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Evaluation of two PCR techniques for detection of Brucella DNA in Contaminated Serum Sample

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Abstract. Brucellosis is a major health problem worldwide.Currently the diagnosis of this zoonosis is based on microbiological and serological tests.PCR has been used to detect DNA from Brucella. PCR techniques and extraction procedures have been previously published for Brucella detection.But only a few of these primers have been used in human samples.and only a few study has been carried out to compare sensitivity between them.In this study,two sets of primers amplifying two different regions of the Brucella genome were compared for detection of Brucella DNA in Contaminated Serum Sample PCR assay to conclude which is most suitable for the clinical diagnostic laboratory.These two pairs of primers amplify:i)a sequence 16SrRNA of *B.abortus*(F4/R2),and(ii)a gene encoding an outer membrane protein(omp-2)(JPF/JPR).The two primers assayed showed a difference in sensitivity for detecting Brucella DNA,ranging between 5Pg and 50Pg for contaminated serum samples.Therefore,the sensitivity of PCR using F4/R2 primers was greater than the PCR using JPF/JPR primers.

Keywords: brucellosis, PCR, contaminated serum, omp-2, 16S rRNA

1. INTRODUCTION

Brucellosis is a widespread zoonosis which is still responsible for economic losses of livestock in many areas of the world.It is transmittable to humans via contact with animals or their products. Half a million new cases are reported worldwide each year, but according to the World Health Organization,these numbers greatly underestimate the true incidence of human disease.Since the disease constitutes a serious infection necessitating treatment with a prolonged course of antibiotics, accuracy and short turnaround time are required for the diagnostic tests.

Global variation ranges from incidence of <1/100,000 population in UK, USA and Australia,to 20_30/100,000 in southern European countries such as Greece and Spain, and up to >70/100,000 in Middle Eastern countries, for example Iran.The countries with the highest incidence of human brucellosis are Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman.Bahrain is reported to have zero incidence.Most human cases are caused by *Brucella melitensis (B. melitensis)*, particularly biovar 3. However, *Brucella abortus (B. abortus*) has been responsible for an increasing number of cases in recent years, e.g. in Yemen. *B. melitensis*

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biovar 3 is the most commonly isolated species from animals in Egypt, Jordan, Turkey and Tunisia. *B. melitensis* biovar 2 was reported in Turkey and Saudi Arabia and *B. melitensis* biovar 1 in Libya and Oman. *B. melitensis* remains the principle cause of brucellosis in Iran[1,3].

Brucellosis is a worldwide reemerging zoonosis and the most frequent laboratory acquired bacterial infection, causing severe disease in humans with unspecific clinical signs affecting numerous organs.Patients suffering from this disease show unspecific symptoms, e.g. fever, chills, malaise, arthralgia, headache, tiredness and weakness. Various other febrile illnesses, e.g. malaria, tuberculosis, typhoid fever and tularemia may present with the same symptoms. Therefore, clinical diagnosis is difficult to establish but effective therapy requires an early diagnosis. A definite diagnosis requires the isolation of Brucellae from blood, bone marrow or other tissues [2,4].

Blood cultures represent the gold standard of laboratory diagnosis, this requires prolonged incubation, blind subcultures, and special growth media due to their comparatively long doubling time. Brucella species grow slowly on primary cultures and subcultures,while their inert biochemical profiles hamper fast identification of isolates, however, the sensitivity of this technique is low, ranging from 15 to 70%. Consequently, detection and identification of Brucella spp. in clinical specimens by cultures may still be a difficult task with significant delays and hazards to lab personnel as Brucella spp.are class III pathogens, since their handling poses considerable risk to laboratory personnel[11].

Thus diagnosis is usually based on indirect serological tests, including several agglutination tests (Rose Bengal, Wright_s tube, Wright_s card, and Wright-Coombs) and indirect immunofluorescence,complement fixation, and enzyme-linked immunosorbent assays(ELISA) [12]. Broad range of test sensitivity, low specificity in areas of endemicity, lack of usefulness in diagnosing chronic disease and relapse, presence of cross-reacting antibodies, and lack of timeliness constitute problems associated with brucellosis serology.To overcome some of these problems, at least two serological tests have to be combined to avoid false negative results.Usually, the serum agglutination test is used for a first screening and complement fixation or Coombs' test will confirm its results[14].

As for other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis. Nucleic acid amplification techniques, like PCR, characterized by high sensitivity and specificity and short turnaround time can overcome the limitations of conventional methodology. Only a few studies in the literature,however, address direct detection of Brucella spp. in clinical specimens of human origin and in these studies the extraction procedures, the use of different primer pairs, a variety of different target genes and different amounts of DNA were applied.Whole blood was used as clinical specimen in all the studies except for one where serum was used instead of whole-blood samples for the diagnosis of human brucellosis by PCR[15].

The present study addresses the issue of comparing 2 reported PCR techniques for diagnosis of brucellosis Contaminated Serum Sample and selecting the one most suitable for a diagnostic microbiology laboratory in terms of sensitivity, specificity, robustness and ease of implementation.

2. MATERIAL AND METHODS

2.1. DNA isolation from bacteria

B.abortus B19 (ATCC 2308) was kindly supplied by the Department of Bacteriology (University of Tarbiat modares). Bacterial strains were resuspended in phosphate buffered saline (PBS), pH 7.4, then an equal volume of propanol was added and the recovered cells were stored at 4^0C [15]. Immediately before use, 500 µl of bacteria were pelleted by centrifugation and resuspended in TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0). The cells were incubated at 50 ^{0}C for 30 min with 400 µl of lysis solution (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0) and 10 µl of proteinase K (10 mg/ ml). Cell wall debris, denatured proteins, and polysaccharides were removed by precipitation with 5 mM NaCl and CTAB NaCl solution and incubated at 65^0C for 10 min [15].DNA was extracted with organic solvents by standard protocol,and precipitated with 95% ethanol [16].The DNA pellet was resuspended in 100 µl of TE buffer.DNA concentration and purity were determined spectrophotometrically [17]. Serial dilutions of purified DNA were made and stored at 32^0C until required for further use.

2.2. DNA extraction from blood samples

The method described in above was employed for the extraction of DNA from blood samples.Briefly, blood was collected in heparinized tubes and stored at 32^0C until further use. Immediately before DNA isolation, samples were thawed and aliquots of 0.4 ml were centrifuged at 4000 g for 3 min. Pellets were resuspended in TE buffer, mixed , and again centrifuged.This step was repeated until leukocyte pellets lost all the reddish coloring (at least three times). In that way, plasma proteins, hemoglobin and heparin were washed-out. White cells were resuspended in lysis solution and treated with proteinase K as earlier indicated, and incubated for 30 min at 50^0C.DNA was purified by a standard organic solvent extraction method [18]. Finally, the DNA pellet was resuspended in 100 µl of TE buffer. DNA concentration and purity were determined spectrophotometrically [20]. Samples were aliquoted and stored at 32^0C until further use.

2.3. PCR amplification

The two pairs of primers chosen amplified regions of two different Brucella genes: (i) a 905 bp fragment was amplified with primers F4(5´- TCG AGC GCC CGC AAG GGG- 3´)and R2 (5´- AAC CAT AGT GTC TCC ACT AA- 3´), which derived from the 16S rRNA sequence on *B.abortus* [20]; (ii) oligonucleotides JPF (5′-GCG CTA AGG CTG CCG ACG CAA-3′) and JPR(5′-ACC AGC CAT TGC GGT CGG TA-3′) amplified a 193 bp fragment from a gene encoding an outer membrane protein (omp-2) [21]. characteristic of primers F4/R2 and JPF/JPR for detecting purified Brucella DNA are shown in Table 1

Table 1. Characteristic of primers F4/R2 and JPF/JPR for detecting purified Brucella DNA.

Source	Fragment	Target gene	Sequences	Primers
(8)	905 bp	16S rRNA	5´- TCG AGC GCC CGC AAG GGG- 3´ 5´- AAC CAT AGT GTC TCC ACT AA- 3´	F4/R2
(17)	193 bp	omp-2	5´-GCG CTA AGG CTG CCG ACG CAA-3´ 5´-ACC AGC CAT TGC GGT CGG TA-3´	JPF/JPR

All amplifications were performed in a total volume of 25 µl, containing serial dilutions of Brucella DNA and in the absence or presence of 200 ng of human genomic DNA. Primer and

Taq polymerase concentrations were chosen according to the original reports. To reaction mixtures containing primers F4/R2, 1.0 units of Taq polymerase (Cinnagen, Germany) was added, whilst to mixtures containing primers JPF/JPR, 1.5 units of Taq polymerase was added[19]. The reaction buffer was composed of 50 mM KCl, 10 mM Tris-HCl(pH 8.4), 1 mM $MgCl_2$, as well as 200 µM each of dATP,dGTP, dCTP and dTTP (Cinnagen). The concentration of primers F4/R2 was 0.5 µM, while the concentration of primers JPF/JPR was 2 µM. The reactions were performed in a DNA thermal cycler (model Vapo.protect; Eppendorf) without mineral oil. Amplification using primers F4/R2 consisted of an initial denaturation at 95^0C for 5 min. The PCR profile was set as follows: 30 s of template denaturation at 95^0C, 90 s of primer annealing at 54^0C, and 90 s of primer extension at 72^0C, for a total of 30 cycles, with a final extension at 72^0C for 6 min. Amplifications with primers JPF/JPR were performed using an initial denaturation temperature of 94^0C for 4 min, followed by 35 cycles with denaturation, annealing and extension at 94^0C, 60^0C and 72^0C, respectively (each for 60 s), and one final extension at 72^0C for 3 min[21].

The negative control contained sterile water instead of DNA template. As positive controls, DNA isolated from *B.abortus* B19 were used. After amplification, the samples were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide (2 µg /ml). DNA bands were visualized under UV light and photographed with Gel documentation (Biorad, Germany).

2.4. Studies of sensitivity

The sensitivity of the two pairs of primers (F4/R2 and JPF/JPR) was evaluated by using serial dilutions of DNA of *B.abortus* B19. To study the influence of human DNA on PCR amplification of Brucella spp., 200 ng of human genomic DNA were added to different dilutions of the above mentioned Brucella spp. This human genomic DNA was extracted, as described above [22], from the blood sample of a healthy individual, who according to clinical, serological and microbiological tests was free of Brucella infection.

As a negative control, PCR amplification was performed using all reaction components but DNA. An additional negative control was carried out using human genomic DNA from the healthy individual mentioned above. Positive PCR controls were performed using genomic DNA from *B.abortus* B19.

2.5. Studies of sensitivity

To evaluation of the specificity of PCR metod were used from standard DNA of the six bacterial strains,include: *Escherichia coli*(ATCC9546), *Yersinia enterocolitica*(9610), *Listeria monocytogenes*(7302) ,*Shigella dysenteriae*(9361), *Francisella tularensis* (25017) , *Salmonella* Typhi (7823) [23].

3. RESULTS

Proper use of materials to maintain the sensitivity and improving the quality of the results are very important.The results of false positive and negative in evaluation of PCR, suggested that the possibility of the existence of contamination or inhibitors in clinical samples and can affect the sensitivity of measurement.

In this study was perused the important and effective factors on the reaction,for example: concentration of $MgCl_2$, quantity of SmarTaq Polymerase,concentration of template

DNA,concentration of nucleotids and annealing temperature and obtained best conditions (Table2)

The comparison of the sensitivity of primers F4/R2 and JPF/JPR for detecting purified Brucella DNA are shown in Fig 2. The most sensitive primers were F4/R2; they amplified 5 pg of purified *B.abortus* B19 DNA(Fig.2D),While, primers JPF/JPR amplified 50pg,of purified genomic DNA from *B.abortus* B19(Fig. 2B).

We investigated the influence of human genomic DNA for the detection of Brucella spp. by PCR using the two primers mentioned above. The sensitivity of primers JPF/JPR was not affected by the presence of human genomic DNA,while with primers F4/R2 the sensitivity decreased .

Table 2. Effective factors on the reaction PCR.

Annealing tem	**Nucleotids**	**SmarTaq**	**$MgCl_2$**	**Factors**
55-65ċ : 193 bp 45-55ċ : 905 bp	100-500 µM	0.5 U-10 U	0.25 mM-2 mM	**Range**
63.9ċ : 193 bp 48.3ċ : 905 bp	200 µM	1.25 U	0. 5 mM	**Optimum concentration**

To evaluation of the specificity of PCR metod were used from standard DNA of the six bacterial strains, but did not any reaction , that is indicating the specificity of the primers and PCR in diagnosis of the brucella(Fig 1)

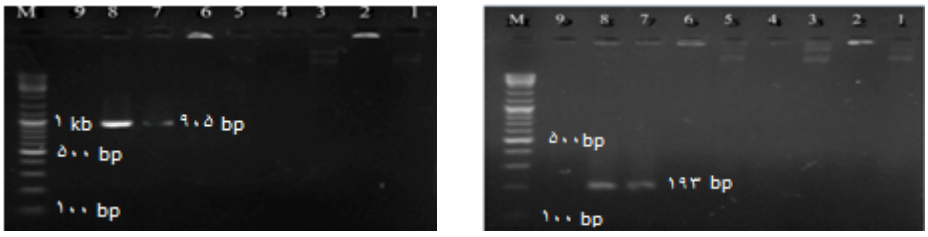

Figure 1. Evaluation of the specificity of PCR method by standard DNA of the six bacterial strains.Right picture: omp-2(193 bp) and left picture: 16S rRNA(905 bp).

Well 7:*Brucella abortus* well8:Control+ well9:Control- wells 1-6 respectively:

Escherichia coli(9546), *Yersinia enterocolitica*(9610), *Listeria monocytogenes*(7302) , *Shigella dysenteriae*(9361), *Francisella tularensis* (25017) , *Salmonella* Typhi (7823). M:Marker(100bp DNA Fermentase).

To determine the sensitivity of PCR method were used from different Serial dilutions of template DNA.the results showed : were observated 193bp band(omp-2) until dilution3(in serum)(50Pg) and in water until dilution3(5Pg).But 905 bp band(16S rRNA) showed until dilution4(in serum) (5Pg) and in water until dilution6(50Fg) (Fig 2).

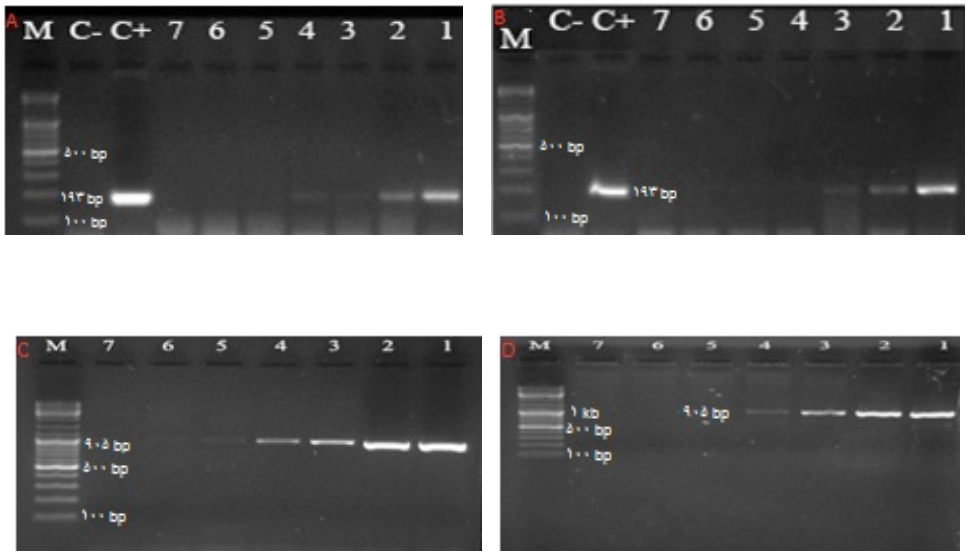

Figure 2. Determine the sensitivity of PCR method by different Serial dilutions of template DNA.

Right pictures(B,D):prepared by serum,Left pictures(A,C): prepared by water.Above pictures(A,B):

omp-2(193bp),Below pictures(D,C):16SrRNA(905bp).

1) 5 ng 2) 500 pg 3) 50Pg 4) 5Pg 5) 500 fg 6) 50Fg 7) 5 fg M)100 bp Marker DNA Fermentase

4. DISCUSSION

Brucellosis is not an emerging disease but rather one that is overlooked by the majority of the scientific community.Brucellosis is the most common reported infectious disease among National Guard soldiers and their families. *Brucella melitensis* is the most pathogenic for humans among the six brucella species[22].

Currently, the diagnosis of this zoonosis is based on microbiological and serological laboratory tests. The diagnostic value of antibody assays is unsatisfactory in the early stages of the disease due to low sensitivity, serological cross-reactions, and the inability to distinguish between active and inactive infection due to antibody persistence after therapy. Some may show false positive serological reactions because of infection by other gram-negative organisms, *Yersinia enterocolitica* in particular. The pathogen can also be detected by blood cultures (which represent the gold standard of laboratory diagnosis). However, false negative cultures could be attributed to antibiotic intake[24].

When performing the techniques exactly according to the published protocols, no bands were obtained and after several trials at modifications of the techniques, positive results were finally acquired. These modifications were then used throughout the study and namely were: the *Taq* polymerase concentration used with the JPF/JPR primers was reduced to 1.25U instead of 2.5U, as well as changing the MgCl2 concentration to the optimum value for each technique as obtained by calibration. The amount of primers used in the JPF/JPR reaction were also reduced

to decrease the intensity of formation of primer dimmers which were consistently observed when using the original concentrations[26]. The F4/R2 primers yielded the least sensitivity and several attempts were made to increase that value including increasing the *Taq* polymerase concentration from 0.5 U per reaction to 1.25 U and using a range of primer concentrations from 0.5 µM up to 2 µM as well as eliminating Triton X-100 from the reaction mixture without success in improving the sensitivity of the technique. It is speculated that increasing the number of cycles has improved the diagnostic sensitivities of all primers used as some weak bands were obtained after increasing the number of cycles that were not detectable using the original cycle numbers published[27].

In the present study, to eliminate the possibility of PCR inhibitors such as heparin (which binds *Taq* polymerase) and EDTA (which chelates Mg+2 ions from the PCR mixture), sodium citrate was used as the anticoagulant. Additionally, the extraction technique was unified so that the results obtained would depend solely on the primer sensitivity. Moreover, during the primary phase of the study, different concentrations of target DNA were tested (ranging from 1µl to 5µl of control DNA) to eliminate the possibility of reaction inhibition due to excess target DNA[27].

The F4/R2 technique was the high sensitive and needed the lower number of cells to give a positive band, all attempts at improving its results were successful.Different specimens, sample pretreatment, and DNA extraction methods could account for discrepant results in comparison to the original reports but not for the differences obtained with analytical sensitivity[29].

In this study, the sensitivity of these primer pairs was different from that described in the original reports. However, considering the complexity of PCR methods and differences between procedures, these results are not surprising. Despite use of the same primer pair, parameters like sample selection, anticoagulants, storage conditions, sample pretreatment methods, extraction methods, and finally the actual PCR assay all were variable. attempts to improve the analytical sensitivity by changing assay parameters were successful.

Only afew reports in the literature have evaluated the application of PCR for the diagnosis of human brucellosis, and most of them used the primers B4/B5. The first study examined samples from 20 brucellosis patients diagnosed by serology. Mononuclear cells were isolated from EDTA-whole blood; DNA was extracted with a lysis buffer containing proteinase K and used directly for PCR without purification. All patients tested positive; however, two successive rounds of PCR were required in order to enhance band intensity, an approach prone to lead to contamination with amplicons.All negative controls were negative.Another study examined peripheral blood samples from 47 brucellosis patients retrospectively. Specimens were collected in sodium citrate, depleted of red blood cells, and digested with a proteinase K-containing lysis buffer, and DNA was extracted by a salting-out procedure.Excellent sensitivity (100%) wasreported in comparison to blood culture and serology (70 and 84%, respectively).Extensive washing of cell pellets, determination and adjustment of the isolated DNA concentration, and incubation of DNA with H2O2 were recommended for avoiding false negatives; however, this method of optimization resulted in a lengthy, complicated procedure. The specificity was 98%[25].

Finally, a short report described a study involving a small number of brucellosis patients that tried to reproduce results obtained with the methodology described above. The use of identical procedures, however, did not reproduce the previous results; the sensitivity and specificity were 50 and 60%, respectively. Different inoculum sizes and degradation of target DNA in clinical samples due to different storage conditions were assumed to account for discrepant results, as did the well-known fact that in-house PCR results are difficult to reproduce in different

laboratories[18].

The excellent sensitivity reported by Matar and Queipo-Ortuno, using the primers B4/B5, in the diagnosis of human brucellosis has not been reproduced by other groups.15,16 In the present study, the diagnostic sensitivity of B4/B5 primers was 98% and not 100% but it was able to detect the 3 relapse cases (which was also the case in Queipo-Ortuno et al, 1997). The reasons why these PCR methods, using the same primers, showed different sensitivities are unknown, but these results are not surprising, considering the complexity of PCR methods and the differences between procedures. In the present study, increasing the number of PCR cycles to 40 rather than 35 as well as increasing the annealing time to 60 seconds instead of 30 seconds had its toll on increasing the detection limit as some weak bands were observed that were not seen when the 35 cycle protocol was followed[28].

Navarro et al in 200225 showed F4/R2 to be the most sensitive primers, which was not the case in the present study. They found that this pair of primers was affected by the presence of human DNA. One study used F4/R2 primers for diagnosis of brucellosis with a sensitivity of 72.1% which was higher than that found in the present work (Nimri, 2003). In accordance with previous results, the B4/B5 PCR assay specificity as well as the other primer pair assays was excellent[17].

The short turnaround time of PCR (less than 4 h) compares favorably with that of blood cultures and Wright_s tube and Wright-Coombs tests (3 to 7 days, 24 h, and 48 h, respectively). Finally, costs of in-house PCR methods are low for laboratories already equipped with the necessary infrastructure[16].

However, since the aim of the present study is to recommend a procedure that would be practical, cost effective, with short turn around time and limited hazards to lab personnel as well as being simple enough to be routinely done in a diagnostic lab, these modifications do not seem suitable.

The evaluation of the sensitivity of primers F4/R2 and JPF/JPR for detecting Brucella DNA were showed that the most sensitive primers were F4/R2; they amplified 5 pg of purified *B.abortus* B19 DNA;While, primers JPF/JPR amplified 50pg,of purified genomic DNA from *B.abortus* B19(Fig.2).

We investigated the influence of human genomic DNA for the detection of Brucella spp. by PCR using the two primers mentioned above. The sensitivity of primers JPF/JPR was not affected by the presence of human genomic DNA,while with primers F4/R2 the sensitivity decreased.

5. CONCLUSIONS

Brucellosis remains a serious public health issue, and much remains to be done to reach the goal of controlling human and animal brucellosis.In the present work, we have compared two different PCR methods for the detection of Brucella spp. in human serum samples. We conclude that primers F4/R2 are the more effective of the two PCR methods evaluated in this study for the detection of Brucella DNA, and they could provide a useful tool for diagnosis of human brucellosis in a clinical diagnostic laboratory setting.

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