucella agar plates, blood agar plates and MacConkey agar plates) for 24- 48h at 37°C with and without 5- 10% CO2. The steps of lysis centrifugation blood culture technique were performed according to the method described by Eltemadi and co- workers (1984) 19 and modified by Mantur and Mangalgi, (2004) 20.

Regarding to Castaneda blood culture technique, the colony appearance of suspected Brucella was investigated on the solid phase of Castaneda bottle. After that by bacteriological and confirmatory tests were performed according to Baron and associates (1994) 21.

Molecular methods

All peripheral blood samples were examined for DNA extraction by Promega kit (TM050) which were assayed by PCR amplification process. Different PCR assays and different primer sets were used in the present study. The reagents required for PCR in this study were; PCR pre mix and primers which supplied by Bioneer Company, Korea. PCR pre mix was consisted of DNA polymerase (1U), each dNTPs (250 µM, Tris-HCl (10µM), KCl (30 µM), MgCl2 (1.5µM, and stabilizer and tracking dye. Different sets of primers and different PCR programs were used in this study. The first PCR assay used primer sets B4 and B5. B4 5’TGG CTC GGT TGC CAA TAT CAA3’ and B5 5’ CGC GCT TGC CTT TCA GGT CTG 3’. The second PCR assay used primer sets Ba, Bmel and IS711. Ba 5’ GAC
GAA CGG AAT TTT TCC AAT CCC 3', Bmel 5' TCG CGT CCT TGC TGG TCT GA 3' and IS711 5' TGC CGA TCA CTT AAG GGC CTT CAT3'. The third PCR assay used primer sets Eri1 and Eri2. Eri1 5' GCG CCG CGA AGA ACT TAT CAA 3' and Eri2 5' CGC CAT GTT AGC GGC GGT GA 3'.

Different PCR programs were used in this study, the first PCR assay was used for detection of genus Brucella. The following PCR components were added in each PCR tube: B4 (2µl, 5pmole), B5 (22µl, 5pmole), DNA template (52µl), and DDW (112µl). All tubes were placed in the thermal cycler and amplification program started with initial denaturation at 93°C for 4 min, 40 cycles: denaturation step at 90°C at 60 sec, Annealing step at 60°C for 60 sec and Extension step at 72°C for 60 sec. the final extension at 72°C for 7 min. The amplified product was resolved using 2% agarose gel electrophoresis that is stained with redsafe and photographed by photo documentation system.

The second PCR assay depends on strain locus specific multiplexing. PCR cocktail was used for detection of B. melitensis and B. abortus by using primer sets Ba, Bmel and IS711. The following PCR components were added in each PCR tube (20 µl reaction volume): Ba (2 µl, 20 pmole), B mel (2 µl, 20 pmole), IS711 (6 µl, 20 pmole), DNA (4 µl) and DDW (6 µl). All tubes were placed in the thermal cycler and amplification program was initial denaturation at 95°C for 4 min, 35 cycles: denaturation step at 95°C at 1.20 min, Annealing step at 55.5°C for 2 min and Extension step at 72°C for 2 min. the final extension at 72°C for 10 min. The amplified product was resolved using 1.5% agarose gel electrophoresis that is stained with redsafe and photographed by photo documentation system.

The third PCR cocktail assay were used primer sets (Ba, Bmel, IS711, Eri1, and Eri 2) to differentiate vaccine strain (B. abortus S19). Each PCR tube (20µl) contain: Ba and Bmel (2 µl, 20 pmole), IS711 (4 µl, 20pmole), Eri 1 and Eri2 primers (2 µl, 20 pmole), DNA (4 µl) and DDW (4 µl). These tested by using previously mentioned program (PCR cocktail program) and the PCR products was detected by previous method. Regarding to serological test, blood culture and PCR, all the controls represented negative results.

Results

In the serological part of this study, out of 124 peripheral blood samples obtained from patients with highly suspicion of chronic brucellosis submitted to (RBT) and (SAT). our results revealed that 36(29%) revealed strong positive results for RBT, while 60(48.4%) and 20(16.1%) represented moderate and weak positive respectively. Also, 8(6.5%) revealed negative results see table 1.

Table (1). The distribution of strong, moderate and weak positive results of slide agglutination test (Rose Bengal test) strong study isolates.

<table>
<thead>
<tr>
<th>Rose Bengal test result</th>
<th>The results No. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>strong positive</td>
<td>36 (29 %)</td>
</tr>
<tr>
<td>Moderate positive</td>
<td>60 (48.4%)</td>
</tr>
<tr>
<td>weak positive</td>
<td>20 (16.1%)</td>
</tr>
<tr>
<td>negative results</td>
<td>8 (6.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>124 (100%)</td>
</tr>
</tbody>
</table>

On the other hand, three diagnostic thresholds were used in this study for STA. When STA≥1/320, 61 (49%) revealed positive results, when STA ≥1/160, 104(83.87%) represented positive cases and when STA titer
≥1/80118 (95.2%) showed positive results see table 2.

Table (2). The distribution of positive and negative cases of standard tube agglutination test with three diagnostic thresholds among study isolates.

<table>
<thead>
<tr>
<th>Standard tube agglutination test</th>
<th>Positive cases No. (%)</th>
<th>Negative cases No. (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA ≥ 1/320</td>
<td>61 (49.2%)</td>
<td>63 (50.8%)</td>
<td>0.857</td>
</tr>
<tr>
<td>STA ≥ 1/160</td>
<td>104 (83.87%)</td>
<td>20 (16.13%)</td>
<td>0.000</td>
</tr>
<tr>
<td>STA ≥ 1/80</td>
<td>118 (95.2%)</td>
<td>6 (4.8%)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The study results of 2ME showed that positive cases observed in 89 (71.8%) and negative cases in 35 (28.2%).

Regarding to blood culture, three techniques were used in this study, monophasic blood culture method, biphasic Castaneda blood culture technique and lysis centrifugation blood culture method. Our results showed that out of 124 patients with suspected brucellosis, brucellae were isolated in 50 (40.3%) and 64 (51.6%) for monophasic and biphasic blood culture techniques respectively. On the other hand, only 40 patients submitted to lysis centrifugation blood culture technique where positive results observed in 35 (87.5%) cases as see in table 3.

Table (3). Distribution of culture positive and negative cases among all study patients for both of monophasic and biphasic blood culture techniques and among 40 study patients who submitted to lysis centrifugation blood culture technique.

<table>
<thead>
<tr>
<th>Type of blood culture technique</th>
<th>Positive cases No. (%)</th>
<th>Negative cases No. (%)</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Monophasic blood culture technique</td>
<td>50 (40.3%)</td>
<td>74 (59.7%)</td>
<td>124</td>
</tr>
<tr>
<td>2 - Biphasic blood culture technique</td>
<td>64 (51.6%)</td>
<td>60 (48.4%)</td>
<td>124</td>
</tr>
<tr>
<td>3 - lysis centrifugation blood culture technique</td>
<td>35 (87.5%)</td>
<td>5 (12.5%)</td>
<td>40</td>
</tr>
</tbody>
</table>

In the molecular part of this study, PCR was positive in 103 (83%) and only 21 (17%) represented negative cases when PCR assay was performed by primer set (B4 and B5) to indicate the presence or absence of Brucella organism as in the following figure.
Brucellosis represents a prevalent disease in humans and animals. The clinical features and presentation of human brucellosis overlap with different infectious and autoimmune or neoplastic processes. Since the symptoms of brucellosis are non-specific, the clinical diagnosis of the disease is difficult.

The most popular serological tests are Rose Bengal test (RBT) and serum agglutination test (STA). Regarding to RBT, the study patients grouped in to three group, strong, moderate and weak positive. The sensitivity of this test is often low. RBT test is valuable, easy to use and cost-effective screening test. On the other hand, false negative and false positive represent serious limitation for this test.

SAT test, which is cost-effective and easy to use is the most appropriate test especially in regions endemic for brucellosis. In this study three diagnostic thresholds were used, the sensitivity and specificity of STA depends on the cut-off value used, the background level of reactive antibodies in the population and endemicity. The titer of SAT of $\leq 1/160$ is problematic in areas of endemicity, since low SAT titers may be present in healthy people who previously suffered the disease, during the first stage of the infection, and in patients suffering chronic brucellosis or a relapse. The SAT titer of $<1/160$ cannot always be disregarded without follow up. Conversely SAT titer of $\geq 1/160$ do not always signify active infection, especially in Brucella endemic areas. SAT is frequently used as a reference to which other serological tests are compared. Nevertheless, cross-reaction with various bacteria have been reported results in false positive results.

Discussion

In the current study new primer set was used, ErI1 and ErI2 which used to detect and differentiate B. abortus S19 vaccine strain. All positive PCR cases submitted to PCR cocktail (2) by using primer sets (Bmel, Ba, IS711, ErI1, and ErI2). Out of 103 positive PCR cases, 77 (74.8%) represented positive results for B. melitensis while 26 (25.2%) showed negative results. These results revealed absence of B. abortus S19 vaccine strain as in following figure.

Figure (2). PCR amplification of B. melitensis DNA and B. abortus DNA by using primer set (Ba, Bmel, and IS711). Lane 1 and 20 represented DNA ladder, Lane 2,3,4,5,6,7,8,9,11,12,16,17,18 and 19: B. melitensis, Lane 10,13,14 and 15: neither B. melitensis nor B. abortus.

Figure (3). Polymerase chain reaction assay results by using primer sets (Bmel, Ba, IS711, ErI1 and ErI2), Lane 1 and 11 represented DNA ladder. Lane 2, 3, 4,5,6,7 and 8 revealed positive result for B. melitensis. Lane 9 and 10 revealed negative results.
other hand, the false negative SAT results may occur in patients with a recent infection or whose serum contains blocking antibodies (Prozone) a commonly reported phenomenon in brucellosis31.

The 2-mercaptoethanol test is taken as evidence for the presence of specific IgG antibodies at which2-ME destroys disulfide bonds of IgM antibodies so only IgG remains as agglutinable antibodies (if present)11,17. This test helps to confirm the stage of the disease and response to the antibiotic treatment. Comparing to SAT and 2ME test results, it was possible to prove which antibodies were present in the sera (IgM, IgG or both). Patients with positive 2ME and SAT positive had only IgG in their sera, while patients with negative 2ME and positive SAT had IgM antibodies (acute stage)33.

Frania, (1985)34 concluded that serological tests proved to be either too sensitive giving false positive results, or too specific giving false negative results, beside misdiagnosis due to cross reactivity of other gram negative bacteria such as Yersinia enterocolitica with smooth Brucella species 23.

The blood culture is method of choice, but specimens should be taken in the early stage of the disease. the study results indicated that biphasic Castaneda blood culture techniques more useful than monophasic blood culture method where the high number of positive cases showed by biphasic blood culture method. Also, forty patients submitted to lysis centrifugation blood culture technique, out of them, 35 (87.5%) revealed positive results. This technique is very useful especially in chronic stage of the disease. On the other hand, The incubation of the blood culture takes a long time, between five to 35 days.

The isolation of the bacterium is proceeding of the biological hazard for the laboratory staff. Brucellosis is one of the most common laboratory acquired infections 25.

As other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis, since detection of specific DNA is a true indication of the presence of a pathogen35. PCR, characterized by high sensitivity and specificity, short turnaround time and less hazardous, this method can overcome the limitations of conventional methodology36. PCR could be a more sensitive technique than blood cultures and it is more specific than conventional serological test. This technique has to be very sensitive and specific to detect small amounts of microbial DNA in eukaryotic gene material 23.

Numerous PCR based assays and different sets of primers have been developed and evaluated for the identification of Brucella species to improve the diagnostic capabilities ranging from general identification of the genus Brucella (Genus-Specific PCR assay), that is designed to explicate a single unique genetic locus that was highly conserved in Brucella (e.g. BCSP31) to specific identification of the Brucella species (differential PCR-based assay), that is depends on strain locus specific multiplexing (e.g. PCR-based on IS711)37.

The first PCR assay was used for detection of genus Brucella, the second PCR assay was used for detection of Brucella abortus and Brucella melitensis, and the third PCR assay was used for differentiation Brucella abortus S19 from other Brucella species. Each of these PCR systems produces a discrete DNA
product, whose length is identical for and specific to all *Brucella* species\(^\text{11}\).

Regarding the first PCR assay which was used primer sets (B4 and B5), to indicate the presence of *Brucella* organism. This primer sets used for amplification was specific for the genus encoding a 31 KDa *Brucella* outer membrane protein (BCSP31) with molecular weight 233 bp. It is conserved in all species and biovars of *Brucella*\(^\text{33}\). In this study, 103(83.06%) revealed positive results, this result reflect the superiority of PCR compared with traditional techniques. Also, Queipo- Ortuno and co-workers (1997)\(^\text{38}\) founded that 100% sensitivity and 98.3% specificity using B4 and B5 primer pair.

PCR could be particularly useful in patients with chronic brucellosis and specific complications such as neurobrucellosis, or other localized infections, since cultural serological testing often fail in such patients\(^\text{11, 39}\).

For species-specific surveillance programs, differential PCR programs and different primer sets are needed\(^\text{32}\). In this study all positive PCR cases using B4 and B5 primers were submitted to PCR cocktail for identification of *Brucella* species. In the second PCR assay, the primer sets (Bmel, Ba and IS711) were used for differentiation *B. melitensis* from *B. abortus* at which Bmel primer indicated presence of *B. melitensis* while Ba primer indicated presence of *B. abortus*. PCR cocktail was based on the existence of specific DNA sequence for each species and IS711 copies which found in unique chromosomal location for each of *Brucella* species\(^\text{33}\).

In this study, out of 103(83%) positive PCR cases submitted to PCR cocktail, 77(74.8%) revealed positive results for *B. melitensis*, while 26(25.2%) represented negative cases. The negative cases could be explained by many factors such as presence of PCR inhibitors, number of *Brucella* organism below the detection threshold, degradation of target DNA in the samples and inefficient DNA extraction\(^\text{40}\).

It is well known that primer cocktail used to identify more number of *Brucella* biovars and also to discriminate between *B. abortus* vaccine strains and other isolates of *Brucella* \(^\text{41}\). So, the third PCR assay was carried out using primer sets (Bmel, Ba, IS711, Eri1, and Eri2). Eri1 and Eri2 were used to differentiate *B. abortus* S19 vaccine strain\(^\text{37}\).

Regarding to our study there was no *B. abortus* S19 (vaccine strain) detected in patients who submitted to PCR cocktail. These results indicating that all positive samples identified as *Brucella* species were *B. melitensis*. Similar results were previously reported by (Gupta *et al.*, 2006; Ica, *et al.*, 2012)\(^\text{40, 42}\). Regarding to the relationship between STA, blood culture and PCR, the study results reflected the superiority of PCR technique. These results prove the role of PCR as good tool for diagnosis of brucellosis when other tests fail in the diagnosis of brucellosis.

**Conclusions**

The study suggested that combination of Rose Bengal test and serum agglutination test ensured the diagnosis of brucellosis. Also Castaneda biphasic blood culture method had improved the rate of isolation and reduce the period of incubation. On the other hand, lysis centrifugation blood culture technique showed increase in the rate of isolation especially in chronic stage. Further, the
current study suggested that PCR has several advantages over the conventional methods for the diagnosis of human brucellosis such as speed, safety, high sensitivity and specificity. Further, PCR is very specific and highly sensitive technique that can be used not only for detection of *Brucella* antigen in any stage of the disease but also in differentiating *Brucella* species by using PCR cocktail which used different sets of primers.

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