RAPID DIAGNOSIS OF HUMAN BRUCELLOSIS FROM BLOOD SAMPLES USING POLYMERASE CHAIN REACTION TECHNIQUE

ABSTRACT:
Brucellosis is a zoonotic disease and its traditional diagnosis is based on blood culture and serological methods, for more sensitive and specific detection, PCR method is recommended. In the present study we targeted the Brucella genus-specific 16S rRNA for detection of Brucellosis using peripheral blood by nested-PCR technique. Fifty-five blood samples were gathered from suspected outpatients from Sulaimani province. Diagnosis was established depending on ELISA and Rose Bengal test and later was examined by PCR method. Brucella spp were detected in Fifty-two patients (1100 bp), while when we targeted the sequences found only in B. melitensis, fifty suspected patients showed a positive PCR product of 958 bp. Fifteen patients were male (27%) and 40 were female (73%), and their ages ranged from 5-65 yr old (mean, 36 yr). The highest number of Brucellosis (Considering ELISA and PCR) cases was found in the >30-40 years age group (20, 36%), while the lowest number was found in <10 years age group and >60 age group (2, 4%) for each. PCR technique is an easy and rapid assay for diagnosis of Brucellosis in suspected cases.

KEY WORDS:
B. melitensis, PCR, 16S rRNA

INTRODUCTION:
Brucellosis continues to be an important zoonosis of economic significance and human suffering. It affects human populations in many developing countries including the Middle East, and Latin America where it is still endemic (Alton, 1991).

According to the World Health Organization, half million of new human cases are reported worldwide each year, they report an annual incidence of human brucellosis of less than one to 78 cases per 100,000 population in the Middle East, with six countries reporting an annual total incidence of more than 90,000 cases (WHO, 1997). These numbers greatly underestimate the true incidence of human disease because the clinical picture of human brucellosis is extremely variable, and it is under declared to local authorities and misdiagnosed by clinicians (Zerva et al., 2001).

Brucellosis is usually transmitted to humans by ingestion of unpasteurized dairy products or by direct contact with infected animals. Occupational disease is contracted by exposure of abattoir workers and veterinarians to infected animals especially aborted fetuses, fluids, membranes or urine (Laila, 2003).

The culture techniques are time-consuming and lack sensitivity for patients with chronic infections, and handling of the organism in the laboratory is hazardous. Conventional serologic techniques are insensitive for patients with chronic infections, although newer techniques may be an improvement. Cross-reactions with other gram-negative bacteria occur, giving false-positive results. In areas endemic, where brucellosis is a significant proportion of the well population may be seropositive (Matar et al., 1996; Ruiz-Mesa et al., 2005). Over the past decade, there have been major advancements in all aspects of molecular diagnosis with regard to human brucellosis. PCR- based tests are proving to be faster and more sensitive than traditional methods (Romero et al., 1995, Navarro et al., 2002 & 2004; Al Dahouk et al., 2003; Gee et al., 2004). The aim of this study was to detect Brucella spp. from peripheral blood using Brucella genus-specific 16S rRNA Nested-PCR technique.
MATERIAL AND METHODS:
Sample collection
Venous blood specimens were collected in 2.5 ml EDTA containing tube from fifty-five suspected brucellosis outpatients over a period of 4 months (February to June 2009) in medical consultant clinic of Shahed Hady and in Sarezh Laboratory inside Sulaimani city, Iraqi Kurdistan Region. Diagnosis was established depending on clinical signs and symptoms as well as ELISA and serological laboratory testing (Rose Bengal test).

DNA extraction:
Bacterial DNA was extracted directly from the blood using QIAamp DNA Mini Kit (Qiagen, Germany), and then the DNA was used as template for PCR.

Primer selection:
The DNA sequences of the primers, the size of PCR and the targeted genes are shown in Table 1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Amplicon Size (bp)</th>
<th>Targeted gene</th>
<th>Primer Synthesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruc1F</td>
<td>ATAGCTGGCTGAGAGGATGATCAG</td>
<td>1100 bp</td>
<td>Brucella spp. 16SrRNA gene</td>
<td>MGW Oligosynthesis (Austria)</td>
<td>Kazemi et al. (2008)</td>
</tr>
<tr>
<td>Bruc1R</td>
<td>TTCGGGTAAACCAATCCCATGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bruc2F</td>
<td>ATATTGGAACATGGGCCGCAA</td>
<td>958 bp</td>
<td>Brucella melitensis biovar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bruc2R</td>
<td>AGCGATTCCAACCTTCATGCA</td>
<td></td>
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</tbody>
</table>

PCR conditions:
Each PCR assay was performed in 0.5 ml eppendorfs, each containing a total volume of 50 µL including: 25 µL PCR master mix, 1 µL (10 pmol/µL) for each primer and 1 µL of the extracted DNA finally 22 µL of H2O (DNase, RNase free) were added. The product of the first reaction was used as a template for the second run with the second set of primer using same quantities.

The amplification was performed in a Thermal Cycler (Genius, Technne, UK). After an initial denaturation cycle of 2 min at 95°C, the reaction mixes were subjected to 35 amplification cycles of 30 sec at 95°C and 30 sec at 60°C and 60 sec at 72°C, and final extension of 5 min at 72°C. Same conditions were used for the second set of the primer (Nested-PCR), except the annealing temperature (50°C instead of 60°C).

DNA from amplified PCR reaction was analyzed after electrophoresis on 1% agarose gel at 90 volts for 1.15 h and stained with ethidium bromide, a molecular marker (100bp DNA ladder, Fermentas) was used to determine the size of the amplicons (Kazemi, et al., 2008).

RESULTS:
Distribution of Brucellosis according to gender of the patients:
In this research, 55 blood samples were examined in suspected cases of brucellosis. Fifty-two cases were positive by PCR method, and 54 cases by ELISA method. Fifteen patients were male (27%) and 40 patients were female (72%), and their ages ranged from 5-65 yr old (mean, 36 yr). Statistically, significant differences were found between male and female patients at p<0.05 (Table 2).

<table>
<thead>
<tr>
<th>Age range in year</th>
<th>Mean</th>
<th>Age range in year</th>
<th>Mean</th>
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<tbody>
<tr>
<td>5-61</td>
<td>30</td>
<td>5-65</td>
<td>38</td>
</tr>
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</table>

Total 55
Age range (5-65 year) Mean (36 year)

* Significant differences between male and female patients (p< 0.05)

Incidence of Brucellosis according to the age groups:
As shown in figure 1, the highest number of Brucellosis (Considering ELISA and PCR results for diagnosis) cases was found in the >30-40 years age group (20, 36%), followed by >20-30 years age group (12, 22%) while the lowest number was found in <10 years age group and >60 age group (2, 4%) for each. There were statistically significant differences between age groups and infection (p<0.05).

Fig. 1. Incidence of Brucellosis according to the age groups.
PCR results:

Fifty-two samples (94%) were positive for the detection of *Brucella spp.* by PCR test using Nest 1 primers (Bruc1F/Bruc1R), in which 1100 bp band were detected after electrophoresis (Fig. 2).

![Image of PCR results](image1.png)

By using Nest 2 primers (Bruc2F/Bruc2R), which targeted the sequences found only in *B. melitensis*, 50/55 (91%) suspected patients showed a positive PCR product of 958 bp. (Figs 3 & 4).

![Image of PCR results](image2.png)

Fig. 2. *Brucella spp.* detected by PCR using Nest 1 primers (Bruc1F/Bruc1R) in which 1100bp band were detected after electrophoresis. M: DNA markers

Fig. 3. *B. melitensis* detected by PCR using Nest 2 primers (Bruc2F/Bruc2R) in which 958 bp band were detected after electrophoresis.
DISCUSSION:

Fifty-five suspicious blood samples were used for diagnosis of Brucella by PCR methods. Fifty-two samples were positive by PCR method and 54 samples by ELISA method. Similar result reported in IRAN by Kazemi et al. (2008), they found seropositive cases (Wright method) were higher than those cases which diagnosed by PCR, this may be due to that the disease is epidemic in our country as well as IRAN and the seropositive cases may indicate past infection or vaccination.

Considering brucellosis in different ages indicated that most infections were in ages >30 to 40. This was in agreement with other studies reported by Kazemi et al. (2008) and Al Sekait (1999), they reported the seropositivity of brucellosis to be higher in the age groups of 30-44 years and >45 years. These age groups include active age groups that are settled in different manners in animal husbandry, dairying, working at home and have connection with livestock and products of livestock.

In current study, the prevalence of brucellosis was significantly higher in females (72%) than in males (27%), probably due to the increased involvement of women in handling animal products and farming domestic animals. Cetinkaya et al. (2005) also indicated that there is relationship between age, sex and positivity \( p<0.05 \).

Consumption of unpasturized milk and their products and direct contact with animals were documented in the present work. Consumption of fresh cheese and milk cream produced from unpasteurized milk has been reported to be a significant risk factor for brucellosis (Al Sekait, 1999; Abu Shaqra 2000; Bikas et al., 2003; Sumer et al., 2003.).

As the clinical picture of human brucellosis is nonspecific, it is necessary to resort to isolation of the germ, by demonstrating high levels of specific antibodies or seroconversion, in order to make a definite diagnosis. However, all these methods have serious limitations (Kiel et al., 2002). In the present study, we investigated the potential use of a PCR assay as a rapid test for the diagnosis of human brucellosis.

The high sensitivity of the technique is probably related to its ability to detect 10fg of bacterial DNA, which equates to approximately two bacteria, a number of microorganisms possibly present in any 1-ml sample of peripheral blood from patients with clinical brucellosis (Queipo-Ortuño et al., 1997).

The method proposed herein can be used with a simple sample of 0.5 to 1 ml of peripheral blood without the need to separate the cells. It enables an easy extraction of the DNA with a high degree of purity. It is not necessary to employ organic solvents like phenol, which described in previous works as able to inhibit Taq polymerase, and it avoids the risks to laboratory personnel associated with handling the microorganism (Queipo-Ortuño et al., 1997). Therefore, we believe that assay could become the method of choice for the diagnosis and follow-up of patients with brucellosis.

One of target genes for determining bacterial identification is 16S rRNA that was used in the present study. This gene has high endurance. DNA sequences in separate types of one genus, they have just small difference with each other (Kazemi et al., 2008). In first set of primer, specific sequences was targeted which exist in all Brucella spp. Determination of species not required for beginning the treatment and dose not effect the type of treatment, although, the severity of the disease effected by the different species, on the other hand, identification of the species level may needed for epidemiological researches and other studies.

For these reasons, the second primer sets were used in order to amplify the specific sequences in all Br. melitensis biovars, and very small differences occur between these biovars, in which we can determine them by DNA sequence analysis of the targeted gene.

Other aspects make the diagnosis of brucellosis by PCR is preferred include:

- Brucella spp. are slow-growing pathogens, culture requires prolonged incubation, which may lead to excessive delays in diagnosis.

-Serological diagnosis lacks adequate specificity in areas where the disease is endemic and its results are difficult to interpret in some slowly evolving focal forms (Ariza et al., 1992).

- The high sensitivity of the PCR assay, even in those patients with prior antibiotic
therapy, seems to relate to the high detection capacity of the technique.

- Moreover, PCR is able to amplify intramacrophagic pathogens, as well as pathogens which are damaged or nonviable as a result of previous treatment and which would be impossible to isolate in conventional cultures.

REFERENCES:


Laila F Nimri. 2003. Diagnosis of recent and relapsed cases of human brucellosis by PCR assay; BMC Infectious Diseases.

- The sample can be stored at -20°C until processing, thereby enabling it to be collected by any physician and processed immediately or else stored and safely sent to another laboratory if required (Lopez-Goni and Moriyon, 2004).

Finally, we conclude that PCR technique was an easy and rapid assay for diagnosis of Brucellosis in suspected cases.
التشخيص السريع لمرض البروسيليا (حمى مالطية) من عينات الدم بواسطة تقنية PCR

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يعتبر مرض البروسيليا من الأمراض المشتركة بين الإنسان والحيوان، والذي يتشخيص عادة بالطرق التقليدية التي تشمل الطرق المصلية ومزاعم الدم. أما تقنية PCR فتُعتبر من الطرق المهمة وذلك لحساسيتها ودقة في تشخيص المرض. الهدف من هذه الدراسة هو تشخيص بكتيريا البروسيليا (Brucella spp) باستخدام تقنية PCR ومن خلال استهداف جين 16S rRNA من عينات الدم مباشرة. تم جمع 55 عينة دم للمريض المشكوّك بإصابتهم بالمرض في محافظة السليمانية/إقليم كوردستان العراق. وقد تم التشخيص باستخدام فحص PCR. وقد تم تأكيد الإصابة باستخدام تقنية BENGAL وELISA.

المحكمون:
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أ.د. رضا حلمي سمير، قسم النبات، علوم طب النبات.

 הפרاكسيس:
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