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Optimization of PCR Conditions for Detection of Human Brucellosis from Human Serum Samples

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Abstract: The conditions of PCR were optimized in order to diagnose brucellosis from human serum samples. For this purpose, 16 serum samples, from confirmed brucellosis cases were examined. The specificity of a polymerase chain reaction assay for detecting *Brucella* DNA using primers specific for the amplification of a 223 bp region of the sequence encoding a 31 kDa immunogenic *Brucella abortus* protein (BCSP31) was evaluated. After modification, factors such as annealing temperature, time, concentrations of magnesium ion, dNTP, Taq and additives like Bovine Serum Albumin (BSA), dimethyl sulfoxide (DMSO), glycerol, gelatin, Tween 20 and Triton X-100 for enhancing PCR reaction were optimized. The optima conditions determined to be: PCR profile with annealing at 60°C for 50 sec optimum concentration of Mg^{2+}(1.5 mM), dNTP(200 μM),Taq(1.25 U), pH 8.3 and the relation between $MgCl_2$ and dNTP concentration, Triton X-100, Tween 20 and BSA were found to be suitable additives.

Key words: PCR, optimization, human, brucellosis, diagnosis, serum

INTRODUCTION

In many developing countries, brucellosis has still an important role in public health and economy (Young, 1997; Corbel, 1997; Ariza, 1999). Each year half a million new cases are reported worldwide, but regarding the World Health Organization report (WHO, 1997), such numbers are much less than the true incidence of this disease in human (Pappas *et al*., 2005). The diagnosis of this disease in human can be established only by laboratory methods and that is because of the variability of clinical forms of human brucellosis. The disease causes a serious infection and the treatment needs a prolonged course of using antibiotics. All required tests need high level of accuracy and short turnaround time (Solera *et al*., 1997). Regarding the heterogeneous nature and poorly specific clinical symptomatology of the disease, a laboratory confirmation is always necessary (Cutler *et al*., 2005). There are some limitations in using conventional microbiological methods for the diagnosis of human brucellosis. Such methods displays poor sensitivity in the early stage of the disease, during which the levels of antibodies may be low. Furthermore, the lack of specificity in areas where the disease is endemic and there can be cross-reaction with other bacteria (Young, 1997; Yagupsky *et al*., 2000). Blood culture gives the best results in microbiological diagnosis, but its sensitivity is considerably diminished in patients with long-term clinical courses or with focal complications (Yagupsky, 1999). *Brucella* sp. are Class III pathogens, so there exists a high level of risk for laboratory personnel (Yagupsky *et al*., 2000; Yagupsky and Baron, 2005). In order to well diagnose infectious diseases caused by slow growing or fastidious bacteria or fungi in clinical laboratories, amplification of DNA by PCR have been recommended (Bogard *et al*., 2001; Kami *et al*., 2001; Kuoppa *et al*., 2002). Assays

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based on the Polymerase Chain Reaction (PCR) have been suggested as a powerful technique for the diagnosis of human infection (Matar *et al*., 1996; Queipo-Ortuño *et al*., 1997; Zerva *et al*., 2001). The development of specific PCR assay, is a recent advance; however, standardization of method is lacking and a better understanding of the clinical significant of the results is still needed (Navarro *et al*., 2004). In this study attempts are made to optimize factors such as annealing temperature, time, pH, concentrations of magnesium ion, Taq DNA polymerase and additives like Bovine Serum Albumin (BSA), dimethyl sulfoxide (DMSO), glycerol, gelatin, Tween 20, Triton X-100 and ammonium sulfate which may effect the PCR final products.

MATERIALS AND METHODS

Clinical Specimens

Sixteen serum samples from patients with acute and chronic brucellosis were collected from Imam Khomeini hospital, Tehran, Iran and stored at -20°C until they were used. All patients were occupationally exposed to *Brucella* (age range, 21 to 74 years [mean, 52 years] disease duration range, 1 week to 90 days [mean, 35 days]).

Isolation of DNA from Serum Samples

DNA was extracted from serum samples employing commercial kit (Cinnagen, Iran) as follows: Five microliter of protease to 100 μL of serum in tube was added, vortexed and placed at 72°C for 10 min then 100 μL of sample with 400 μL of lysis solution was mixed, vortexed for 20 sec, addition of 300 μL of precipitation solution was followed and vortexed for 5 sec and placed at -20°C for 20 min, then centrifuged at 12000 g for 10 min, decanted by gently inverting the tube and placed it on tissue paper for 3 sec, down ward. One milliliter wash buffer was added to pellet, mixed gently for 5 sec and centrifuged at 12,000 g for 5 min and the supernatant was decanted. The pellet was dried at 65°C for 5 min, pellet was suspended in 30 μL of solvent buffer by shaking and placing it at 65°C for 5 min, unsolved materials were precipitated by centrifuge at 12,000 g for 30 sec. The purified DNA was quantified spectrophotometrically by reading the optical density at A_{260} and A_{280}.

Extraction of Genomic DNA from *B. abortus* S-19

Bacterial cells were washed twice with phosphate-buffered saline (PBS) and pelleted by centrifugation. The pellet of bacteria was suspended in a solution containing 68 μL of 20 mg mL^{-1} lysozyme (Cinnagen, Iran), 40 μL of 10% sodium dodecyl sulfate, 80 μL of lysis buffer (375 mM NH_4Cl, 120 mM Na_2-EDTA [pH 8.0]) and 157 μL of sterile Milli-Q water, mixed and incubated for 30 min at 37°C. After incubation, 40 μL of 10 mg mL^{-1} proteinase K (Cinnagen, Iran) was added, mixed gently by inverting the tube several times and then incubated for 30 min at 55°C. Purification and precipitation of bacterial DNA were done as for the serum samples (described above). The concentration of the DNA was then determined spectrophotometrically by reading the optical density at A_{260} and A_{280}.

DNA Amplification

A 223 bp fragment from the conserved region of the gene which encodes an immunogenic membrane protein of 31 kDa of *B. abortus* specific to the *Brucella* genus and present in all its biovars (Mayfield *et al*., 1988) was amplified. A pair of 21-nucleotide primers, B4 (5' TGG CTC GGT TGC CAA TAT CAA 3') and B5 (5' CGC GCT TGC CTT TCA GGT CTG 3'), described by Baily *et al*. (1992) used in the amplification process. PCR was performed in a 50 μL mixture containing template DNA; PCR buffer (10 mM Tris HCl [pH 8.4], 50 mM KCl, 1.5 mM $MgCl_2$); A 200 nM concentration of each of the PCR primers; a 200 μM concentration (each) of dATP, dCTP

and dGTP; 190 µM dTTP; 10 µM digoxigenin-11'-dUTP and 1.25 U of *Taq* polymerase (Cinnagen, Iran). The reaction was performed in a DNA thermal cycler without mineral oil. PCR consisted of a preheating at 93°C for 5 min; 35 cycles of 90°C for 1 min, 60°C for 30 sec and 72°C for 1 min and incubation at 72°C for 7 min. Positive controls based on DNA from *B. abortus* S-19 were included in all the tests, as were negative controls which contained all of the elements of the reaction mixture except DNA. Fifty micro liters of each PCR-amplified sample was loaded onto each lane on a 2% agarose gel and stained with 2 µg of ethidium bromide mL^{-1} to determine the sizes of the amplified products. To guarantee the reliability of the results, all samples were processed in duplicate.

RESULTS AND DISCUSSION

Effect of Magnesium Ions

Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA polymerase. It exists as dNTP-Mg complexes that interact with the sugar-phosphate backbone of nucleic acids (Blanchard *et al*., 1993). So altering the concentration of magnesium ions can lead to one primer/template pair behaving significantly different from another under identical conditions. Therefore, optimizing the concentration of magnesium ions for PCR performance is important (Innis and Gelfand, 1990). To check the optimal concentration of magnesium ions to be used in the amplification reaction, different concentrations of $MgCl_2$ were used. All other factors of the PCR were kept unchanged. The optimum concentration of $MgCl_2$ in the amplification of 31 kDa gene found to be 1.5 mM. Lower $MgCl_2$ concentration (0.5 mM) failed to yield visible bands and higher magnesium concentration (1 and 2.0 mM) yielded adequate but somewhat less amplification product (Fig. 1). Some of the bands were also present at 2.5, 3 and 3.5 mM $MgCl_2$, while at 1.5 mM $MgCl_2$ the band was the sharpest.

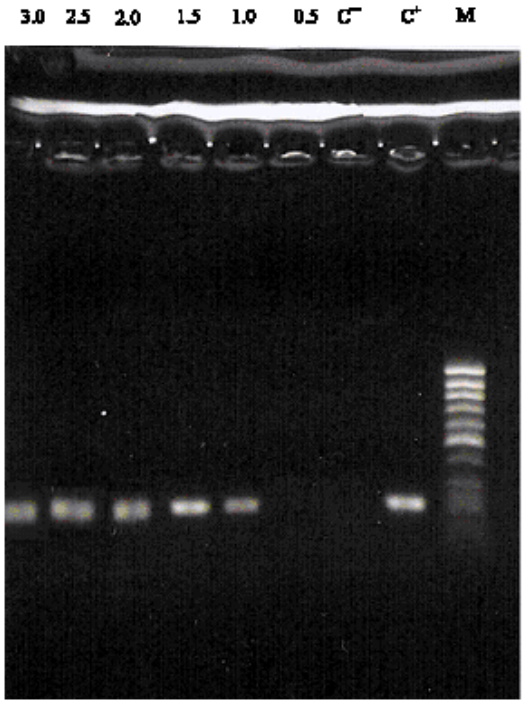

Fig. 1: Effect of Mg^{++} concentration on amplification of 31 kDa gene

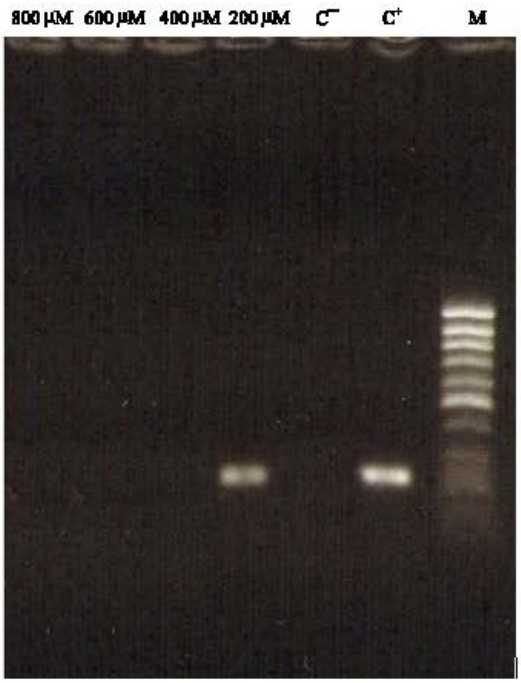

Fig. 2: Effect of varying dNTPs concentrations on amplification of 31 kDa gene

Also this test confirmed that any increase in dNTP concentration requires an increase in the concentration of magnesium ions in order to proceed the reaction. The relation between the concentration of magnesium ion and that of the dNTPs was investigated by performing PCR with a primer in reaction mixture that contained 200, 400, 600 and 800 µM of dNTP, combined with 1.5, 2, 3 and 4 mM $MgCl_2$. Most efficient amplification is seen at concentrations of 200 µM each dNTP. Further increase in the dNTP concentration inhibits the reaction when $MgCl_2$ is kept constant. Lower Mg^{2+} ions concentration resulted in a low yield of PCR product and higher concentration caused the yield of non-specific products and promoted misincorporation.

Effect of Taq DNA Polymerase

The effect of different concentrations of Taq DNA polymerase on the amplification of the 31 kDa gene was evaluated. The amount of Taq polymerase varied from 1-2.0 U per 50 µL reaction. Concentrations higher than 2 units/50 µL can generate non-specific products and may reduce the yield of the desired product (Saiki, 1989). However, if inhibitors are present in the reaction mix (e.g., if the template DNA used is not highly purified), higher amounts of *Taq* DNA Polymerase (2-3 u) may be necessary to obtain a better yield of amplification products. The 2% agarose gel electrophoresis revealed PCR products of faint intensity in reactions amplified with 1.25 U/50 µL of the enzyme.

Effect of Deoxynuclectide Tri-Phosphates (dNTPs)

The concentrations of dNTPs used in a reaction mixture were determined by the affinity of *Taq* DNA polymerase for dNTP as a substrate. Thus dNTPs at 200 µM (final concentration) are appropriate (Fig. 2). Higher concentration of dNTP concentration inhibits/reduces the activity of *Taq* DNA polymerase and also higher magnesium ions in the reaction mixture will be required.

Effect of pH

In carrying out the optimization process, the pHs of Tris-HCl buffer were in ranges of 8.3, 8.6 and 8.9. It was observed that the buffer having pH 8.3 was optimal. It is understood that the pH of Tris buffer decreased at high temperatures, long-template PCR requires more time at high temperatures and increased time at lower pH may cause some depurination of the template, resulting in reduced yield of specific product. The ideal pH for PCR is (8.3) and for long templates, a higher pH [pH 9.0] is suggested. The pH of the Tris buffer in the reaction mix will decrease in high temperatures. The lower pH may cause depurination of the template, resulting in a lower yield of amplicons.

Effect of Annealing Temperature/Time

The optimal annealing temperature often varies from the estimated Tm, even when using pairs of primers with a similar Tm value. As a starting point, an annealing temperature 5°C below the Tm can be used. This is usually then adjusted to improve specificity and yield in a series of tedious optimization experiments. The requirement of an optimal PCR reaction is to amplify a specific locus without any unspecific by-products. Although several annealing temperatures used in the thermal cycling profile produced some products, the protocol (57-63°C) appeared to produce the optimal results. With increasing temperatures from 57-66°C (57, 60, 63, 66 and 69°C), the amount of visible product decreased, with almost no product at 69°C. The PCR protocol produced the most intense bands on polyacrylamide gels, possibly due to the formation of specific products at the higher temperatures, followed by the more efficient primer binding at the progressively lower temperatures. Stringent annealing temperature, especially during the first several cycles, is recommended for increased specificity (Innis and Gelfand, 1990). The PCR protocol with a range of annealing temperatures is advantageous during the initial optimization steps and ultimately reduces the number of temperature profiles to be tested. We used annealing temperature of 60°C as PCR protocols during optimization with high success rates.

The *B. abortus* DNA isolated from serum sample was amplified for 35 cycles. The annealing temperature was kept at 60°C, but the time of amplification was varied for 3 different times (10, 30 and 50 sec). Therefore, 50 sec was chosen as the optimum annealing time for this target in all further amplification experiments.

Effect of Enhancing Reagents

A variety of PCR additives and enhancing agents can be used to increase the specificity and consistency of yield, in PCR reactions. There are number of additives that may have beneficial effects on some amplification so it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers (Kovarova and Daber, 2000). Higher yields can be achieved by stabilizing/enhancing the polymerase activity with enzyme-stabilizing proteins such as bovine serum albumin (BSA or gelatin), enzyme-stabilizing solutes such as enzyme-stabilizing solvents (glycerol), solubility-enhancing solvents dimethyl sulfoxide (DMSO) has been shown to improve reaction yield during PCR (Sidhu *et al*., 1996).

Triton X-100, Tween 20 may increase yield but may also increase non-specific amplification. $(NH_4)_2SO_4$ affect the denaturing and annealing temperature of the DNA, as well as the enzyme activity.

Recommended final concentrations are: up to 5% for DMSO, 0.1% for Triton X-100, Tween 20, 0.01% for gelatin and 20 mM for $(NH_4)_2SO_4$, in addition 5% for glycerol failed to yield visible bands. We observed that BSA (100 μg mL^{-1}), Tritonx-100 and tween 20 had the best PCR enhancing properties at a concentration of 0.1% in all PCR samples, whereas glycerol was less effective (Fig. 3).

In conclusion, PCR conditions for *B. abortus*, since PCR conditions, such as the annealing temperature/time, concentrations of Mg^{2+}, Taq, dNTP, pH and the relation between Mg^{2+} ions and

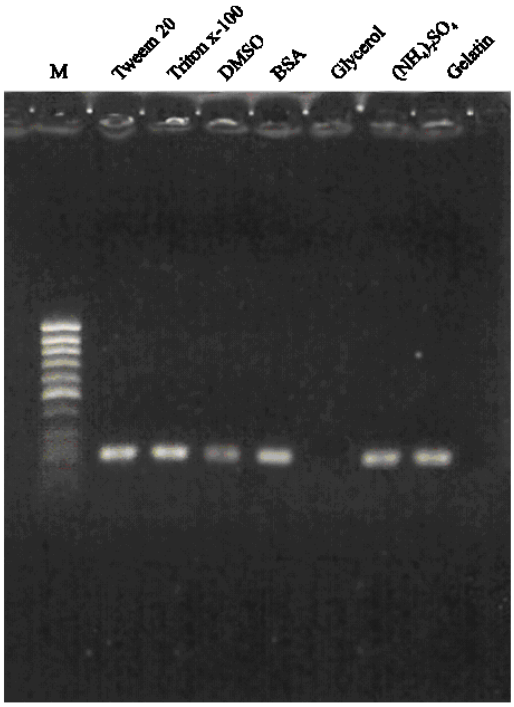

Fig. 3: Effect of additives on amplification of 31 kDa gene

Table 1: Optimized PCR conditions

Reagents	Optimum
Tris-HCL buffer	pH 8.3
$MgCl_2$	1.5 mM
dNTP	200 μM
Taq DNA Polymerase	1.25 U
Annealing temperature	60°C
Annealing time	50 sec
Bovine Serum Albumin (BSA)	100 μg mL^{-1}
Dimethylsulfoxide (DMSO)	5%
Gelatin 1%	0.01%
$(NH_4)_2SO_4$	20 mM
Tween 20	0.1%
Triton x-100	0.1%

dNTP can affect the final products of PCR, the optimized PCR conditions for primers specific for *B. abortus omp* 31 were determined. As shown in Table 1, the optimized annealing temperature, time, pH and the optimized concentrations of Mg^{2+}, Taq DNA polymerase and dNTP for PCR with primers for *omp 3*1 were 60°C, 50, 8.3 and 1.5 mM, 1.25 U, 200 μM respectively. In addition the concentrations of additives like bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), gelatin, Tween 20, Triton X-100 and $(NH_4)_2SO_4$ for enhancing PCR reaction were optimized to be 100 μg mL^{-1}, 5, 0.01, 0.1, 0.1% and 20 mM, respectively.

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