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Evaluation of Real-Time PCR Method for Rapid Diagnosis of Brucellosis with Different Clinical Manifestations

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Abstract

In this study, we tested the advantages of TaqMan real time PCR technique and compare it to conventional methods using serum samples from patients with different clinical forms of brucellosis. A total of 50 patients were included in the study. Blood culture using BACTEC 9240 system, Standard Wright's tube agglutination, and real time PCR methods were used. Control blood samples from 30 people with no history of brucellosis or exposure to *Brucella* spp. were examined too. Serological assay was positive for 49 patients (98%). Forty-four (88%) of the 50 patients had a positive PCR result, whereas *Brucella* spp were isolated from blood cultures of 18 patients (36%). STA test was all positive for focal brucellosis. Real time PCR test was positive in 9 patients with focal disease (90%), whereas blood culture was positive only in 4 patients (40%). The sensitivity, specificity, positive and negative predictive values of the real time PCR method were calculated as 88%, 100%, 100%, and 83%, respectively. Our results suggest that the high sensitivity and specificity of real time PCR method make it a useful tool for diagnosis of brucellosis with different clinical manifestations.

Key words: brucellosis, diagnosis by PCR, serological diagnosis

Introduction

Brucellosis is the most common bacterial zoonosis worldwide. According to the World Health Organization, half a million of new human cases are reported each year (World Health Organization, 2001). The disease is endemic in many countries especially around Latin America, Mediterranean and Middle East countries (Pappas *et al.*, 2006, Franco *et al.*, 2007). Brucellosis is also endemic in Turkey, a Mediterranean country located between Europe and Asia. In 2006, 10 810 new cases were reported to the Ministry of Health, with the incidence rate being 16.43/100 000 (Ministry of Health of Turkey, 2006). Despite high morbidity rates in many developing countries, brucellosis often remains underdiagnosed and underreported.

The timely and accurate diagnosis of brucellosis continues to challenge clinicians because of the some limitations of conventional microbiological methods (Franco *et al.*, 2007). Blood culture is accepted as the gold standard for diagnosis. However, the sensitivity of this technique is low, ranging from 15 to 70%, and a long incubation period is necessary and represents a great risk of infection for laboratory technicians (Navarro *et al.*, 2004). The diagnostic value of antibody assays is unsatisfactory in early disease due to low sensitivity, serological cross-reactions and the inability to distinguish between active and inactive infection, due to antibody persistence after therapy (Navarro *et al.*, 2004). Most significantly, there is no standardization of antigen preparation and methodology, even for the standard Wright's tube agglutination test (STA). The diagnosis of focal forms of brucellosis is much more difficult than the diagnosis of systemic disease, as the yield of conventional cultures of non-blood samples is as low as 10–40% among all cases (Morata *et al.*, 2001). Furthermore, false negative serological results may occur in case of focal infections. PCR based assays have been proposed as a useful tool for the diagnosis of human brucellosis in recent years. They have proved to be faster and more sensitive than conventional methods (Navarro *et al.*, 2004).

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The aim of the study was to evaluate the yield of TaqMan real time PCR technique with serum samples from patients with systemic and focal clinical forms of brucellosis compared with that of conventional methods.

Experimental

Materials and Methods

Patients and blood samples. A total of 50 patients diagnosed with brucellosis over a period of 24 months in the Infectious Diseases Clinic of Ataturk Training and Research Hospital and Celal Bayar University Hospital were included in the study. All patients presented clinical signs consistent with brucellosis. The diagnosis of brucellosis was established according to either of the following criteria: (i) isolation of *Brucella* species from culture of blood (ii) and/or presence of a compatible clinical picture, together with the demonstration of specific antibodies at significant titers or seroconversion. Significant titers were considered to be STA test result of ≥1:160 or a Coombs antibrucella test titer of ≥1/320. All patients with suspected brucellosis had two or more blood cultures and a STA test. A 3.5 ml peripheral blood sample was also taken for real time PCR analysis. The samples were taken before the onset of adequate antimicrobial therapy. Control blood samples were obtained from 30 randomly selected volunteer blood donors with no history of brucellosis or exposure to *Brucella* sp.

Demographic and other relevant data such as occupation, clinical presentations, serologic and blood culture results and treatment history of the patients was recorded by the clinicians. Cases with clinical symptoms less than two months old were considered as acute cases, those that lasted more than six months before treatment was initiated were considered as chronic cases. Also different focal involvements presenting with specific clinical symptoms were noted. The study was approved by the University's ethic committee.

Bacteriological and serological techniques. Blood cultures were processed in a semiautomatic BACTEC 9240 system (Becton Dickinson Diagnostic Instrument Systems, USA). If no growth was detected within the usual five day protocol, incubation was maintained for 15 days, and blind subcultures were plated on Brucella agar (Becton Dickinson, USA) after 7 and 15 days. These subcultures were incubated at 37°C in 5–10% CO_2 atmosphere for three days. If growth appeared, the suspected colonies were identified by colony morphology, Gram staining, oxidase, catalase, urease tests and positive agglutination with specific antiserum. Serotyping of the bacteria was not performed. STA test and Coombs antibrucella test were performed according to previously described techniques (Moyer *et al.*, 1995).

DNA extraction and TaqMan Real time PCR. Serum samples were taken at the time of blood cultures and preserved at –20°C until processing. For the PCR assay, DNA extraction from 200 ml of serum was done using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the supplier's manual.

Real time PCR assay was performed using the RoboGene Brucella Detection Kit (Roboscreen, GmbH, Leipzig, Germany) and ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. RoboGene kit provides primers and TaqMan probes necessary for amplification and specific detection of the 16S rRNA target gene and is adapted for real time PCR. Two positive (high copy and low copy *Brucella* plasmid DNA) and a negative control were included the assay. Briefly 5 ml of extracted DNA, 0.5 ml of forward and reverse primers for 16s rRNA of *Brucella*, 0.5 µl of Brucella TaqMan probes were completed to 20 ml with reaction mixture. Cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 30 s, annealing and extension at 60°C for 90 s with a ramping time of 20°C/s. Fluorescence radiated from TaqMan probes was recorded during PCR procedures.

Statistical analysis. Data were analyzed with SPSS 10.0 for Windows statistical package (SPSS Inc, Chicago, IL, USA). Observed agreement and Kappa value were calculated to assess the reliability of three methods. Sensitivity, specificity, and positive and negative predictive values were calculated.

Results

Of the 50 patients included in the studies, 29 (58%) were males and 21 (42%) were females. The mean age of the group was 37.7 ± 18.3 (range; 12–80 years). Twenty-eight patients (26 farmers, 2 veterinarians) had usual contact with sheep, goats or cows. Of the 50 patients, 34 (68%) were living in rural areas. All patients had clinical signs of the disease. Of the 50 patients, 18 (36%) had a history of treatment, but none of them had received antimicrobial treatment for at least 35 days during the collection of blood samples.

Serological assays were positive in 49 patients (98%); STA and Coombs antibrucella tests were positive in 43 and 6 patients, respectively. STA test result was negative in one patient diagnosed by clinical findings only. The patient was a farmer and presented with signs and symptoms of acute brucellosis. Blood culture and PCR results for this patient were also

Table I
Clinical manifestations of the patients with brucellosis and positive test results

Clinical manifestations	No of patients (%)	Positive Test Results		
		STA No (%)*	Blood Culture No (%)*	Real time PCR No (%)*
Acute brucellosis	34 (68.0)	33 (97.1)	13 (38.2)	31 (91.2)
Chronic brucellosis	6 (12.0)	6 (100)	1 (16.7)	4 (66.7)
Focal organ involvements	10 (20.0)	10 (100)	4 (40.0)	9 (90.0)
Meningitis	4 (8.0)	4 (100)	1 (16.7)	4 (100)
Epididymoorchitis	4 (8.0)	4 (100)	2 (50.0)	3 (75.0)
Osteomyelitis	1 (2.0)	1 (100)	1 (100)	1 (100)
Spinal epidural abscess	1 (2.0)	1 (100)	0	1 (100)
Total	50 (100)	49 (98.0)	18 (36.0)	44 (88.0)

* Percentages were calculated along the rows.

negative. Forty-four (88%) of the 50 blood samples from the patients with brucellosis had a positive PCR result, whereas *Brucella* spp. were isolated from blood cultures of only 18 of these patients (36%). Bacteria were isolated within 7 days of incubation in all positive cases.

Table II
Sensitivity, specificity, positive and negative predictive values of the three methods

	STA %	Real time PCR %	Blood culture %
Sensitivity	98.0 (49/50)	88.0 (44/50)	36.0 (18/50)
Specificity	100 (30/30)	100 (30/30)	NA
PPV	100 (49/49)	100 (44/44)	NA
NPV	96.7 (30/31)	83.3 (30/36)	NA
Patient (n = 50)	+49, –1, t 50	+44, –6, t 50	+18, –32, t 50
Control (n = 30)	–30, t 30	–30, t 30	Not done
Total (n = 80)	+49, –31, t 80	+44, –36, t 80	

PPV: positive predictive value, NPV: negative predictive value, NA: not-applicable; + positive, – negative, t – total

Ten patients had focal brucellosis including meningitis (4 cases), epididymoorchitis (4 cases), osteomyelitis (1 cases), and spinal epidural abscess (1 cases). STA test was positive in all focal brucellosis cases. Real time PCR test was positive in 9 patients with focal disease (90%), whereas blood culture was positive in only 4 patients (40%). Control blood samples tested similarly for brucellosis were all negative by both STA and PCR. Clinical manifestations of the patients and positive test results are presented in Table I.

Table II shows the diagnostic yield of real time PCR method, compared with STA and blood culture method (gold standard). Sensitivity, specificity, positive and negative predictive values of the methods were calculated.

Observed agreement or reliability of the real time PCR, blood culture and STA methods are presented in Table III. Observed agreement was 90% between real time PCR and STA tests ($p = 0.006$), but the rate between real time PCR method and blood culture method, which is accepted the gold standard, was only 48%.

Discussion

Although human brucellosis is an endemic disease in many countries including Turkey, cases of brucellosis often remain unrecognized and are treated as another disease labeled fever of unknown causes (Doganay *et al.*, 2003). Blood culture still represents the gold standard of laboratory diagnosis. However,

Table III
Reliability of the three methods for diagnosis of brucellosis

	Agreement				Disagreement				Observed agreement	Kappa value	P Value
	Positive		Negative		+/–		–/+				
	No	(%)	No	(%)	No	(%)	No	(%)			
Blood culture / Real time PCR	18	(36)	6	(12)	0	(0)	26	(52)	48%	0.142	0.05
Blood culture / STA	18	(36)	1	(2)	0	(0)	31	(62)	38%	0.023	0.4
Real time PCR / STA	44	(88)	1	(2)	0	(0)	5	(10)	90%	0.260	0.006

STA – tube agglutination test

automated blood culture systems are not available in many rural areas and therefore clinicians rely on serological diagnosis. In this study, we used BACTEC 9120 automated blood culture system and microorganisms were isolated from 18 cases (36%). In studies (Ozkurt *et al.*, 2002, Ozturk *et al.*, 2002) that used automated culture systems, blood culture positivity rates were reported between 48 to 82%. The low rates of our results may be due to the fact that 40% of the cases had focal brucellosis or patients could have given incorrect information about antibiotic usage for various diseases. The sensitivity of culture method could be enhanced by using bone marrow specimens but bone marrow aspiration remains an invasive and painful technique, therefore blood samples were preferred for culture.

STA test is important when the disease can not be detected by culture. STA is widely used in Turkey for the diagnosis of brucellosis, because it is inexpensive, easy-to-perform, and rapid in comparison to culture (Mert *et al.*, 2003). In this study, STA test was positive in 49 patients (98%), and all 10 patients with focal brucellosis could be diagnosed with serology. However, serologic assays have some disadvantages. Their interpretation can be difficult, particularly during the early stage of the disease, re-infections and relapses, in endemic areas, in exposed professionals, and in patients with chronic brucellosis (Vrioni *et al.*, 2004). Therefore PCR based assays are promising alternatives for the diagnosis of brucellosis (Navarro *et al.*, 2004, Franco *et al.*, 2007, Queipo-Ortuno *et al.*, 2005). Real-time PCR assays quantitatively monitor PCR products as they accumulate during thermal cycling (Navarro *et al.*, 2004). Since this technology does not require post-amplification handling, the results are obtained much faster, in less than two hours. Also, the risk of laboratory contamination and false positive results by this method is less than conventional PCR due to closed tube system. In this study we performed TaqMan real time PCR assay with serum samples as templates. Of the 50 patients, 44 (88%) were positive by this method. All of the control samples were negative. Rates of positive results in acute brucellosis and localized infection were 91.2% and 90.0%, respectively. Only one patient with epididymoorchitis was negative with real-time PCR, and was diagnosed only with serology. Despite the satisfactory results in acute and localized disease, PCR test results were only positive in 66% of patients with chronic disease. This lower rate might be due to low organism load in the blood of the patients with chronic disease or limited number of chronic cases in this study (12%). Three patients with acute brucellosis were negative by PCR but positive by STA test, a result that might be attributed to the possibility of temporary absence of bacteremia or presence of some inhibitors in the samples. Since Brucella spp. are intracellular pathogens and the amount in specimens is usually small, most studies of PCR assays on brucellosis have been undertaken with whole blood samples to decrease false negative results (Queipo-Ortuno *et al.*, 2005). In our studies we used serum samples in the study, because some reports of increased sensitivity with serum samples in comparison to whole blood samples (Queipo-Ortuno *et al.*, 2005a; Zerva *et al.*, 2001). Serum offers several advantages for PCR assays. Inhibition by anticoagulants, hemoglobin, human DNA, or any other substances present in whole blood but not in serum is circumvented (Zerva *et al.*, 2001; Navarro *et al.*, 2002).

The sensitivity and specificity of real time PCR compared to the gold standard, *i.e.* blood culture were found to be 88% and 100%, respectively. In the literature, sensitivities and specificities of real time PCR assay in blood or serum samples varies between 66.7 to 93.3% and 94.6 to 100%, respectively (Queipo-Ortuno *et al.*, 2005a; Queipo-Ortuno *et al.*, 2005b; Debeaumont *et al.*, 2005; Kattar *et al.*, 2007). However, there is no uniformity among studies in technique such as extraction method, primers, and target sequences, storage conditions of samples or experimental setup. Therefore different results have been reported in the studies. The reliability of real time PCR method was 48% by blood culture while it was 90% by STA test. The high agreement between STA and PCR methods suggests that PCR test results can be particularly important in patients who demonstrate clinical signs and symptoms with negative serological results, allowing early and rapid confirmation of the disease.

In conclusion, although the number of cases was limited in the study, our results suggest that the high sensitivity and specificity of real time PCR make it a useful tool for diagnosis of brucellosis with different clinical manifestations. Due to its cost, it can be used primarily for patients whose blood culture and STA test are negative or inconclusive but the clinical picture is consistent with brucellosis.

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