Original Article

The Efficacy of multiplex PCR in comparison with agglutination and ELISA in diagnosis of human Brucellosis

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Abstract

Objective: Brucellosis is a zoonotic disease of which diagnosis is based on clinical symptoms and positive laboratory findings. 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.
In this study, three diagnostic methods were compared in suspected patients with brucellosis in the endemic area of Mianeh, Iran.

Patients and Methods: In this descriptive study, results of standard agglutination test (SAT) and specific immunoglobulin (Ig) G and IgM by enzyme-linked immunosorbent assay (ELISA) were compared with Multiplex PCR in 100 patients with suspected brucellosis referred to the Imam Khomeini Hospital, Mianeh, 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 B. melitensis and B. abortus based on IS 711 in the brucella chromosome.

Results: We found 28 cases with positive results for B. melitensis by Multiplex PCR technique which was significantly different from of SAT ($P<0.05$). Six samples were positive for B. abortus by PCR.

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.

Keywords: SAT, enzyme linked immunosorbent assay, B. melitensis, B. abortus, B. suis, Multiplex PCR.

Introduction

Brucellosis is a zoonotic disease which is relatively common among humans and animals. Brucellosis is an important problem in public health and a community infection with seroprevalence of about 1-2% in many parts of the world. This infection can involve young people and often economically healthy ones that may reduce their productivity (1). Since the presentations are non-specific and can be imitated by many other febrile illnesses, the diagnosis will be confirmed by bacteriological and/or serological test results (2-4). Moreover, efforts to isolate Brucella from cultures which are often unsuccessful and require long time (about four weeks for subculture) have been helpful in diagnosing for 50% only (5,6). Serum agglutination test (SAT) is considered as the most common diagnostic method for brucellosis, but sometimes, especially early in course of the disease, its misleading results can limit its application to studies with large sample size and therefore, PCR can be considered instead of culture (7,8). However, despite high specificity of PCR (up to 98% in some studies), sensitivity of this technique varies between 50 – 100%. Noticeably, however, use of serum instead of whole blood will increase sensitivity of PCR for about 50%. Some limitations of PCR include relatively high costs and technical constraints in some laboratories (9-11). The goal of this study was determination of the efficacy of multiplex PCR with agglutination and ELISA for diagnosis of human brucellosis.

Patients and Methods

This descriptive study is performed in 2010 on 100 patients who were suspected of brucellosis and referred to Imam Khomeini Hospital in Mianeh (East Azerbaijan province, Iran). Patients were chosen without regardless of age, sex and condition. For each case, a blood sample was obtained by venipuncture needle and all samples were considered for SAT and 2-Mercaptoethanol (2ME) methods. These tests were performed using laboratory

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instructions proposed by WHO reference standards in Iran (12). 2ME antigen and buffers were provided from Pasteur Institute of Iran. SAT with ≥1/80 was considered positive. All sera were also examined for IgG and IgM by ELISA (IBL International GmbH, Germany). DNA was extracted from serum samples (GeNet Bio company, Korea) and examined by Multiplex PCR involving specific primers for B. melitensis and B. abortus based on IS 711 in the brucella chromosome. The sequences of the forward and reverse primers were:

F 5'-CATGCGCTATGTCTGGTTAC -3',
R 5'GGCTTTTCTATCACGGTATTC-3', for B. abortus;
5'-AGTGTTTCGGCTCAGAATAATC-3', for B. melitensis

The PCR cycle condition was 1 cycle consisting of 2 min at 95 °C for preliminary DNA denaturation, followed by 35 cycles consisting of 2 min 95 °C for DNA denaturation, 45 s at 59 °C for DNA annealing, and 30 s at 72 °C for polymerase-mediated primer extension. This was followed with one last cycle of 72 °C for 7 min. PCR was performed in an Ependorfe Thermocycler and then 10 µl of the amplified PCR product was analyzed by agarose 1% gel electrophoresis, after which the gel was stained with 0.5 mg/ml ethidium bromide and DNA fragments were visualized by UV-transilluminator.

Working with Brucella could be very dangerous. Therefore, to prevent personnel of laboratory from being infected, strong biosafety measures should be applied, at least containment level 3.

Results

Of 100 patients suspected for brucellosis, 59% were female and 41% were male. SAT were positive in 27% of patients, IgG in 39% of patients and IgM in 23% of patients. There is no statistical difference between sexes ($p>0.05$). Age range of the patients was between 2 to 70 years and most cases with positive test results were in 10-35 age group (The comparison of ELISA and SAT results are shown in Table 2).

Table 2: Results of ELISA and SAT positive cases in 100 patients suspected of brucellosis

Ab / Test	IgM+IgG	IgG	IgM
ELISA	%16	%39	%7.2
SAT	%16	%27	0

Comparison between the results of tube Wright (SAT) and IgG ELISA **(Table 3)** shows that %14 patients had negative Wright test results while ELISA was able to detect IgG antibodies in these patients. Additionally, Wright test was positive only in three cases in whom IgG ELISA was negative. The sensitivity and specificity of the SAT test for our patients were 63% and 98.6% respectively and the positive and negative predictive values were 63% and 98.5% respectively.

Table 3: Comparison of results of Wright and IgG ELISA in 100 patients suspected of brucellosis

IgG ELISA		SAT
_	+	
%0.8	%24	+
%60	%14	_

In general, IgG and IgM (in acute infection) were detected in 16% cases by ELISA and SAT while serum IgG (in sub-acute disease) was detected in 39% of cases. Moreover, SAT was positive just in 27% of patient while ELISA was able to detect Brucella IgM in 7.2% of cases however 2ME was not able to detect the infection ($p<0.05$). For our study population, SAT, PCR, IgG and IgE were detected in 27%, 34%, 39% and 23%, respectively. Multiplex PCR technique applied to serum samples was positive for B. melitensis in 28 cases and we were able to isolate the bacteria in more cases compared with SAT ($P<0.05$). Six of examined sera samples with PCR were positive for B. abortus (Fig. 2).

Table 1: Comparison of the results of PCR amplification procedure with those of routine SAT and ELISA for the diagnosis of 100 cases of patients suspected of brucellosis.

Method	Positive(%)	Negative(%)
SAT	27	73
ELISA IgG	39	61
ELISA IgM	23	77
PCR	34	66

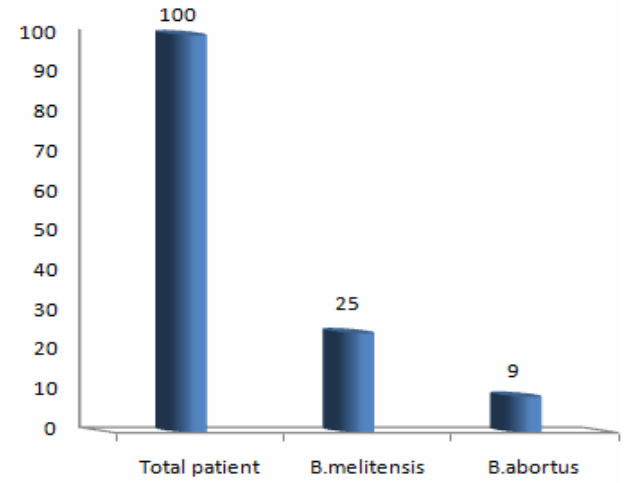

Fig- 1: The prevalence of positive cases by PCR method in suspected patients to brucellosis

Fig- 2: Electrophoresis of *Brucella PCR product on 1%* agarose gel
Lane 2,3,5,7,8: 252 bp as PCR product of *B. melitensis gene*
Lane 10, 11, 13: 113bp as PCR product of *B. abortus gene*
Lane 1,15: 100 bp DNA ladder marker

Table 4: Comparison between PCR and ELISA IgM

Diagnostic tool	Sensitivity(%)	Specificity(%)	PPV(%)	NPV(%)
PCR	64.4	77.5	48.8	86.8
ELISA IgM	69.3	90.4	68.8	88.1

PPV: positive predictive value; NPV: negative predictive value

Discussion

An accurate diagnosis of brucellosis is very important for treatment, control and eradication of this disease. We have developed rapid, species-specific PCR-based assays that can be accomplished in less than 25-30 min when killed cells or extracted DNA samples were used to supply the template DNA. The primers used in these assays are derived from IS711 of Brucella spp. and species or biovar-specific chromosomal loci. IgG and IgM were detected in 23 of our 100 patients with suspected brucellosis by ELISA and SA. IgM was detected by ELISA in 7.2% cases, while 2ME was not able to detect it ($p<0.05$).

Morshedi et al (13) 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 39.1% while SAT was positive in 14% of patients (13). Ismail-zadeh et al (14) studied 176 patients and confirmed brucellosis in 72 cases (40%) and 24 cases (13.6%) with ELISA and SAT, respectively (13). There was no significant difference between ELISA findings in this study and ours.

Isalatmanesh et al designed diagnostic value on 31 patients with suspected brucellosis and revealed sensitivity of ELISA for IgG and IgM as 100% and specificity of 63.3% and 72.7%, respectively (15). According to that study, because sensitivity of ELISA was very high and specificity was not low, ELISA can be used as a sensitive test. In our study sensitivity of SAT and ELISA (IgG and IgM) were 63% and 69.4% and their specificity were 98.7% and 95.2%, respectively. Apart from that, Gad et al (16) studied 135 patients suspected to brucellosis in Saudi Arabia and reported 25 cases with negative SAT but positive ELISA (16). In our study, however, there were 44 cases with negative SAT but positive ELISA. This may be due to increased use of the new kit which may have higher sensitivity. In addition, ELISA was preferred for diagnosis of brucellosis over SAT according to other studies as well (16 - 19). It seems that large number of studied individuals had close contact with animals such as goats and consumption of non-pasteurized dairy products. So it is recommended that ELISA test for diagnosis of human brucellosis should be done in parallel with other tests such as SAT, Coombs and Wright. Additionally, it is possible that seronegative patients suffer from some forms of brucellosis such as chronic. Therefore, thorough studies using molecular methods to assess diagnostic value of ELISA and SAT in different forms of the illness are necessary. Hence the samples were studied by Multiplex PCR for determination of species. In this study, the sensitivity and specificity of PCR were 64.4% and 77.5%, respectively. That is in contrast with a study conducted by Al-Attas et al in 2000 who showed PCR is a rapid test for diagnosis of human

brucellosis with sensitivity and specificity of 100% and 98.3% (20). Moreover, our study is also different from Al-Nakkas's study done by nested –PCR that indicated the sensitivity of 97% which helped detection of chronic infection (21). Kazemi et al studied blood samples of 104 patients with suspected brucellosis by serology, culture and PCR of which73, 15 and 84 samples were detected by PCR, culture and SAT, respectively (18). They proposed the molecular method to diagnose patients with suspected brucellosis similar to ours. On the contrary, Amirzargar and colleagues used different molecular and serological methods for diagnosis of acute human brucellosis in hospitalized patients and achieved more sensitivity. In their study PCR was positive in 48.8% of patients (22).

Conclusion

There is general agreement that ELISA is a more sensitive method than traditional techniques used in diagnosis of brucellosis. Furthermore, SAT does not discriminate between immunoglobulin classes (IgG and IgM). The detection of specific immunoglobulins by a single, simple and rapid test is a major advantage of ELISA. The main problem with widespread use of ELISA in our country has been the lack of a definite cutoff value. In summary, the accuracy of PCR and ELISA methods in aiding the diagnosis of brucellosis in 100 suspected patients were compared. ELISA is proven to be a more appropriate diagnostic method with sensitivity, specificity, positive predictive value and negative predictive value of 69.3%, 90.4%, 68.8% and 88.1%, respectively. If future studies with larger sample sizes confirm our findings, PCR will be expected to be replaced by ELISA soon for diagnosis of brucellosis in our country.

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