

The Efficacy of Multiplex PCR in Comparison with Agglutination and ELISA in Diagnosis of Human Brucellosis

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Article information	Abstract
<p>Article history: Received: 4 Jan 2013 Accepted: 13 Feb 2013 Available online: 5 May 2013 ZJRMS 2014; 16(4): 24-28</p> <p>Keywords: Brucella abortus Brucella melitensis ELISA Multiplex PCR</p> <p>*Corresponding author at: Young Researchers Club, Veterinary Medicine, Islamic Azad University, Shahr-e-Kord Branch, Shahr-e-Kord, Iran. E-mail: reza.vet64@gmail.com</p>	<p>Background: Human brucellosis is an endemic disease in many countries including Iran. Exact diagnosis of brucellosis is not just based on clinical symptoms, because it will be considered in differential diagnosis of other diseases. Therefore, defining organism in culture or identification of organism by serological and molecular methods for confirming clinical diagnosis is necessary. Our aim was to develop a diagnostic PCR assay and define the optimal clinical specimen for this test.</p> <p>Materials and Methods: This cross-sectional and descriptive study was from February 2011 to November 2012. Results of standard agglutination test (SAT) and specific immunoglobulin IgG and IgM by enzyme-linked immunosorbent assay (ELISA) were compared with multiplex PCR in 116 patients with suspected brucellosis referred to the Ali Ebn-e-Abitaleb hospital, Rafsanjan, Iran. Their sera were collected and tested by SAT, ELISA and multiplex PCR. DNA was extracted from serum samples and examined by multiplex PCR involving specific primers for <i>Brucella melitensis</i> and <i>Brucella abortus</i> based on IS711 in the brucella chromosome.</p> <p>Results: Brucellosis was confirmed in 116 patients (75% male and 25% female) based on applied diagnostic methods and clinical features. Results of ELISA, the SAT, and PCR were positive in 116, respectively. <i>B. abortus</i> and <i>B. melitensis</i> were detected in 101 and 15 patients.</p> <p>Conclusion: The results of present study showed that multiplex PCR assay is a rapid and sensitive technique for diagnosis of brucellosis compared to SAT. However it is more accurate when coupled with conventional methods.</p> <p>Copyright © 2014 Zahedan University of Medical Sciences. All rights reserved.</p>

Introduction

Brucellosis is a transmissible zoonotic disease. This disease is worldwide, especially in the Mediterranean basin, the Middle East, India, and central and south America. Human brucellosis is an asepticemic febrile illness or localized infection of bones, tissues, or organs. It is transmitted by the ingestion of raw or unpasteurized milk and other dairy products, by direct contact with infected animal tissues, or by accidental ingestion, inhalation, or injection of the brucella culture [1]. Since the disease needs a serious necessary infection treatment with a prolonged course of antibiotics, accuracy and short turnaround time are required for these tests. The variable symptoms, the paucity of distinctive physical signs, and the occurrence of subclinical and atypical infections in both the acute and the chronic stages make the clinical diagnosis of human brucellosis difficult. Clinicians therefore rely substantially on laboratory confirmation, even though they are confident in most of the cases that the clinical picture is highly suggestive of brucellosis [2]. Several agglutination tests (Rose Bengal, Wright's tube, Wright's card, and Wright-Coombs) and indirect immune fluorescence, complement fixation, and

enzyme-linked immune sorbent assays are also available for diagnosis of brucellosis. The standard, with which all other methods should be compared, is Wright's tube agglutination test. A 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 time lines constitute problems associated with brucellosis serology [3]. Most significantly, though, there is no standardization of antigen preparations and methodology, even for the standard Wright's tube agglutination test. 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. Since serology tests are not specific and sensitive enough, polymerase chain reaction (PCR) can be an alternative method in making the final decision in suspicious cases [4]. There have been many studies

regarding isolation of brucella spp. Such as Zeinali and Shirzadi [4], Hajia et al. [5], Queipo-Ortuno et al. [6]. Our aim was to investigate PCR assay to diagnose human brucellosis and compared it with conventional diagnostic methods.

Materials and Methods

This cross-sectional and descriptive study was done from February 2011 to November 2012 on 116 patients who were suspected of brucellosis and referred to Ali Ebn-e-Abitaleb hospital, Rafsanjan (North Kerman, Iran). The volume of sampling 116 cases were determined by $N=Z^2 \times P \times (1-P)/d^2$. Overall, 116 serum samples were collected from 64 males and 52 females. Sampling was conducted using different clusters and simple sampling. An informed consent was taken from all subjects, besides; a questioner was filled out for each case including various factors such as age, sex, job, locality, literacy etc. For serology, 5 ml venous blood with the consent and ethics were transferred to plain tubes and serum was separated from clotted blood by centrifuging at 1200 rpm for 10 min. Separated serum was collected in a screw capped sterilized plastic vial and stored at -20°C until use. For blood culture and PCR 5 ml of whole blood was aseptically transferred to screw-capped sterilized vials containing anticoagulant sodium citrate and stored at -20°C until use. Patients were for each case, a blood sample was obtained by vein puncture needle and all samples were considered for SAT and 2-mercaptoethanol (2-ME) instructions proposed by WHO reference standards in Iran [7]. 2-ME antigen and buffers were provided from Pasteur Institute of Iran. SAT with $\geq 1/80$ was considered positive. All sera were also examined for IgG and IgM by ELISA (IBL international GmbH, Germany). The PCR procedures used in this study have been previously described Bricker and Halling [8]. DNA was extracted the DNA was then extracted from serum samples by using a genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol and examined by multiplex PCR involving specific primers for *B. melitensis* and *B. abortus* based on IS711 in the brucella chromosome. The sequences of the primers were: the forward and reverse primers were:

F 5'-CATGCGCTATGTCTGGTTAC-3',
R 5'GGCTTTTCTATCACGGTATTC-3', for *B. abortus*;
And 5'- AGTGTTCGGCTCAGAATAATC-3', for *B. melitensis*.

The primers were supplied by Cinnagen company, Iran. PCR amplification of DNA using . The reaction mixture contained 20 pmol of each primer, 0.2 mM dNTPs (10 mM), 1x PCR buffer (10 x), 1.5 mM MgCl₂, 0.5 U Taq DNA polymerase, and 10 µl of template DNA. The cycling conditions were optimized at: initial denaturation at 93°C for 5 min, 40 cycles of template denaturation at 90°C for 1 min, 30 sec of primer annealing at 58°C and 60 sec of primer extension at 72°C with final extension at 72°C for 7 min. In each PCR run, positive and negative

controls were included to monitor performance of the run and absence of cross contamination. Amplification reactions were carried out using a DNA thermal cycler (Master Cycle Gradient, Eppendorf, Germany). Were used as the positive controls and DNase free water was used as the negative control. Ten microliter of amplified products were analyzed in 1.5% agarose gel containing ethidiumbromide at a final concentration of 0.5 mg/ml after electrophoresis as per the method described by Queipo-Ortuno [6]. After transferring blood samples to laboratory, samples that were considered for culture, they were kept in period of 3 weeks at 37°C inside of crystal container containing medium for culturing liquid of soya bean casein medium or tripton soya broth with microaerophilic condition. For making microaerophilic condition, candle and gas pack were used. After 3 weeks, each sample was cultured on two agar plates. One of the plates was kept in aerobic condition and another one in microaerophilic condition at 37°C, and after 48 h their results were obtained. Data were transferred to microsoft excel spreadsheet (Microsoft Corp., Redmond, WA, USA). For analysis using SPSS-17.1 statistical software (SPSS Inc., Chicago, IL, USA), chi-square test and fisher's exact two-tailed test analysis were performed and differences were considered significant at values of $p < 0.05$.

Results

Of the 116 patients included in this study 87 (75%) were male and 29 (25%) were female. Difference faced between sex and infection by brucellosis was not considerable. The mean age of the group was 37.7 ± 18.3 years (range 12-80) 86 patients (84 farmers, 2 veterinarians) had usual contact with sheep, goat or cows of the 116 patients, 97 (83.6%) were living in rural areas. Between age groups and infection to brucellosis, considerable difference was not faced. All patients had clinical signs of the disease. Of the 116 patients 12 (10.34%) had history of treatment but none of them had received antimicrobial treatment for at least 35 days during the collection of the blood samples. Of the 116 patients with brucellosis, 94 (81.3%) acquired their infections through direct contact with livestock, 18 (15.51%) acquired their infections by consuming non pasteurized dairy products, 2 (1.72%) acquired their infections possibly from either of these two sources of infection, and the remaining 2 (1.72%) acquired their infections from an unknown source. Information on medical history of the cases and PCR positivity are shown in table 1. In this research, 116 blood samples were examined in suspected cases of brucellosis. One hundred and fifteen (100%) cases were positive by PCR method (Fig. 1), 84 (72.41%) cases by culture and 103 (88.79%) cases by serological methods. The prevalence of positive cases by PCR method in suspected patients to brucellosis shown in (Fig. 2) . Distribution of samples under study based on PCR results, culturing and serology have shown in tables 2. Most of infected cases with brucellosis were (concerning on results obtained from PCR) in age group

between 30 to 39 year and the least infected cases with brucellosis were in age group of 12 to 18 year. Between age groups and infection to brucellosis, considerable difference was not faced ($p>0.05$). The sensitivity and specificity of PCR technique were compared to that of blood culture as gold standard (Table 3). Lane 2, 3, 5, 7, 8, 9, 10, 11, 12: 113bp as PCR product of *B. abortus* gene, Lane 16, 17: 252 bp as PCR product of *B. melitensis* gene, Lane 1, 18: 100 bp DNA ladder markers

Table 1. Medical history of cases at presentation

Presentation mani festation	PCR positive* N (%)
Fever	116(100)
Headache, back pain, arthralgia and myalgia	87(75)
Fatigue, weight loss	57(49.13)
Night sweating	78(67.24)
Chills	78(67.24)
Sweating	78(67.24)
Orchitis	15(12.93)

* Results expressed as the number of brucellosis-positive samples/number of samples analyzed (%).

Table 2. Comparison between PCR and other technique

Diagnostic tool	Positive PPV N(%)	Negative NPV N(%)	Sensitivity (%)	Specificity (%)
PCR	116(100)	0(0)	100	-
ELISA	107(92.24)	9(7.76)	78	28
IgG				
ELISA	105(90.51)	11(9.49)	80	34
IgM				
SAT	103(88.79)	13(11.21)	81	40
Blood culture	84(72.41)	32(27.59)	*	*

PPV: positive predictive value; NPV: negative predictive value

*:Blood culture was gold standard test.

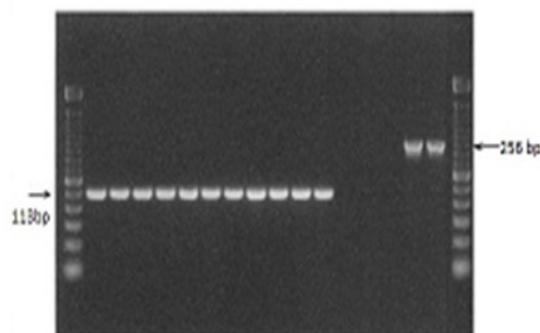


Figure 1. Electrophoresis of brucella PCR product on 1.5% agarose gel

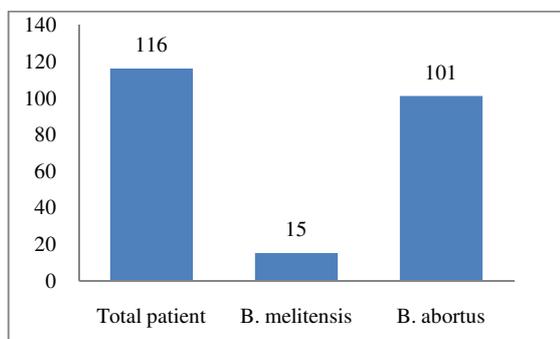


Figure 2. The prevalence of positive cases by PCR method in suspected patients to brucellosis

Discussion

Although human brucellosis is an endemic disease in many countries including Iran [1]. Cases Of brucellosis often remain unrecognized and are treated as another disease labeled fever of unknown causes. Exact diagnosis of brucellosis is not just based on clinical symptoms, because it will be considered in differential diagnosis of other diseases such as malaria, typhoid and leptospirosis. Therefore defining organism in culture or identification of organism by serological and molecular methods for confirming clinical diagnosis is necessary [9]. We used 116 blood samples because microorganisms of the brucella genus are facultative intracellular pathogens and the inoculum found in patients is normally small. Most studies of PCR assays involving human brucellosis have been undertaken with whole blood samples. However, eighty-four samples were grown on culture, 103 samples were positive by Wright method, 105 samples were Elisa IgM, 107 samples were Elisa IgG and 116 samples by PCR method.

Morshedi et al. [10] diagnosed brucellosis in Iran by ELISA and found differences in patients in acute, chronic and sub-acute phases of the disease. They also reported that 22.2% of SAT negative patients have positive results with ELISA. In this study, in cases with acute disease there was no difference between the two methods, but in sub-acute and chronic phases, IgG ELISA was positive in 90.51% while SAT was positive in 88.79% of patients [10]. Ismailzadeh et al. [11] studied 176 patients and confirmed brucellosis in 72 cases (40%) and 24 cases (13.6%) with ELISA and SAT, respectively [11]. There was no significant difference between ELISA findings in this study and ours. Gad et al. [12] studied 135 patients suspected to brucellosis in Saudi Arabia and reported 25 cases with negative SAT but positive ELISA [12].

In our study, however, there were 4 cases with negative SAT but positive ELISA. This may be due to increased use of the new kit which may have higher sensitivity. In Greece, according to the National Epidemiological Surveillance Center (Ministry of Health), more than 85% of all human brucellosis cases are diagnosed by serology only [13]. Khosravi et al. reported that none of the examined specimens tested positive for *B. abortus* [14]. However, Doosti et al. [15] collected specimens from the provinces of Isfahan and Chaharmahal Va Bakhtiari and reported that, among 76 PCR-positive cases, 41 had tested positive for *B. abortus* and 6 had tested positive for *B. melitensis*. They reported that the frequency of *B. abortus* was higher than *B. melitensis* in Chaharmahal Va Bakhtiari, compared with Isfahan. Hence, the frequency of brucella species can vary by region in Iran. Hajia et al. ELISA displayed the highest level of efficiency. Also, *B. melitensis* showed a higher frequency rate than *B. abortus* [15]. Queipo-Ortuno et al. [6] reported the sensitivity of our PCR assay was 100% [6]. Roushan et al. [16] diagnosed brucellosis in Iran by Rose Bengal method and reported that 62.5% were positive. These cases were followed by 2-ME and Wright methods.

They considered cut-off for 2-ME equivalent to 1/160 and for Wright test equivalent to 1/320 and 37.7% became positive [16]. Elfaki et al. diagnosed much positive brucellosis by agglutination tests, while there were 40% and 70% positive by culture and PCR methods. They believe that producing antibody against brucellais not related to disease condition and for following disease have to use blood culture and PCR [17]. By considering brucellosis epidemiology that was made by Hassanjani-Roushan et al. [18] in Babol city, highest risk factor was from using of dairy products (fresh cheese). In aforementioned study, job (such as veterinarian) is not introduced as risk factor. Infection level was higher in rural areas and men were infected more than women [18]. In our study considering brucellosis in different ages indicate that most infections are in ages 30 to 39 (29%) and after that (17.8%) related to ages 20 to 29 and 40 to 49 year. These age groups include active age groups. These peoples are settled in different manners in animal husbandry, dairying, working at home and have connection with livestock and products of livestock. At all there is not considerable difference in outbreak of disease in adults and children. Therefore, there is not any rational relationship between age and having brucellosis. Salariet al. considered 792 cases for brucellosis with serological method and they believe that outbreak of disease in men is more than women [19].

Karimi et al. considered brucellosis out-break in 415 healthy people including butchers and slaughterers by serology method and con-firmed contribution of job in this disease [20]. But, Hajia et al. considered brucellosis serologically and indicated that there is relationship between age, sex and positivity. In consideration of job groups, most infection is between housekeeping and animal husbandry jobs, since these jobs (in villages) have direct contact with livestock and livestock products. Women housekeepers in village are subject to have connection with livestock because of daily activities and even some times they attempt to help animals to born and

without usage of gloves they remove aborted fetus from their wombs by hand [21]. A large number of different tests have been used for the serological diagnosis of brucellosis, thus demonstrating the lack of an ideal technique. The sensitivity of these serological tests ranges from 65% to 95%, but their specificity in areas of endemicity is low, because of the high prevalence of antibodies in the healthy population. Moreover, most serological tests can produce cross-reactions with other bacteria, and they also have important limitations during the early phases of the disease, in persons whose professions involve exposure to brucella species, in patients with a recent history of brucellosis, and in patients who experience relapse.

The PCR method is more sensitive and specific than culture and serology for diagnosis of brucella from blood in suspected cases. Thus, PCR is a promising diagnostic tool for routine investigation and surveillance of brucellosis which is the key element for management of prevention and control programmers. But patient condition before testing, optimal clinical specimen, sample volume used, simple and efficient DNA extraction protocol are the points of concern for PCR to be used as a routine test in clinical laboratory practice.

Acknowledgements

Many thanks are due to Rafsanjan young researchers club and veterinary administration who accompanied us in developing this study.

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

Islamic Azad University, Shahr-e-Kord Branch.

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