

## Molecular Diagnosis of *Brucella* species in Baghdad

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### Abstract:

Brucellosis is possess a significant public health problem in Baghdad. In this study, we investigated the potential role of the PCR assay in detection of *Brucella* species, from patients suspect to have brucellosis, using blood samples in both human and animal.

To establish a PCR technique for diagnosis of active brucellosis in our samples, DNA extraction was carried out using a commercial kit, and a laboratory extraction procedure. PCR amplification was done using 1 set of primers: B4/B5 for *Brucella* species. Extraction of *Brucella* DNA using the commercial kit was successful. The laboratory extraction was successful and more economic.

A total of 178 peripheral blood specimens were collected from patients with high suspected brucellosis, and 15 samples from animals. When PCR technique was applied to blood samples, 13 cases for patients blood and 9 cases for animals blood, were positive for *Brucella* species.

**Key Words: Brucellosis, PCR, Baghdad.**

### Introduction:

Brucellosis possess a significant public health problem in Baghdad and many developing countries which requires fast and accurate diagnosis. Brucellosis is a chronic infectious disease caused by bacteria of the genus *Brucella* that affects animals and humans. Each species of *Brucella* has its preferred host: *B. abortus* infects cattle, *B. melitensis* infects sheep and goats, *B. suis* infects swine, *B. canis* infects dogs, and *B. ovis* infects sheep, although they can also infect other animals [1]. Brucellosis in sheep and goats is endemic in the Mediterranean region but is spread throughout Asia, Africa, and Central and South America [2, 3]. Along with tuberculosis and rabies, brucellosis is the most important bacterial zoonosis and remains an important public health and economic concern All *Brucella* species can cause infection in human exception

of *B. ovis* and *B. neotomae*. New *Brucella* species pathogenic for humans – *B. cetaceans* and *B. pinnipedialis* – have recently been discovered in marine mammals [4]. Which is transmitted to humans either by direct contact with the infected animals or by consuming infected milk or fresh cheese [1].

Another major infection route is through occupational exposure to infected live stock, ie, inhalation of contaminated secretions of infected animals or contamination through skin cuts or abrasions [5, 6]. Clinically, brucellosis can be classified into subclinical, acute, subacute, and chronic relapsing forms. Clinical polymorphism is very common and for this reason brucellosis is often unrecognized in primary health care settings [7]. Exact diagnosis is based on the clinical picture, epidemiological

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data, and different laboratory tests, such as bacterial culture, agglutination, and polymerase chain reaction [8, 9].

PCR assay has been used in diagnosis both animal [10, 11] and human [10, 12, 13]. PCR technique provides rapid diagnosis of brucellosis, which is necessary for starting a specific patient treatment. In this study, we investigated the potential role of the PCR technique in the diagnosis of human and animal brucellosis using whole blood.

### Materials and Methods:

A total of 178 peripheral blood specimens were collected from patients with high suspected of brucellosis, referred to Al-Yarmook, Al-Karama, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him) and Al-Kadhmiya Hospitals in Baghdad, and 15 peripheral blood and milk specimens were collected from animals, referred to Al-Fudhailiyah and Al-Husainia regions in Baghdad, during the period from November 2009 till November 2010. Samples were taken from patient suspected to be with brucellosis depending on clinical picture [14], a positive Rose Bengal test titer of  $\geq 1:160$ , and/or a positive blood culture, moreover demographic, occupational, clinical, and risk factor details were recorded for each patient, and serological diagnosis was carried by positive Rose Bengal test and ELISA test.

### Culture and biochemical testst:

Five milliliters of blood were taken from each patient and animals and divided into identical parts. One part was collected in EDTA and the serum was separated from the second part, was aliquot and store at  $-20^{\circ}\text{C}$  until processing. The first part of the blood with anticoagulant was inoculated into: Blood Agar Base, *Brucella* agar, trypticase soya agar and trypticase soya broth culture medium containing

both a solid and a liquid phase [15]. Then it was subculture on duplicate agar plates and incubated one in air and the other in an atmosphere at  $37^{\circ}\text{C}$  in the presence of 5-10%  $\text{CO}_2$ . After 7-30 days, colonies grown in the solid phase, were identified by inoculation into *Brucella* agar, blood agar and trypticase soya agar.

### DNA extraction from blood samples:

Genomic DNA was extracted from blood and bacterial culture of *Brucella* spp. using a Wizard Genomic DNA Purification Kit / Promega – company (USA). This kit is designed to isolate genomic DNA from white blood cells.

### PCR for *Brucella* species:

Two PCR methods were used. Samples were tested in duplicate in most cases. The primer B4 (5'-TCGGTTGCCAATATCAA-3') and B5 (5'-CGCGCTTGCCTTTCAGGTCTG-3') [16], were used to amplify a target sequence of (223bp) in a gene encoding a 31-KDa in *B.abortus*. This sequence has been shown to be common to all *Brucella* biovar. The PCR was performed in 50  $\mu\text{l}$  reaction mixture containing: 31.75  $\mu\text{l}$   $\sim$  32  $\mu\text{l}$   $\text{H}_2\text{O}$ , 10  $\mu\text{l}$  10x PCR buffer, 1  $\mu\text{l}$  (dNTPs) mix (200 mM) deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, dTTP), 3  $\mu\text{l}$   $\text{MgCl}_2$ , 2  $\mu\text{l}$  for each oligonucleotides B4 and B5 (100 nM each), 0.25  $\mu\text{l}$  of Taq polymerase, 2  $\mu\text{l}$  of samples DNA.

The PCR was started as in the following steps:-

1-Denaturation 1 at  $93^{\circ}\text{C}$  for 5 min.

2- 35cycles of:-

A-Denaturation 2 at  $90^{\circ}\text{C}$  for 2 min.

B-Annealing at  $60^{\circ}\text{C}$  for 2 min.

C-Extension 1 at  $72^{\circ}\text{C}$  for 2 min.

3- Extension 2 or final extension at  $72^{\circ}\text{C}$  for 4 min.

The last step is (extension 2 or final extension) according to [17].

The PCR products plus 100 bp DNA Ladder were subjected to electrophoresis on 2% agarose gel stained with Ethidium bromide. Electrophoresis was performed at 70 V for 60 min. The gel was visualized on UV transilluminator at 320 nm and was photographed by polaroid system.

### Results:

A total of 178 peripheral blood specimens have been collected from suspected brucellosis patients. The diagnosis of brucellosis was established by clinical findings confirmed by Rose Bengal test, ELISA, blood culture and molecular methods by PCR technique. They were divided into two groups, acute cases with duration of symptoms less than 6 months and

chronic cases with duration of symptoms more than 1 year. Thus, among these patients, 10 (5.61%) were chronic cases and 22 (12.35%) were acute cases of total 178 cases. In addition 15 samples were obtained from animals. The diagnosis of brucellosis was based on abortion fetus or infected new born and confirmed by serological test (Milk ring test), blood culture and molecular methods by PCR technique.

All patients gave a history for their disease. SO 150(84.26%) were lived in epidemic area and consuming unpasteurized milk and milk products;

and 28(15.73%) were farmers or butchers or they are lived near animals. A total of 178 (89%) blood samples were cultured using plates of BAM and TSAM for isolation of *Brucella* species, showed no results for culture but a total of 15 blood samples were cultured using plates of BAM and TSAM for isolation of *Brucella* species, only 3 were positive. The round, glistening, small, convex colonies on plates of BAM were suspected to be of *Brucella*. The isolates were streaked on blood agar (BA) plates. The non-haemolytic and have a small pale shape isolates on BA. In present study DNA extracted from 178 human blood samples, 178 (100%) samples were positive results and 15 animals, 15 (100%) blood samples and 3 (20%) blood culture samples were positive results by the Wizard Genomic DNA Purification Kit (Promega – company –USA) was used and subjected to PCR using different primer pairs.

When PCR technique was applied to blood samples, 13cases (7.30%) of total 178 patients and 9 cases (60%) of total 15 blood animals, were positive and gave (223bp) for *Brucella* specific-species bands (figure-1 and 2) Figure-1: the diagnostic results of PCR technique for 3 cases for patients for B4 and B5 (223bp). Figure-2: the diagnostic results of PCR technique for 9 cases for animals for B4 and B5 (223bp).



Figure 1: Agarose Gel Electrophoresis for Human of *Brucella* 31-KDa Gene 223 bp Specific PCR Products Amplified with Primer B4/B5.

Lane M, molecular weight DNA ladder (100bp), lane 1: negative control, lane 2: B4/B5 positive control (223bp), lanes 4, 5, 6: positive samples, lane 3, 7: negative samples.

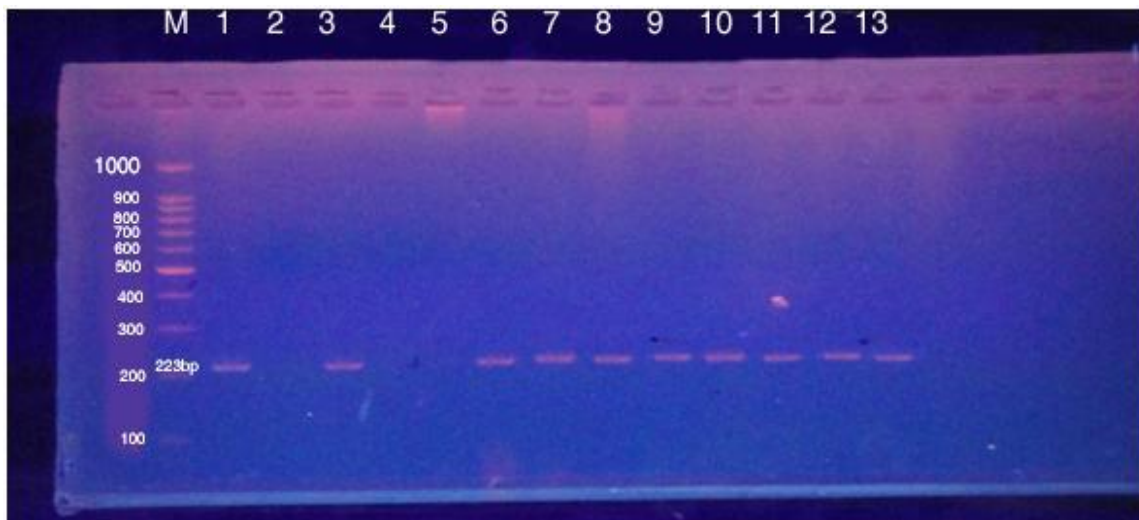


Figure 2: Agarose Gel electrophoresis for Animal of *Brucella* 31-KDa Gene 223 bp Specific PCR Products Amplified with Primer B4/B5.

Lane M, molecular weight DNA ladder (100bp), lane 1: B4/B5 positive control, lane 2: negative control, lane 3, 6→13: positive samples, line 4, 5: negative samples.

**Discussion:**

Brucellosis has a worldwide distribution and remains a major problem in human and animals. Brucellosis is also a health problem for humans and animals and causes economic loss due to the loss of animals. The study revealed that brucellosis increases in rural area about 115 (64.60%) more than urban area about 63 (35.39%) according to ELISA for both

IgM 10 (5.61%) and IgG 22 (12.35%). Therefore, people in rural area were lived in epidemic area, contact with animals like: owner of the herds, their family members because were used unpasteurized milk, cheese and milk product from the infected cows or by the long exposure time of the people to the infected animals inside their house; and pupils in urban area were farmers, butchers or they lived near animals, or pupils which drink milk without boiling or eat unboiled cheese were exposed to this disease. The emphasizes that the farmers and butchers working with livestock were infected with *Brucella*. This helps us to realize that boiling milk and cheese before consumption which is a very simple and uncostly step can reduce disease infection.

Human blood samples showed no results for culture out of 178 samples, because all patients use different long-term antibiotic treatments for various diagnostic suspicions in other clinical sectors, so that affected culture method. Also [18] (2004) concluded that there are clinical and serological finding to support the presence of brucellosis in patients with negative blood culture and positive PCR. While culture as a reference, 3 (20%) samples were positive by culture and 12 (80) samples were negative out of 15 animal's blood samples which their causes from aborting fetus or infected new born. The explanation for the low

yield of conventional culture in present study appears to be related more to the low number of pathogen in the blood sample and use of the different antibiotic treatments for various diagnostic suspicion in other clinical sectors. In spite of difficulty of isolation *Brucella* species from animals blood samples. Hence makes PCR more sensitive than culture while the specificity was 100% and identical to culture so; suggest that PCR could replace blood culture as the gold standard for the diagnosis of animals blood samples. [19] (2004) who analyzed samples obtained from 67 aborted bovine fetuses by means of bacteriological methods and PCR and also found that the samples that were positive by PCR (34/67) more than that of culture (26/67), so he concluded that PCR was more sensitive than culture. Using blood culture as a gold standard, the PCR technique gave a100% sensitivity, which is in agreement with other authors [12, 20]. Although most investigators prefer using commercial kits for extraction of *Brucella* DNA [13, 21, 22]. We successful to extract DNA by a commercial kit. We used a laboratory extraction procedure according to Wizard Genomic DNA Purification Kit / Promega – company –USA.

Our results showed that the sensitivity of the PCR assay using blood samples for patients and using blood samples for animals was far superior 13 (7.30%) for patients and 9 (60%) for blood animals, were positive and gave (223bp) for *Brucella* species This very good sensitivity, confirm that the PCR assay could be a useful tool for the diagnosis of human brucellosis as other investigators showed by using whole blood [12, 23] or serum samples [20]. Finally, in addition to the high yield of the PCR assay for the diagnosis of human brucellosis according to present study, and focal complications in such

patients as previously reported [23], other important aspects are: 1) PCR is fast, providing results in 24 hours, which is much less than the time required for conventional methods to rescue a fastidious microorganism such as *Brucella* spp., 2) the technique almost completely obviates the necessity for direct handling of the pathogen, thus drastically reducing the risk of infection of laboratory personnel, and 3) the samples can be stored at -20°C until processing, thus enabling it to be collected by any physician and processed immediately, or else stored and safely sent to another laboratory if necessary.

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## التشخيص الجزيئي لجنس البروسيلا في بغداد

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### الخلاصة:

يشكل داء البروسيلا مشكلة صحية عامة في بغداد. في هذه الدراسة، نحن تقصينا عن الدور الكامن للـ PCR في الكشف عن جنس البروسيلا، من المرضى المشتبه بهم داء البروسيلا، باستخدام عينات الدم في الإنسان والحيوان. بنى العمل على تقنية الـ PCR لتشخيص داء البروسيلا في كل العينات، إستخلاص الدنا باستخدام الكت التجاري. تضخيم الـ PCR باستخدام نوع من البادئات: B4/B5 لجنس البروسيلا. من مجموع 178 عينة دم جمعت من المرضى المحتمل إصابتهم بداء البروسيلا، و 15 عينة من الحيوانات. أظهرت النتائج باستخدام تقنية الـ PCR، إن النتائج الأيجابية للعينات كانت 13 من دم المرضى من مجموع 178 و 9 من الحيوانات من مجموع 15 كانت مصابة بداء البروسيلا.