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RESEARCH ARTICLE

Isolation of *Brucella melitensis* and *Brucella abortus* from brucellosis patients and animals by culture method and molecular method (PCR).

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Abstract

Objective: Isolation of *Brucella melitensis* and *Brucella abortus*, from patients suspect to have brucellosis and animals by conventional culture and comparison with PCR technique in diagnosis of human and animal brucellosis.
Duration and place of study: Samples were obtained from suspected brucellosis patients, referred to many hospitals in Baghdad city, which include: Al-Yarmook, Al-Karama, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him) and Al-Kadhmiya Hospitals, while samples were obtained from animals, referred to Al-Fudhailiyah and Al-Husainia regions in Baghdad, during the duration from (November 2009 to November 2010).
Methodology: A total 178 peripheral blood samples were collected from patients with high suspect brucellosis and 15 peripheral blood samples were from animals. The samples were inoculated into *Brucella* agar or trypticase soy agar and blood agar and after 7-30 days, the isolated colonies were identified by inoculation into *Brucella* agar or trypticase soy agar and identified by biochemical tests. DNA extraction was carried out using a commercial kit and a laboratory extraction procedure and examined by PCR involving specific primers for *B.melitensis* and *B.abortus* based on IS711 in the *Brucella* chromosome.
Results: We identified 3 isolates of *Brucella* form animals using culture and biochemical methods but no isolation from patients. When PCR technique was applied to patients and animals blood, 3 patient blood samples were positive for *B.melitensis* and 10 patient blood samples were positive for *B.abortus* but 3 animal blood samples were positive for *B.melitensis* and 6 animal blood samples were positive for *B.abortus*.
Conclusions: The results of present study showed that PCR assay is a rapid and sensitive technique for diagnosis of brucellosis compared to culture method. However it is more valuable when coupled with conventional methods.

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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].

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].

Exact diagnosis is based on the clinical picture, epidemiological data, and different laboratory tests, such as bacterial culture, agglutination, and polymerase chain reaction [7, 8]

Amplification of DNA by polymerase chain reaction (PCR) has been in use for decades to diagnose several infectious diseases caused by fastidious or slowly growing bacteria. Previous studies have detected the small amounts of *Brucella* DNA in pure cultures of human and animal samples by means of PCR [9, 10].

PCR assay has been used in diagnosis both animal [9, 11] and human [9, 12, and 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 detection of *B.melitensis* and *B.abortus* from human and animal brucellosis using whole blood and compared its sensitivity with culture diagnostic method.

Materials and Methods

A total of 178 peripheral blood specimens were collected from patients with high suspected of brucllosis, referred to Al-Yarmook, Al-Karama, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him) and Al-Kadhmiya Hospitals in Baghdad, The samples were taken from patient suspected to be with brucellosis after adequate antibiotic treatment, and 15 peripheral blood specimens were collected from animals, referred to Al- Fudhailiyah and Al-Husainia regions in Baghdad, during the period from November 2009 till November 2010.

The diagnosis of brucellosis was established by the presence of a compatible clinical picture [14] including undulant fever, night sweat and serological diagnosis was carried by positive Rose Bengal test titer of ≥1:160 and ELISA test, moreover demographic, occupational, clinical, and risk factor details were recorded for each patient.

Culture and biochemical tests

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°C until processing. The first part of the blood with anticoagulant was inoculated into: Blood agar, *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°C in the presence of 5-10% CO_2 .After 7-30 days, colonies grown in the solid phase, were identified by inoculation into *Brucella* agar or trypticase soya agar and taken the growth of colonies by loop and spreaded on the surface of plates containing blood agar media and performance of biochemical tests [16].

DNA extraction from blood samples

Genomic DNA was extracted from blood and colonies grown on solid media (bacterial culture) of *Brucella* spp. using a Wizard Genomic DNA Purification Kit / Promega – company (USA).

PCR for *Brucella melitensis* and *Brucella abortus*

Oligonucleotide primers specific for IS711 *B. melitensis* and *B. abortus* which were used in this study, were: 5’-AAA TCG CGT CCT TGC TGG TCT GA and 5’-TGCCGA TCA CTT AAG GGC CTT CAT for *B. melitensis* and 5’- GAC GAA CGG AAT TTT TCC AAT CCC and 5’-TGCCGA TCA CTT AAG GGC CTT CAT for *B. abortus*.[17]. PCR assay was performed in a final volume of 25 µl mixture containing 13.75µl~ 14µl H_2O, 5µl 10x PCR buffer, 0.5µl (dNTPs) mix (200 mM), 1.5µl $MgCl_2$, 1 µl for each oligonucleotides *B. melitensis* and *B.abortus* (0.5 µM each), 0.25µl of Taq polymerase, 2 µl of samples DNA. The amplifications were carried out in a thermocycler USA, with the following steps: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 2 min, 55°C for 2 min and 72°C for 2 min with a final extension at 72°C for 4 min. The last step is (extention 2 or final extention) according to [18]. The products were analyzed by electrophoresis through a 2% (w/vol) agarose gel was performed at 70 V for 60 min, after which the gel was stained with 2 µl ethidium bromide, and DNA fragments were visualized by UV transilluminator at 320 nm and was photographed by polaroid system. Positive and negative controls of PCR were included in each experiment. Negative control, containing all the reagents but lacking template DNA was processed exactly as has been described to monitor for contamination with *Brucella* DNA. All

were negative in all experiments. Positive controls with 100ng of genomic DNA isolated from a suspension of *B. melitensis* and *B. abortus* were also included.

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. 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 27 (15.16%) were farmers or butchers or they are lived near animals and one (0.56%) patient (female) causes aborted fetus. 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. (Figure 1).

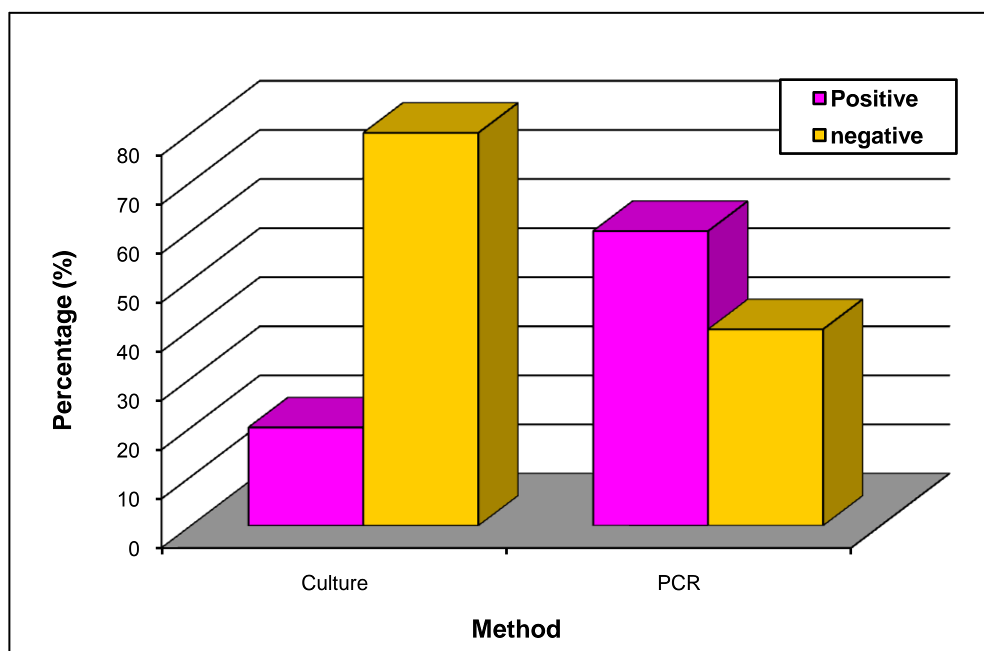

Figure 1: Comparison between PCR amplification & blood culture for diagnosis of 15 samples of the Animals.

In present study DNA extracted from 178 human blood samples, 178 (100%) samples were positive results (Figure 2) and 15 animals, 15 (100%) blood samples (Figure 3) and 3 (20%) blood culture samples (Figure 4) were positive results by the Wizard Genomic DNA Purification Kit (Promega – company –USA) was used and subjected to PCR using IS711 primer for *B.melitensis* and *B.abortus*.

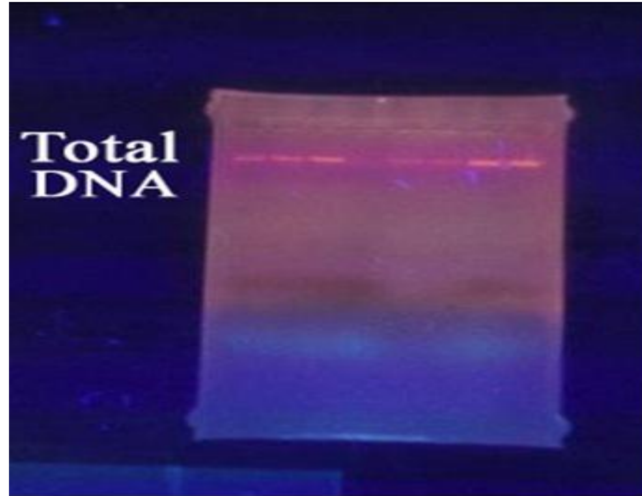

Figure 2: A total DNA extracted from human samples. The seven bands of isolated DNA from human blood. Separated by electrophoresis on 0.8% agarose gel stained with ethidium bromide at 70 volts for 90 min.

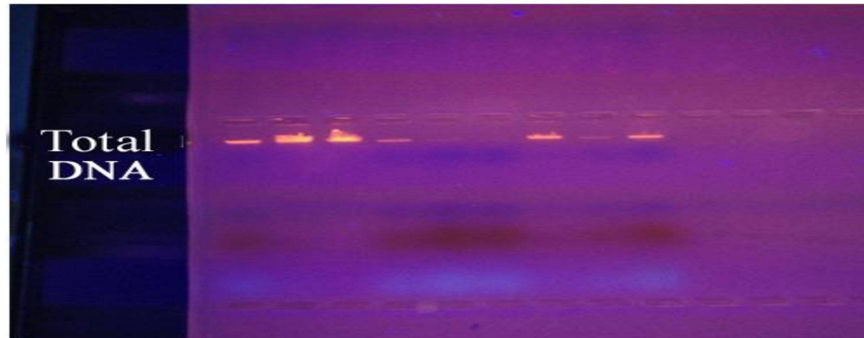

Figure 3: A total DNA extracted from animals blood samples. The seven bands of the *Brucella* chromosomes. Separated by electrophoresis on 0.8% agarose gel stained with ethidium bromide at 70 volts for 90 min.

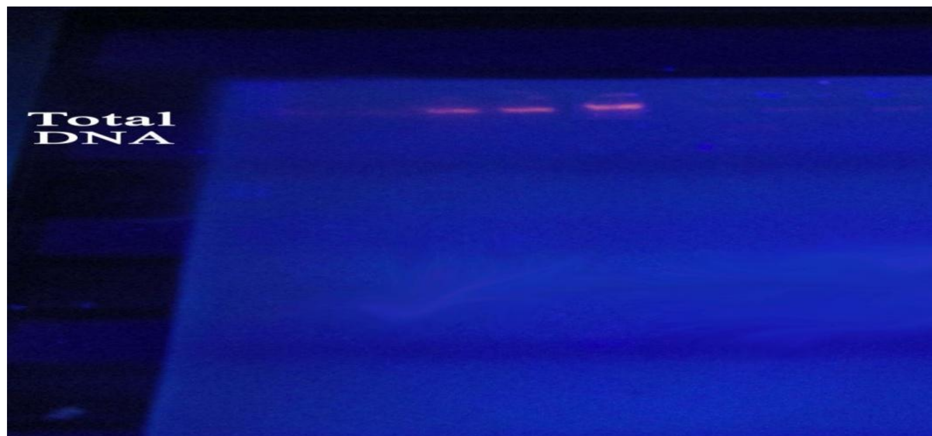

Figure 4: A total DNA extracted from animal blood culture. The three bands of the *Brucella* chromosomes. Separated by electrophoresis on 0.8% agarose gel stained with ethidium bromide at 70 volts for 90 min.

When PCR technique was applied to blood samples, 10 (5.61%) were found positive and gave (731bp) *Brucella abortus,* whereas 3 (1.68%) were found positive and gave (498bp) *Brucella melitensis* of total 178 patients and 3 (20%) were found positive for cows and gave (731bp) *Brucella melitensis* and 6 (40%) were found positive for

buffalos and gave (498bp) *Brucella abortus* of total 15 blood animals. (Figure 5, and 6).

Figure 5: Agarose gel electrophoresis for human of PCR products amplified with Primer IS711 *Brucella melitensis* (731bp) and *Brucella abortus* (498bp).

Lane M, molecular weight DNA ladder (100bp), lane 1: negative control. Lane 3: positive control for *Brucella aboruts,* lane 4: positive sample for *Brucella aboruts,* lane 5: positive control for *Brucell melitensis*, lane 6: positive sample for *Brucella melitensis*, lanes 2, 7: negative samples.

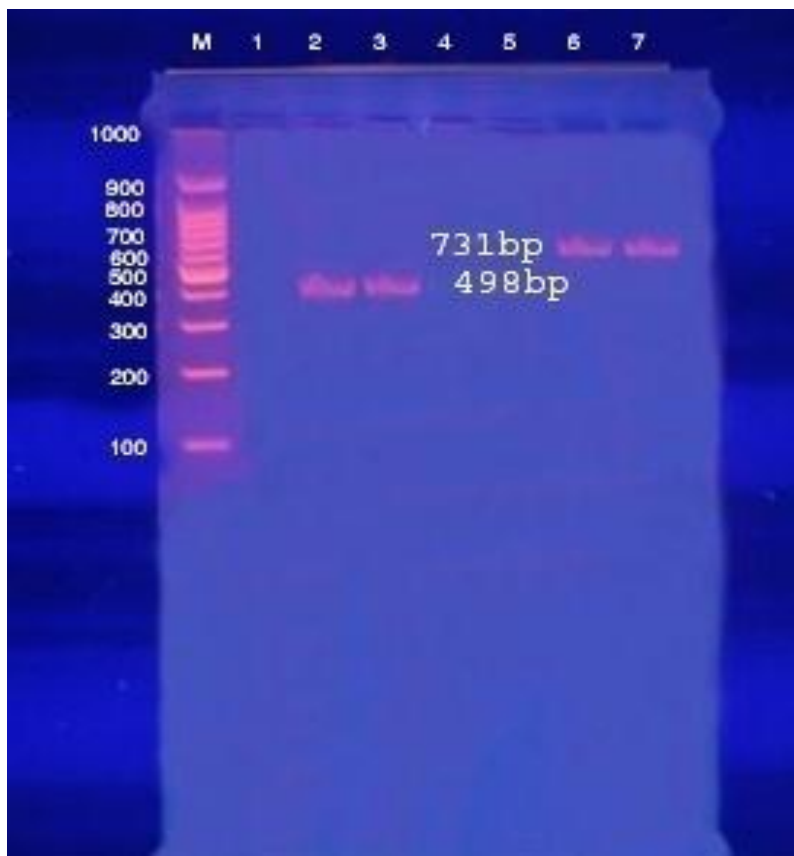

Figure 6: Agarose gel electrophoresis for animal samples of PCR products amplified with primer IS711 *Brucella melitensis* (731bp) and *Brucella abortus* (498bp). Lane M, molecular weight DNA ladder (100bp), lane 1: negative control, lane 2: positive control for*Brucella aboruts,* lane 3: positive sample for *Brucella aboruts,* lane 6: positive control for *Brucella melitensis*, lane 7: positive sample for *Brucella melitensis*, lanes 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. 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 [19] (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. In spite of difficulty of isolation *Brucella* species from animals blood samples. Hence makes PCR more sensitive than culture; so suggest that PCR could replace blood culture as the gold standard for the diagnosis of animals blood samples. [20] (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, 21]. 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 10 (5.61%) were found positive and gave (731bp) *Brucella abortus,* whereas 3 (1.68%) were found positive and gave (498bp) *Brucella melitensis* for patients and 3 (20%) were found positive for cows and gave (731bp) *Brucella melitensis* and 6 (40%) were found positive for buffalos and gave (498bp) *Brucella abortus* for animals. This very good sensitivity, confirm that the PCR assay could be a useful tool for the diagnosis of human and animal brucellosis as other investigators showed by using whole blood [12, 23] serum sample [21].

Finally, in addition to the high yield of the PCR assay for the diagnosis of human and animal brucellosis according to present study, and focal complications in such patients as previously reported [24], other important aspects are: (1) PCR is fast or it is use to confirm the diagnosis of brucellosis in a short period , providing results in 24 hour, 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. In this study molecular diagnosis of brucellosis by PCR technique in comparing with culture method which consider to newer and superior along with culture was evaluated.

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