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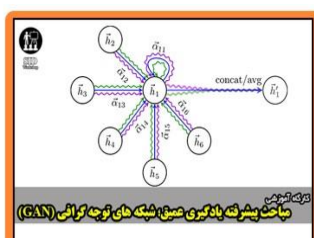

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Comparing Rapid and Specific Detection of *Brucella* in Clinical Samples by PCR-ELISA and Multiplex-PCR Method

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ABSTRACT

Background: Rapid diagnosis and differentiation of *Brucella* is of high importance due to the side effects of antibiotics for the treatment of brucellosis. This study aimed to identify and compare PCR-ELISA as a more accurate diagnositc test with other common molecular and serological tests.

Methods: In this experimental and sectional study, during March 2014 to Sep 2015, 52 blood samples of suspected patients with clinical symptoms of brucellosis were evaluated in medical centers all over Iran with serum titers higher than 1:80. Using two pairs of specific primers of *Brucella abortus, B. melitensis* and DIG-dUTP, Fragment IS711 (The common gene fragment in *B. melitensis* and *B. abortus*) was amplified. DIG-ELISA was performed using specific probes of these 2 species of Brucella and patterns were subsequently analyzed, then positive responses were compared by detecting gel electrophoresis.

Results: PCR-ELISA method detected all 28 samples from 52 positive samples. Its sensitivity was 6.0 pg concentration of genomic DNA of *Brucella*. In gel electrophoresis method, 22 samples of all positive samples were detected. PCR-ELISA was more efficient than PCR and bacterial culture method at *P*-value <0.05.

Conclusion: PCR-ELISA molecular method is more sensitive than other molecular methods, lack of mutagenic color and also a semi-quantitative ability. This method is more effective and more accurate compared to PCR, serology and culture of bacteria. PCR-ELISA does not have false responses. The limitation of this method is detection of bacteria in the genus compared to Multiplex PCR and Gel Electrophoresis.

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Introduction

Brucella are Gram-negative coccobacilli, non-motile, non-spore and non-capsules and obligate parasites of animals and humans. They are often intracellular and have disabled metabolic (1). These microorganisms cause systemic infectious diseases called brucellosis. *Brucella* pathogen

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lives naturally in animals body including livestock and humans mostly infected accidentally by eating dairy products and animal foods. So far, ten strains of this genus have been identified and *B. melitensis* was the most important one responsible for most significant zoonosis in humans (2, 3). *B. abortus* causes human infection and has less pathogenesis than *B. melitensis*. In developing countries, the epidemic of these two common types causes many damages (4, 5).

This disease with debilitating complications causes impressive injuries and even death, which necessiates timely and early detection (6). Diagnosis of this infection is only based on laboratory methods (7). Cultrue is used to diagnose the infection, but because of the danger of these bacteria, it requires class 3 bio-safety and trained personals (8). Serological methods can also be used for diagnosis based on detection of antibodies to *Brucella,* which may yield false results. Serological tests have low sensitivity, especially in early stage of disease where antibody production is low (9).

PCR is the most reliable method to diagnose the disease from infected tissue samples. PCR test results would be evaluated in different ways (10, 11), the most common of which is gel electrophoresis (10). This method has some restrictions for diagnosis including detection of presence or absence of a specific gene (12). These restriction made scientists to use diagnostic methods and new equipment such as Real-time PCR (4). In 1980 to 1982, immune detection techniques of DNA became popular because of these limitations (13). Among the immuno detection methods of DNA, Coutlée et al. presented a diagnosis method using RNA probes Biotinylated (biotinylated RNA) (14). Since then, numerous studies were performed based on this method and immunosorbent techniques (15-17). PCR methods were used by enzymes connection immunosorbent (immunosorbent assay (PCR-ELISA) (15). This method is a combination of both PCR and ELISA as an analytical technique, which has similar wide range of usage like ELISA, but in this method, the nucleic acid is used instead of protein (13, 15). Therefore, considering the advantages and limitations of PCR-ELISA and Multiplex-PCR, this study compared rapid and particular detection of *Brucella* in clinical samples and eventually evaluting the results of study by gold standard culture.

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Materials and Methods

Preparation and Culture of Clinical Samples

In this experimental and sectional study, 52 blood samples of suspected patients with clinical signs of brucellosis during March 2014 to Sep 2015 were collected from medical centers all over Iran. Samples of people who showed symptoms of brucellosis in serum higher than 1:80 titers were performed by experts. Samples were incubated 21 d in blood culture (BacT/Alert, Biomerieux, France) at 37 °C. After this period, subculture was done on brucella agar (Merck, Germany) and incubated at 37 °C for 48-72 h.

Table 1
Probes and Primers (Forward and Reverse) Brucell abortus and B. melitensis

Primer Name	Oligonucleotides 5' → 3'	References
B.a-F	GACGAACGGAATTTTTCCAATCCC	(18)
B.m-F	AAATCGCGTCCTTGCTGGTCTGA	(18)
IS711-R	TGCCGATCACTTAAGGGCCTTCAT	(18)
Probe. *B. abortus*	Biotin-CGGCTTTTCTATCACGGTAT	-
Probe. *B. Melitensis*	Biotin-GGAGTGTTTCGGCTCAGAAT	-

R (Reverse *B. abortus* and *B. melitensis* is the same and the primer gene fragment IS711).

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Genome Extraction and Primers

Extraction of DNA samples of *Brucella* was conducted by proteinase K and phenol-chloroform method according to previews study (2). This research was conducted on the gene fragment IS711 (common gene fragment between *B. abortus* and *B. melitensis*). First by using bioinformatics software, specific primers and probes were designed (2), then they were synthesized by TakapouZist (Iran) company.

Multiplex PCR

Multiplex PCR was set up in a final volume of 25 µL in a GenAmp PCR system (Eppendorf, USA) with 15 µL Master Mix Red 2X (Ampliqon, Denmark) that contained Tris-HCl pH 8.5, (NH4) 2S04, 3 mM MgCl2, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 units/µL Ampliqon Taq DNA polymerase, Inert red dye and stabilizer, 10 pmol of each primer, 1 µl template DNA (0.5 µg) and sterile distilled water up to 25 µL. Following polymerase activation (95 °C for 8 min), 35 cycles were run with 30 sec denaturation at 95 °C, 30 sec annealing at 66 °C and 45 sec extension at 72 °C. The final extension was at 72 °C for 5 min. The double-stranded PCR product was analyzed using the electrophoresis technique on 1.5% agarose gel for one h at 85 V and 25 mA, stained by SYBER green and visualized under UV transilluminator. Standard strains of *B. abortus* S19 and *B. melitensis* 16M were used as standard samples.

PCR–ELISA

Multiplex PCR program was used to perform PCR-ELISA. DIG-dNTP was used instead of 0.4 mM dNTPs. Furthermore, unlike the Multiplex-PCR method, primer pairs of *B. abortus* and *B. melitensis* were used separately.

Identification of PCR products by Digoxigenin-ELISA

In this method, the plates commercially coated by 2.5 µg/mL of streptavidin were used. At first, 5 microliter of DNA products labeled with DIG was diluted with 95 microlitres of PBS (pH of PBS solution should be 5.7). Then probe was added into wells coated with streptavidin, the labeled product was diluted with PBS (mentioned above) and added to the wells and placed for 2 h at 37 °C.

After this time, 100 µL anti-digoxin added to the well and placed at 37 °C for 30 min, then after 30 min the contents of the wells emptied and washed with PBS for three times. After the washing step, tetramethylbenzidine was added and placed in dark place for 30 min, after this time the color would be visible. Finally, by adding 100 µL 2 M sulfuric acid diluted, color reaction stopped and the results read by ELISA reader at a wavelength of 450 nm. The test was repeated three times for each sample. Distilled water was used as negative control. DNA of *B. melitensis and B. abortus* were used with 100 ng as positive control.

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Determining the Sensitivity of PCR-ELISA and Multiplex-PCR:

Five dilutions (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1) of, 955 ng / µL, DNA of *B. abortus* and *B. melitensis* provided and color reaction were compared with Multiplex-PCR results.

According to the initial concentration of standard prototype strains of *B. abortus* S19 and *B. melitensis* 16M which was 955 ng/µl , calculating of the sensitivity of the reaction, the number of dilutions were prepared and then PCR method was carried out for several prepared dilutions .

Statistical Analysis

Data were analyzed using SPSS software, version 16.0 for Windows (Inc, Chicago, IL, USA). Sensitivity, specificity, positive and negative predictive values, likelihood ratios and 95% CIs were calculated.

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Results

Distribution of Samples and Bacterial Cultures:

Preliminary results were checked after 72 h to 10 d .Among 52 blood samples from 52 patients suspected to brucellosis (36.5%) 19 ,had positive results ,in which all 19 patients(100%) were identified as *B .melitensis* by the colony characteristics on *Brucella* Agar, Gram staining and biochemical and differential tests.

PCR Test Results

Of fifty two blood samples from individuals suspected to brucellosis, after performing Multiplex PCR and gel electrophoresis, 22 (42.30%) had positive and 30 (46.70%) negative results. In DNA samples, the band related to *B. melitensis* was observed in 20 cases and the band belonged to *B. abortus* observed in two cases (Figure 1).

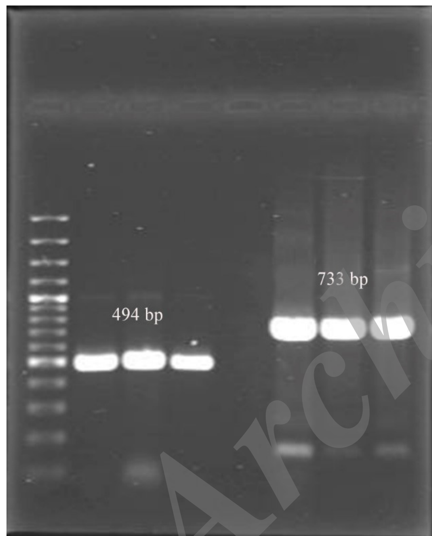

Fig. 1
Multiplex-PCR reaction results in clinical samples: Lane 1 (marker DNA ladder, 100 bp), Lanes 2 to 4 (*B. abortus* to 494bp), Lane 5 (negative control) and Lanes 6 to 8 (*B. melitensis* with 733bp)

The Results of PCR-ELISA

Of 52 samples of *Brucella*, 28 samples had positive results. The test is designed as a semi-quantitative. Some positive wells were highlighted than standard samples indicated a higher concentration of DNA during replication.

The Results of Tests to Determine the Sensitivity of PCR-ELISA

The results determine the sensitivity, below 0.6 pg dilution of the DNA of both bacteria could be identified in this way (Figure 2).

Furthermore, for determining sensitivity of Multiplex-PCR, according to concentration of genomic DNA as 955 ng /µL for standard strains *B. abortus* S19 and *B. melitensis* 16M, the best product was obtained from 10^{-2} (equivalent 9.55 ng / µL) of genomic DNA.

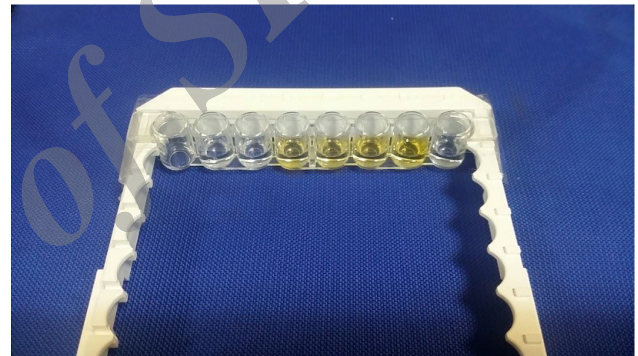

Fig. 2
From right to left, the first well is negative control and there are respectively dilutions of DNA of *Brucella*: 955 ng /µL, 95.5 ng/µL, 9.55 ng/µL, 0.95 ng/µL, 0.095 ng/µL

Discussion

Over the past decade, major advances in molecular diagnosis of brucellosis (especially in clinical samples) have been achieved (18, 19). Detection methods based on nucleic acids are promising tools for detection and eradication of disease (4). The possibility of contamination in molecular biological methods is very low than traditional methods such as bacteria cultures and the results can be achieved in a very short period (1). The method based on a piece of DNA replication has a special use. The use of a pair of

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primers attached to the specific region of DNA sides that causes mass replication in this area by DNA polymerase is basis of PCR (13). Specificity features, high sensitivity and ability to perform faster in the molecular diagnostics field causes to be extensive (20). Detection of *Brucella* by PCR is more sensitive than culture and there is also less risk of disease for laboratory staff and even it is able to detect low levels of bacteria in the samples. Our study showed that the test had lower accuracy (*P*-value <0.05) than Multiplex-PCR and PCR-ELISA. These results are in accordance with other researchers as follows.

Kazemi et al. and Shapouri et al. identified 14% of clinical samples by culture (21, 22). Blood samples, liquid culture broth (Soya bean casein) and tryptophan soy broth were used in Kazemi's et al . study (21). Hosseini Doust et al. used samples of animal tissue in culture as well (23). In this study, the proportion of identified cases by culture method was lower, so that the success of blood culture with *Brucella* broth and agar was 36.5%. Differences of culture results between this study and researchers' results mentioned above can be related to the types of samples and different used culture media.

We used PCR-ELISA and DIG-labeled products, conducted by micro well capture hybridization assay. In fact, this method is more sensitive than agarose gel electrophoresis. The high sensitivity of this method is because of specific hybridization and enzymatic colorization that affect the probe-bound PCR products. It is also safe because researchers do not use Ethidium Bromide color, which causes mutation. This study indicated that PCR-ELISA method was more accurate than Multiplex-PCR detection products (*P*>0.05).

The results of this study, similar to Kumar et al., showed that Multiplex PCR molecular method had some advantages than other methods to identify and differentiate *Brucella* spp. (24).

Evaluation of the sensitivity of PCR-ELISA showed that the test is a semi-quantitative analysis tool, but the amount of color appears in the microplates also shows different concentrations in clinical samples (Figure 2). Formation of a complex hybrid of peroxidase-conjugated anti-DIG antibody and peroxidase substrate (ABTS) allows measuring the amount of connection of probe to the DNA in semi-quantitative position. However, this technique cannot show the result in PCR like Real-time PCR technique, our test has a high accuracy. PCR- ELISA technique due to the limitations of creating a color in this study was not able to differentiate the two species of *B. melitensis* and *B. abortus*.

Conclusion

PCR-ELISA was used for detection of *B. melitensis* and *B. abortus* from clinical samples. This technique is more accurate than gel electrophoresis. However the Multiplex-PCR techniques are more accurate than culture technique, which can reveal results faster than other methods.

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Financial Disclosure

The authors declare no conflict of interest.

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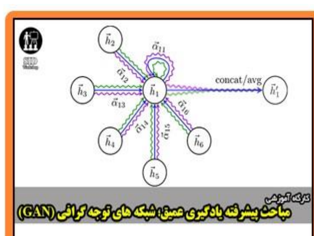

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