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Comparison of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Agglutination Assays in Diagnosis of Brucellosis in Golestan Province, North of Iran

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ABSTRACT

Introduction: Brucellosis is one of the most common zoonotic infections worldwide. The clinical symptoms of brucellosis are similar to a wide range of diseases; hence, reliable diagnostic and laboratory methods are required to identify the causative agent. Iran is an endemic region of brucellosis, and many patients are misdiagnosed due to the nature of the infection. In this study, we aimed to evaluate and compare the use of the conventional Wright test and quantitative polymerase chain reaction (qPCR) for the diagnosis of brucellosis. **Methods:** Diagnosis of brucellosis was performed using serological tests and PCR amplification of a gene encoding 31-kDa immunogenic *Brucella abortus* protein (BCSP31). Data were analyzed using the Chi-square test. **Results:** Brucellosis was diagnosed in 45 (69.23%) and 22 (38.8%) patients using the Wright test and qRT-PCR, respectively. The results of Wright and qRT-PCR assays were consistent in patients with negative results (90%). Moreover, qRT-PCR detected brucellosis in 25% of patients with Wright test titers <1/160, while 55.2% of the patients were positive with titers ≥1/160. No significant association was detected between positive PCR results and age, gender, and clinical symptoms. **Conclusion:** qRT-PCR showed a reliable diagnostic method capable of detecting the infection in suspected individuals with negative Wright results or with Wright test titers <1/160. Also, the positive qRT-PCR assays were in agreement with the Wright test titer. Regarding the financial and availability issues as well as technical problems, the agglutination test remains the preferred method in Iran.

INTRODUCTION

Brucellosis is a significant zoonotic infection and a global public health concern [1]. This disease is transmitted to humans in various ways, including the consumption of contaminated dairy products and direct contact with infected animals, placental membranes, and aborted fetuses [2]. The clinical symptoms of this disease, although non-specific, are classified into acute, subacute, and chronic. This infection may have a variety of clinical presentations, including chills, fever, sweating, malaise, myalgia, and even arthralgia, which overlap a wide range of diseases, such as tuberculosis, enteric (typhoid) fever, and viral infections. Therefore, the application of an accurate and reliable laboratory diagnostic technique is necessary to identify the causative agent [2].

Some laboratory tests are available for diagnosis of brucellosis, including isolation of *Brucella* species from different specimens (*e.g.*, blood, tissues, body fluids, and bone marrow) using culture methods, serological tests for detection of anti-*Brucella* antibodies, and molecular methods for detection of *Brucella* DNA [3]. Isolation of bacteria is the gold standard method, although it depends on many factors that make this method difficult, time-consuming, and costly [4]. Therefore, serological tests play an essential role in the diagnosis of brucellosis. However, these methods have their shortcomings, such as false-negative results in the early stage of infection, the presence of blocking antibodies, and even cross-reactions or false-positive reactions [4].

With the advent of molecular techniques, polymerase chain reaction (PCR) assays were developed for the diagnosis of brucellosis. PCR is rapid, sensitive, and specific methods for the detection of *Brucella* species in peripheral blood and other tissues [5]. Fekete et al. (1990), for the first time used this method for the diagnosis of brucellosis [6].

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This method could detect bacteria in patients with negative serological tests and post-treatment follow-ups [5, 7-9]. Various PCR-based methods are available for the diagnosis and identification of *Brucella* species [10, 11].

The identification of bacteria is possible through using several genes. In this study, we used the bcsp31 *Brucella*-specific gene as a molecular target due to the application of similar antigen in the Wright test. Iran is an endemic region for brucellosis, and patients, due to the nature of the infection, might be misdiagnosed by the conventional detection methods. In Iran, there is not much data comparing serological and PCR assays. Therefore, the present study aimed to assess and compare the accuracy of serological and real-time PCR (RT-PCR) assays.

MATERIAL AND METHODS

Clinical specimens. This cross-sectional study included 65 febrile patients suspected with brucellosis admitted to a medical clinic of Golestan University of Medical Sciences in Gorgan city from June 2016 to May 2017. The patients underwent a physical examination by an infectious disease specialist, and a questionnaire, including demographics and results of physical examination and laboratory tests, was completed for each patient. The study was performed based on the tenants of the Declaration of Helsinki. For sample collection, written consent was obtained from all patients or their guardians. The ethics committee of Golestan University of Medical Sciences approved the study (No. IR: GOUMS.REC1395.122).

Wright test. The Wright test was performed for all patients [12], even for those with negative results. The Coombs and 2-mercaptoethanol (2ME) tests were carried out accordingly [12]. The results were defined as negative for titers ≤1/160 and positive for titers >1/160.

DNA extraction and qPCR assay. Two mL of the peripheral blood sample was taken from patients and collected in tubes containing EDTA. DNA extraction from blood samples was performed by a commercial DNA blood extraction kit (Stratech, UK) according to the manufacturer's instructions. The extracted DNA samples were stored at -20°C until used. A qPCR assay was performed using specific primers and a probe designed by others [13-15] (Table 1) that amplify a 223 bp sequence of the *bcsp31* gene. qPCR reactions, 25 µl each, contained 12.5 µl PCR Master Mix (Applied Biosystems, France), 0.3 µM of forward and reverse primers, 0.2 µM TaqMan probe, 2 ng (~ 4 µl) of DNA template, and double-distilled water to the final volume. Amplifications were programmed in a thermocycler (Bioer real-time PCR, China) for an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s and annealing and extension at 60°C for 45 s. DNA of *B. abortus* IRIRBA (vaccine strain) and PCR water were included as positive and negative controls, respectively.

Statistical analysis. The data were analyzed using descriptive statistics in SPSS version 10 (IBM, SPSS, USA). The Chi-square test was used to compare the results of the PCR assay and the Wright test. The level of significance was considered to be $p< 0.05$.

Table 1. The primers and the probe used in the qRT-PCR assay

Gene	Sequence (5' to 3')	Tm Temperature (°C)	Amplicon size (bp)
BCSP31	F.P: TGGCTCGGTTGCCAATATCAA R.P: CGCGCTTGCCTTTCAGGTCTG Probe: FAM-ACGGGCGCAATCT-MGB-NFQ	64	223

RESULTS

Our study included 65 patients suspected of brucellosis. The mean age of the participants was 42.1 ± 13.9 years. Thirty-nine (60%) patients were male, and 26 (40%) female. Fourteen (21.5%) patients had a history of treatment. Only 10 (15.4%) patients had animal-related jobs, including veterinarians, farmers, butchers, and shepherds. Also, 28 (43.1%) patients reported exposure to livestock; 57 (87.7%) had consumed non-pasteurized dairy products, and 7 (10.7%) had consumed raw or medium-cooked liver. Table 2 presents a summary of the clinical symptoms of the patients suspected of brucellosis. Night sweating was the most common symptom (91.5%), followed by fatigue (64.6%). The result of the Wright test was positive in 45 (69.23%) patients, while PCR assay detected *Brucella* DNA in 22 (38.8%) individuals.

About 90% of the patients with negative Wright test results had a negative PCR result as well. PCR detected *Brucella* DNA in 25% and 55.2% of patients with Wright titers <1/160 and ≥1/160, respectively. The results of the PCR and the Wright test concerning gender, age, and history of treatment are presented in Table 3. In cases with titer ≥1/160, there seemed to be a significant agreement between both the Wright and the PCR tests (Table 3). No significant association between PCR results and other factors, such as age, gender, or clinical symptoms, was observed.

DISCUSSION

In the present study, we aimed to assess and compare the reliability of PCR and conventional Wright test in the diagnosis of *Brucella*. Our findings showed that this disease is still a public health challenge in Iran. The cryptic nature of brucellosis, the shortcomings, and the insufficiency of conventional diagnostic methods, particularly serological tests, have convinced physicians and laboratories to use molecular techniques.

In this study, we evaluated the validity of the qRT-PCR assay for the diagnosis of brucellosis along with serological tests. The PCR turned positive in 10% of patients with negative Wright test results, while the corresponding rate was 69.23% in patients with positive Wright test. There was a significant association between qRT-PCR and Wright titer. Our findings also showed an association between the positivity of PCR assay and increased titers in the Wright test. Therefore, as indicated in previous reports, the titers below

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1/160 should not be ignored without a follow-up test such as PCR or qPCR. On the other hand, titers ≥1/160 may not be indicative of an active infection, especially in *Brucellosis* endemic areas [8, 16, 17].

The results of the present work were not in agreement with those of some similar studies. Garshasebi *et al.* (2014) reported that 123 (96.1%) out of 128 Wright-positive patients were positive for brucellosis using the PCR method [18]. Moreover, in a study by Elfaki and colleagues (2005), the positivity rate of PCR reached 96% using the primers derived from a gene encoding a 31-KDa *Brucella abortus* antigen [19]. Our literature review showed that the sensitivity of PCR using *bcsp31* for the detection of *Brucella* DNA in the human blood or serum ranged from 50% to 100% [20-23]. The variations in the results might be attributed to technical issues in DNA preparation procedure and running PCR assay. Besides, the number of bacteria in the sample may not be sufficient to yield a suitable DNA amount. Also, the type of species of *Brucella* can affect the PCR results. Elfaki *et al.* (2005) and Garshasebi *et al.* (2014) showed that the different species of the *Brucella* and the number of bacteria in the patient specimen could affect the PCR results [18, 19]. The false-positive results may be related to regular exposure to these organisms in occupations, such as farmers and veterinarians, that require direct human contact with animals [24]. However, the results of the present study must be interpreted with caution due to some limitations, *e.g.*, lack of identification of the organism type. Further studies with other genetic markers might provide more information about the status of the infection in Iran.

Table 2. Clinical symptoms of 65 patients suspected of brucellosis in Golestan Province, Iran

Symptoms	Number of patients	Frequency (%)
Fatigue	42	64.6
Fever for less than two weeks	36	56.2
Night sweats	43	91.5
Low back pain	34	52.3
Weakness	31	47.7
Loss of appetite	30	46.2

Table 3. The results of qRT-PCR in brucellosis patients

Variables	PCR results (number)		*P*-value*
	Positive	Negative	
Wright test results			
Negative	2	18	0.03
≤1/80	4	12	0.03
≥1/160	16	13	0.08
History of treatment**			0.08
Yes (treated)	2 (%)	12 (%)	
No (un-treated)	20 (%)	43 (%)	

* The Chi-square test was used to compare the results of the PCR assay and the Wright test. ** The qRT-PCR assay was performed based on the patients' history of treatment and Wright test results. The frequency of negative qRT-PCR results was significantly higher than the frequency of positive results.

In conclusion, PCR showed to be a reliable diagnostic technique for the detection of infections in suspected individuals with brucellosis. However, in the present study, except for a limited number of patients with titers <1/160, the two methods showed almost similar results. Therefore, regarding the financial and availability issues as well as technical problems, the agglutination test remains the preferred method in Iran.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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