Original Article

Zahedan Journal of Research in Medical Sciences

Journal homepage: www.zjrms.ir

Comparison of Culture and Multiplex PCR Technique for Detection of Brucella abortus and Brucella melitensis from Human Blood Samples

Reza Mirnejad,[1] Ali Reza Vahdati,[2] Ali Ahmadi,[1] Seied Mojtaba Mortazavi,[3] Vahhab Piranfar*[4]

1. Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2. Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland
3. Health Station, Kerman University of Medical Sciences, Kerman, Iran
4. Department of Microbiology, Tonekabon Branch, Islamic Azad University of Tonekabon, Tonekabon, Iran

Article information

Article history:
Received: 27 Nov 2012
Accepted: 9 Jan 2013
Available online: 12 Feb 2013
ZJRMS 2013; 15(12): 5-8

Keywords:
Brucella abortus
Brucella melitensis
Multiplex PCR
Culture method

*Corresponding author at:
Department of microbiology, Tonekabon branch, Islamic Azad university of Tonekabon, Tonekabon, Iran.
E-mail:
vahab.p@gmail.com

Abstract

***Background*:** To compare culture methods with multiplex PCR technique for identification of *Brucella abortus* and *Brucella melitensis* from suspicious patients with clinical history of brucellosis and positive serological test (Rose Bengal test and serum agglutination test).

***Materials and Methods*:** In this study, 160 blood samples from patients suspected of Brucellosis with high serum titers of 1/80 were studied. All samples were cultured in Brucella-specific media. Brucella species were identified by using microbiological methods. DNA was extracted with Phenol-chloroform DNA extraction method. IS711 was amplified simultaneously using three specific primers and obtained patterns were analyzed.

***Results*:** From 160 samples, 47.5% (76) were culture positive cases from which 43 cases were *B. melitensis* and 33 were *B. abortus* With the PCR technique 108 were detected positive from which 45.3% were *B. abortus* and 54.6% were *B. melitensis*. It should be noted that all 76 samples with positive culture were also identified by PCR.

***Conclusion*:** Generally, use of the molecular technique multiplex PCR in addition to increased speed and accuracy and less false results than bacterial culture method, is able to identify different species of brucella. This will facilitate the treatment process.

Introduction

Brucella is an intracellular parasite of the disease brucellosis throughout the world [1]. This Gram-negative coccobacillus consists of 10 species, of which *B. abortus*, *B. melitensis*, *B. ovis*, *B. canis* and *B. suis* are pathogenic for humans. Also, *B. microti*, *B. inopinata*, *B. ceti* and *B. pinnipedialis* are isolated from animals but can occasionally cause disease in man [2]. Among these species, the main pathogens for humans are *B .melitensis* and *B. abortus* [3, 4]. *Brucella melitensis* is a highly contagious disease in sheep and goat [5]. This species is the most important zoonosis in humans [1]. Human infections due this species are widespread. The epidemics caused by these two species in developing countries is common and causes great damages [3].

This disease has extremely debilitating side effects that can even cause death. So timely and accurate diagnosis is an important factor to identify bacteria, in addition to clinical epidemiology and positive signs, we need related to the laboratory evidence [6, 7]. Current tests used in diagnosis of brucella include: 1. Culture methods, 2. Polymerase chain reaction and 3. Serological methods based on detecting antibodies against brucella.

Definitive diagnostic of brucellosis can be made with blood culture, lymph, bone marrow and other body fluids and secretions [8]. But what has been reported from previous studies is that blood culture and body fluids haven low sensitivity and also serum analysis need long periods of incubation [9, 10]. Moreover, serological tests have high false positive and false negative rates [9].Since serological tests have cross-reactions with common infections such as *Vibrio cholerae* and even *Yersinia enterocolitica* and *Francisella tularensis*, these tests are not very practical [11]. We have chosen to test polymerase chain reaction method for detection of Brucella because of the deficiencies in traditional methods [12, 13]. Multiplex PCR is a method that can identify small nucleotide differences and from small amounts of samples, therefore is very time and cost efficient [7, 14]. In this study we compare culture and multiplex PCR technique for detection of *brucella abortus* and *B. melitensis* from human blood samples.

Materials and Methods

Clinical specimens

Clinical specimens were collected from suspicious patients with clinical history of brucellosis and positive serological test (Rose Bengal test and serum agglutination test) who had measurable antibody titers 1/80. Ten ml blood was taken from each patient. Five ml for culture and 5 ml for extracting DNA. 5 ml of blood was mixed with EDTA and transferred to laboratory. It should be

mentioned that we considered 1/80 level of Wright test, which is equivalent to four positives, that is more than 200 international units of antibody, to determine if someone was patient.

Bacterial cultures: Five ml blood drawn of patients was added to deionize distilled water containing 0.5% sodium citrate. Gently mixed and centrifuged at 4000×g for 15 minutes. The supernatant was discarded and the pellet was transferred to Brucella agar plates (Germany's Merck brand). It was incubated at 37°C with 5% carbon dioxide for 7 days [15, 16].

After 7 days, colonies were analyzed by grams staining, Ziehl Neelsen staining, microscopy and colony morphology. Also hydrogen peroxidase and catalase and oxidase tests were performed. Fuchsin dye in the presence of growth was evaluated. All colonies were confirmed to be brucella spp. by multiplex polymerase chain reaction (multiplex PCR).

Isolation of DNA from clinical blood samples: We used a modification of the method described by Queipo-Ortuno [17]. Briefly, 0.5 ml of blood with 1 ml of erythrocyte lysis solution (320 mM Saccharose, 5 mM $MgCl_2$, 1% Triton X-100, 10 mM Tris HCl [pH=7.5]) was mixed and centrifuged at 15,000×g for 2 min. The supernatant was discarded, and above steps were repeated for four times until the pellet lost all reddish coloring. Four hundred micro liters of nucleic lysis buffer (10 mM Tris-Hcl, 1% SDS, 10 mM EDTA, 10 mM Sodium acetate [pH=8]) containing proteinase K (10 mg/ml) was mixed and incubated for 30 min at 55ºC in shaker incubator. Then 100 ml of ammonium acetate (7.5 M) was added and centrifugated at 15,000×g for 10 min. Two volumes of absolute ethanol were added to the supernatant, and after centrifuging at 15,000×g for 10 min; the pellets were dissolved in 25 µl of TE buffer (pH 8.0) and stored at 4ºC for PCR or at –20ºC for long-term storage.

Primers: The presence of the mobile genetic element IS711 (Gen Bank accession no.M94960) has been a useful target for molecular characterization of classical terrestrial Brucella species based on the number and distribution of IS711 copies within the bacterial genomes [14]. The following primers were selected for simultaneous detection of *B. abortus, B. melitensis* [18]:

IS711: 5'-TGCCGATCACTTAAGGGCCTTCAT-3'
B1-F: 5'-AAATCGCGTCCTTGCTGGTCTGA-3'
B2-F: 5'-GACGAACGGAATTTTTCCAATCCC-3'

PCR amplification: Each PCR reaction mixture contained 15 µl master mix 2X (Ampliqon Co, Denmark) that contained 1X PCR buffer, 1.5 mM $MgCl_2$, 1 µl template DNA (0.5 µg), 0.15 mM dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers and sterile distilled water up to 50 µl.

PCR were performed in a Gen Amp PCR system (Eppendorf, USA) according to the following program: pre-denaturation for 5-min at 94ºC followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 66°C for 45 sec and extension at 72ºC for 60 sec, followed by final extension at 72ºC for 5 min.

Then, the PCR products were analyzed using electrophoresis technique on 1.5% agarose gel for 1 hour at 85 volt and 25mA, stained by SYBER green and visualized under UV transilluminator. Finally, amplification products were further evaluated by sequencing and restriction digestion procedures.

Extracted genomes of vaccine strains of *B. abortus* B-19 and *B. melitensis* Rev-1 were used as positive control and a suspension containing all of the reagents except template as negative control. All PCRs were carried out in duplicate.

Statistical analysis: The results were analyzed as positive or negative PCR amplification reaction for each bacterium separately, as well as for two or three bacteria simultaneously. Descriptive analyses were performed and results are presented as numbers.

Statistical analysis was conducted to determine how many samples were positive for each bacterium, as well as those positive for two bacterial species. Perspective analyses were performed and data rounded numerical values (percentage) was documented.

Results

In this study, 160 suspicious patients had shown symptoms of the disease for on average 20 days. There was no significant difference between sex and rate of infection by brucellosis. Most of patients were in direct contact with livestock.

Time to positive culture in the samples that grew was 3±0.7 (mean±SD) days. 47.5% of tests were positive, among which 56.5% (43 cases) were *B. melitensis* and 44.5% (33 cases) were *B. abortus*. After 10 days, plates without any colonies were considered as negative. 108 (67.5%) were positive by multiplex PCR method (Fig. 1). The isolation rate for *B. melitensis* was 54.6% (59 cases) and 45.3% (49 cases) for *B. abortus* (Table 1).

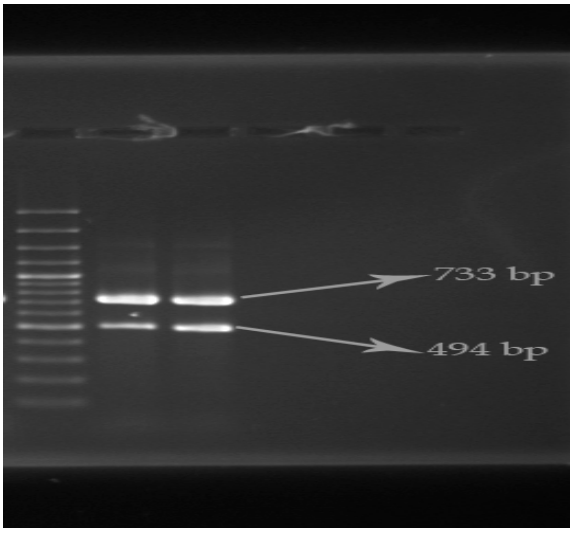

Figure 1. Agarose gel electrophoresis of PCR amplified products generated from DNA samples. Lane 1 shows DNA size marker (100bp DNA ladder). Lanes 2 and 3 show 733 bp *B. melitensis* and 494 bp *B. abortus* amplification product. Lane 4 is negative control

Table 1. Result of PCR and culture method in blood specimens of suspicious patients to brucellosis

Organism	PCR N(%)	Culture N(%)
Positives results	108(67.5)	76(47.5)
B. abortus	49(45.3)	33(44.5)
B. melitensis	59(54.6)	43(56.5)
Negative Results	52(42)	167(52.2)
Total	160(100)	160(100)

Discussion

Brucellosis is a major health problem in developing and Mediterranean countries [8]. This disease can spread directly and indirectly from infected animals to humans. Since brucellosis has no specific symptoms in humans, it is important to be diagnosed early by laboratory methods [19, 20].

Bacterial isolation and culture is always required for diagnosis and biotyping of strains. For the definitive diagnosis of brucellosis, the choosing a tissue sample for diagnosis depends on the clinical signs observed.

In the case of clinical brucellosis, valid samples include aborted fetuses (spleen, lung, and stomach), vaginal secretions, fetal membranes, milk, colostrum, sperm, blood and fluid collected from arthritis. For liquid samples (blood or milk), sensitivity is increased by the use of a specific medium like the brucella medium, originally described for use with human blood cultures. Growth may appear after 3 days, but if cultures did not grow were usually considered negative after 10 days of incubation. But the new methods for identification and sometimes typing of brucella have been developed which are in use in certain diagnostic laboratories such as PCR based methods [18]. Nevertheless, as a general rule, brucellosis multiplex PCR techniques show a lower diagnostic sensitivity than culture methods,

In this study, 160 blood samples of suspected patients with brucellosis were evaluated with two methods. Accuracy and speed of multiplex PCR method was confirmed in this study.

In a study by Yu et al. [21], molecular methods based on polymerase chain reaction was evaluated and routine use of PCR for diagnosis was recommended in the clinical laboratories. A rapid and sensitive PCR method was reported that does not require laboratory biosafety L_3. They showed multiplex PCR to be an efficient method our study also show the same performance for multiplex PCR method. In another study by Kang et al. [22], multiplex PCR was introduced as an accurate, sensitive and rapid detection for brucella was introduced. Our research also shows it to be more sensitive than culture methods.

In this study there was no significant difference between sex and infection rate by brucellosis. Cetinkaya et al., brucellosis serological methods for detection of brucellosis, found that there is relationship between age, sex and infection rate [23, 24].

According to the results, the multiplex PCR technique was better at all stages of diagnosis disease. Multiplex PCR method is more efficient, faster and more accurate than culture methods.

Acknowledgements
This study was supported by a fund of the Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, with grant number BMSU/MBRC- 90-8.

Authors' Contributions
All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest
The authors declare no conflict of interest.

Funding/Support
Molecular Biology Research Center, Baqiyatallah University of Medical Sciences.

References

1. Pappas G, Papadimitriou P, Akritidis N, et al. The new global map of human brucellosis. Lancet Infect Dis 2006; 6(2): 91-99.
2. Foster G, Osterman BS, Godfroid J, et al. Brucella ceti sp. nov. and Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol 2007; 57(Pt 11): 2688-2693.
3. Franco MP, Mulder M, Gilman RH and Smits HL. Human brucellosis. Lancet Infect Dis 2007; 7: 775-86.
4. Guerra, H. The brucellae and their success as pathogens. Crit Rev Microbiol 2007; 33(4):325-31.
5. Ilhan, Z, Aksakal A, Ekin IH, et al. Comparison of culture and PCR for the detection of Brucella melitensis in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. Lett Appl Microbiol 2006; 46(3): 301-306.
6. Ewalt DR, Bricker BJ. Validation of the abbreviated Brucella AMOS PCR as a rapid screening method for differentiation of Brucella abortus field strain isolates and the vaccine strains, 19 and RB51. J Clin Microbiol 2000; 38(8): 3085-3086.
7. Lopez-Goni I, Garcia-Yoldi D, Marin CM, et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all Brucella species, including the vaccine strains. J Clin Microbiol 2008; 46(10): 3484-3487.
8. Gwida M, Al Dahouk S, Melzer F, et al. Brucellosis regionally emerging zoonotic disease? Croat Med J 2010; 51(4): 289-295.
9. Aliskan, H. [The value of culture and serological methods in the diagnosis of human brucellosis] Turkish [Abstract]. Microbiyol Bul 2008; 42(1): 185-95.
10. Haque N, Bari MS, Hossain MA, et al. An overview of Brucellosis. Mymensingh Med J 2011; 20(4): 742-7.
11. Gall D, Nielsen K. Serological diag-nosis of bovine brucellosis: A review of test performance and cost comparison. Rev Sci Tech 2004; 23(3): 989-1002.
12. Bricker BJ, Ewalt DR, et al. Evaluation of the Brucella abortus species-specific polymerase chain reaction assay, an improved version of the Brucella AMOS polymerase chain reaction assay for cattle. J Vet Diagn Investig 2003; 15(4): 374-378.

13. Garcia-Yoldi D, Marin CM, De Miguel, et al. Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1. Clin Chem 2006; 52(4): 779-781.
14. Bricker BJ, Halling SM. Differentiation of Brucella abortus bv. 1, 2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv. 1 by PCR. J Clin Microbiol 1994; 32(11): 2660-2666.
15. Etemadi H, Raissadat A, Pickett MJ, et al. Isolation of Brucella spp. from clinical specimens. J Clin Microbiol 1984; 20(3): 586.
16. Stack JA, Harrison M, Perrett LL. Evaluation of a selective medium for Brucella isolation using natamycin. J Appl Microbiol 2002; 92: 724-728.
17. Queipo-Ortuno MI, Morata P, Ocon P, et al. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. J Clin Microbiol 1997; 35(11): 2927-2930.
18. Mirnejad R, Doust RH, Kachuei R., et al. Simultaneous detection and differentiates of Brucella abortus and Brucella melitensis by combinatorial PCR. Asian Pac J Trop Med 2012; 5(1): 24-28.
19. Moreno E, Cloeckaert A, Moriyon I. Brucella evolution and taxonomy. Vet Microbiol 2002; 90(1-4): 209-227.
20. Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: A re-emerging zoonosis. Vet Microbiol 2010; 140(3-4): 392-398.
21. Yu WL, Nielsen K. Review of detection of Brucella spp. by polymerase chain reaction. Croat Med J 2010; 51(4): 306-313.
22. Kang SI, Her M, Kim JW, et al. Advanced multiplex PCR assay for differentiation of Brucella species. Appl Environ Microbiol 2011; 77(18): 6726-6728.
23. Cetinkaya Z, Aktepe OC, Ciftci IH and Demirel R. Seroprevalence of human brucellosis in a rural area of Western Anatolia, Turkey. J Health Poul Nutr 2005; 23(2): 137-141
24. Baddour MM, Alkhalifa DH. Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood. Can J Microbiol 2008; 54(5): 352-357.

Please cite this article as: Mirnejad R, Vahdati AR, Ahmadi A, Mortazavi SM, Piranfar V. Comparison of culture and multiplex PCR technique for detection of Brucella abortus and Brucella melitensis from human blood samples. Zahedan J Res Med Sci (ZJRMS) 2013; 15(12): 5-8.