Iranian Journal of Microbiology
Volume 13 Number 3 (June 2021) 319-324

ORIGINAL ARTICLE

Serological and molecular investigation of human brucellosis in participants of Famenin brucellosis cohort study, Hamadan, Iran

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Received: January 2021, **Accepted:** March 2021

ABSTRACT

Background and Objectives: *Brucella* is an intracellular pathogen that causes brucellosis in humans and animals. This study aimed to assess the results of brucellosis seroprevalence among participants of the Famenin brucellosis cohort with molecular investigation technique and determine *Brucella*-approved species.

Materials and Methods: Following the first phase of the Famenin brucellosis cohort in 2016 which investigated the seroprevalence of brucellosis among 2367 participants in Famenin city, a total of 575 people including all seropositive and some seronegative people were examined again by wright serological tests in 2019. The PCR assay was accomplished on all cases that have wright titers ≥ 1/20 for tracing *Brucella* DNA using BCSP31 target gene and IS711 locus.

Results: Out of 575 studied cases, 145 people had wright titers ≥ 1/20. The PCR reactions of these 145 blood samples were positive in 63/145 (43.44%) tested samples using primers (B4/B5) for *Brucella* genus detection. In the second PCR assay using specific-primers for *Brucella abortus* and *Brucella melitensis*, 18/63 (28.57%) of the samples were diagnosed as *B. abortus*, and 18/63 (28.57%) were diagnosed as *B. melitensis*.

Conclusion: In this study, using the selected specific genes for the diagnosis of *Brucella* in the genus and species levels, the PCR technique was evaluated as a promising method for the rapid and safe detection of brucellosis besides the serological test for more accurate detection of brucellosis especially in cases that are not definitive.

Keywords: Polymerase chain reaction; Serological test; *Brucella abortus*; *Brucella melitensis*; Brucellosis

INTRODUCTION

Brucellosis is one of the most important zoonotic and economically important disease in productive animals worldwide that causes abortion and infertility (1). Brucellosis in Iran, like in many developing countries is endemic. Of the 12 *Brucella* species which have been known until now, only four species (*Brucella melitensis*, *Brucella abortus*, *Brucella suis*, and *Brucella canis*) are pathogenic to humans in decreasing order of severity (2). Brucellosis usually occurs when humans are exposed to infected food or product of animals infected by brucella (3). In humans, brucellosis is a systemic disease that can affect many organs and tissues (4, 5). Due to diversities of

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clinical symptoms of brucellosis, rapid and accurate diagnosis based on laboratory findings is necessary (6, 7).

Although the definitive diagnosis of brucellosis always entails the isolation of the organism from clinical specimens, serological procedures may be the only tests available in many settings (8). There are some obstacles in confirmation of the agent through a blood culture. The growth of *Brucella* is very slow and due to the high risk of transmission it poses many hazards to personnel (9). Also, the sensitivity of blood culture is significantly diminished in patients with long-term clinical eras and the chance of successful isolation of the organism decreases over time. It can be isolated only in 70% of cases with acute phase of the disease and 25% of chronic cases (10, 11).

Another laboratory technique for diagnosis is measuring the antibody titers against *Brucella* antigens (12, 13). The standard agglutination test (14), with the classic Huddleson, Wright, and/or rose Bengal, 2-mercaptoethanol (2-ME), antihuman globulin (Coombs'), and indirect enzyme-linked immunosorbent assay (ELISA) can be used to identify antibodies associated with chronic disease (8, 15). But due to the structural similarity of *Brucella* cell-wall with some other Gram-negatives, IgM class cross-reaction is common. Also despite the blocking antibodies in the IgA and IgG fractions, a false-negative serum tube agglutination test may occurr too (14). Also in serological tests of the disease, determining the difference in titers between two serum samples at least two weeks apart is very valuable, which unfortunately is not always possible. Nowadays, recent advances in molecular biology have enabled the rapid and accurate detection of *Brucella* bacteria with high sensitivity and specificity in detecting both primary infection and relapse after treatment. PCR technique facilitates the detection and tracking of these slow-growing bacteria and authorizes differentiation between species and strains (16). So far, many studies have been accomplished on the identification of the *Brucella* genus and the isolation of its various species using PCR-based assay on blood or serum samples of humans and animals (17-19).

The objective of this study was to investigate the result of seroprevalence of brucellosis in the 2nd phase of the Famenin brucellosis cohort study by PCR and to identify the organism at species level. Using PCR, all samples with titers ≥1/20 in Wright's sero-agglutination test, the accuracy, and efficiency of serological tests performed in the Famenin brucellosis cohort, particularly in seropositive cases were investigated. This is the first report of PCR-based diagnosis and identification of human pathogenic species of *Brucella* in a large population in a cohort study in Iran. Also, by determining the species of *Brucella* isolated from the participants in the brucellosis cohort, we could have more detailed information about the prevalence of *Brucella* in special areas and provide appropriate treatment and health strategies in the future.

MATERIALS AND METHODS

Subjects and data collection. Following the first phase of the Famenin brucellosis cohort in 2016, which we had investigated the seroprevalence of brucellosis among 2367 participants in Famenin City (20), in the second phase at 2019, a total of 575 participants in Famenin brucellosis cohort, including all seropositive and some seronegative people were recalled from urban and suburban parts of Famenin (a city located in Hamadan, west of Iran). We first investigated all 575 cases with Wright's sero-agglutination test. Based on clinical rules in Iran, all people who had wright titer ≥1/80 were regarded as serologically positive samples. Here in this study we more investigated all the samples with titers ≥1/80 and < 1/80 by molecular test for better interpretation.

Serological techniques. The serological diagnosis was established by Wright's tube agglutination test (*Brucella* Antigen produced by Razi Vaccine and Serum Research Institute, Iran). A titer equal to or greater than 1/80 was considered significant. Antigen mixed with physiological serum was used as the negative control. The positive serum sample was diluted (1:5) with physiologic serum and used in combination with a specific antigen as positive control (20).

DNA isolation from serum samples. Two ml of whole blood samples from all people who had wright titers ≥1/20 were used for isolation of the *Brucella* DNA. In this study, using a manual protocol, 250 µL of blood was mixed thoroughly with 1000 µL of erythrocyte lysis buffer in a 1.5-mL microfuge tube. After vortexing, it was centrifuged at 13,000 rpm for 3 minutes. Then after adding 300 µL WBC lysis buf-

fer and 30 µL SDS 10%, the microtube was vortexed briefly. Then 30 µL of protein kinase was added and the cell lysate was incubated at 56°C for 4 hrs and the suspension was chilled before adding 200 µL of NaCl (6M). Then it centrifuged at 13,000 rpm for 6 minutes to precipitate residual proteins as a tight pellet. Subsequently, the 300-400 µL of supernatant containing DNA was poured into a clean 1.5-mL microfuge tube, gently mixed with absolute cold ethanol, and finally centrifuged at 13,000 rpm for 3 minutes to precipitate purified DNA. The DNA precipitate was washed twice with ethanol 70% and dissolved in sterile distilled water, then stored at -20°C foe future tests.

***Brucella* genus-specific and species-specific DNA detection.** Three PCR assays were developed in 2 steps: one represented the diagnostic assay for the detection of *Brucella* genus, while the next two PCR, was arranged to be used for confirmation of results obtained in the first assay for the identification of *Brucella* species.

The specific primers used for the molecular detection of *Brucella* genus using PCR were *BCSP31-B4-F* 5′-TGGCTCGGTTGCCAATATCAA-3′, and *BCSP31-B5-R* 5′-CGCGCTTGCCTTTCAGGTCTG-3′ (16). This PCR assay amplifies a 223-bp sequence of the gene *bcsp31* encoding an immunogenic outer membrane protein of 31 kDa of *B. abortus*, which is conserved in all *Brucella* species.

Species-specific DNA segments of *B. abortus* and *B. melitensis* were targeted for amplification by specific primers. Forward primers were derived from insertion sequence 711 (IS711) and are unique for the identification of both *Brucella* species but the reverse primers are different, and were derived from *B. abortus* and *B. melitensis* specific locus on chromosomal DNA. The PCR assays using the *B. abortus* specific-primers (*IS711,B.a*-F 5′TGCCGATCACTTAAGGGCCTTCAT3′, and *IS711, B.a*-R 5′GACGAACGGAATTTTTCCAATCCC 3′) and The *B. melitensis* specific-primers (*IS711, B.m*-F 5′TGCCGATCACTTAAGGGCCCTCAT3′ and *IS711, B.m*-R 5′ AAATCGCGT CTTTGCTGGTCTGA 3′) produced 498-bp and 731-bp sequences in *B. abortus* and *B. melitensis* species, respectively (21).

PCR condition and detection of the amplicons. PCR was performed in a total volume of 12.5 µL mixture containing 6.25 µL Master mix RED (amplicon, Denmark), 4 µL DNA template, 0.5 µL of each primer, and 1.25 µL distilled water. The first PCR step was performed at 95°C for 3 min and followed by 35 cycles of denaturation (95°C for 90 s), annealing (64°C for 1 min), and extension (72°C for 1 min). The last step was performed at 72°C for 5 min. The amplified PCR products were analyzed using 2% agarose gels and stained with GelRed® nucleic acid stain. DNA of strains of *B. abortus* (ATCC 23455) and *B. melitensis* (ATCC 23457) were used as positive controls and physiological serum used as the negative control in all reactions.

Ethical considerations. The study was approved by the Ethical Committee of Hamadan University of Medical Sciences, Iran (IR.UMSHA.REC.1397.83), and all subjects signed written informed consent. Blood sampling had no side effects for the patients and was routine for diagnosis of the disease.

RESULTS

Serological titers. Of the 575 blood samples which collected in the second phase of the Hamadan Brucellosis Cohort Project, 145 samples had the wright serological titers (≥1/20) which were included in this study. Table 1 shows the primary results of Wright serological tests of participants in the 2nd phase of the Famenin brucellosis cohort study.

***Brucella* genus detection with BCSP31-PCR.** PCR results of the evaluation of *Brucella* genus DNA with B4 and B5 primers are demonstrated in Fig. 1. As expected, the size of amplicon for *bcsp31* 223 bp. Among the 145 cases that had Wright titers ≥ 1/20, 63 (43.44%) samples were positive by *B4* and *B5* primers, and the others were negative based on PCR results in this step.

***Brucella* species detection with *IS711* –PCR.** At the next step, among 63 samples which were positive by the first PCR reaction of *Brucella* genus, a 498 bp (Fig. 2) and 731 bp (Fig. 3) PCR-fragments were seen following the electrophoresis, corresponding well to the expected size of partial genes of *B. abortus* and *B. melitensis*, respectively. The isolation rate for *B. melitensis* was 28.57% (18/63 cases), and for *B. abortus* was 28.57% (18/63 cases). No bands were amplified in remaining samples using *B. melitensis* and *B. abortus*-specific primers.

Table 1. The results of Wright serological tests of participants in the 2nd phase of the Famenin brucellosis cohort study and molecular investigation of the all samples with titers ≥1/20 in Wright test.

		Urban No. (%)	Suburban No. (%)	Total No. (%)	Brucella genus No. (%)	*B. melitensis* No. (%)	*B. abortus* No. (%)
Wright test	Negative	173 (83.98)	257 (69.65)	430 (74.78)	Not checked	Not checked	Not checked
	1/20	18 (3.47)	67 (18.16)	85 (14.785)	36 (6.26)	6 (91.04)	9 (1.56)
	1/40	10 (4.85)	32 (8.67)	42 (7.30)	9 (1.56)	3 (0.52)	0 (0)
	1/80	4 (1.94)	11 (2.98)	15 (2.61)	15 (2.6)	9 (1.56)	6 (91.04)
	1/160	1 (0.49)	2 (0.54)	3 (0.52)	3 (0.52)	0 (0)	1 (0.17)
	1/320	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	1/640	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	1/1280	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total	206 (100)	369 (100)	575 (100)	63 (10.95)	18 (3.13)	16

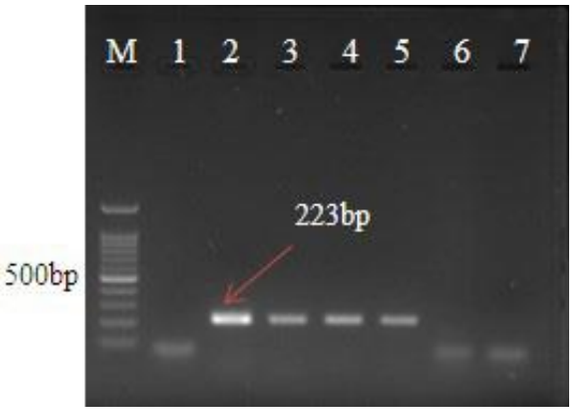

Fig. 1. The PCR assay for detection of *Brucella* genus DNA. M: 1 kb Ladder, Lane 1: Negative control (physiological serum), Lane 2: Positive control (*B. abortus* ATTCC 23455), 3-5: Samples with Wright titer ≥1/20, 6 and 7: samples with no Wright titer

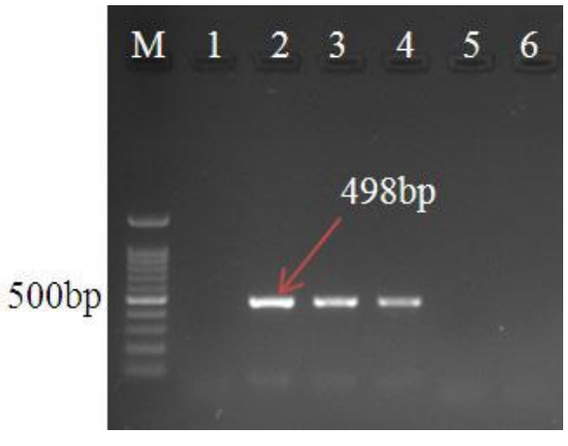

Fig. 2. The PCR assay for the detection of *B. abortus* DNA. M: 1 kb Ladder, Lane 1: Negative control (physiological serum), Lane 2: Positive control (*B. abortus* ATTCC 23455), 3 and 4: Samples with Wright titer ≥1/20, 5 and 6: samples with no Wright titer.

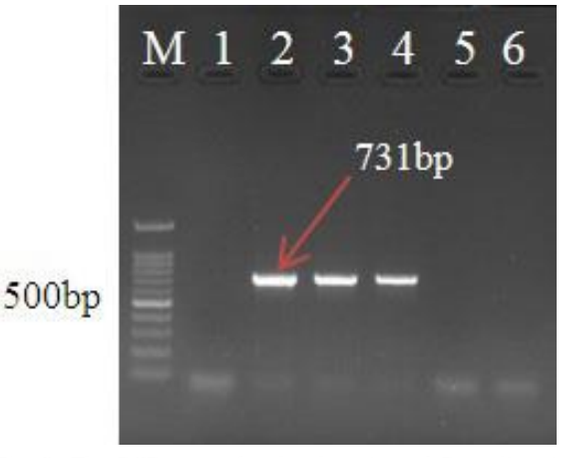

Fig. 3. The PCR assay for the detection of *B. melitensis* DNA. M: 1 kb Ladder, Lane 1: Negative control (physiological serum), Lane 2: Positive control (*B. melitensis* ATCC 23457), 3 and 4: Samples with Wright titer ≥1/20, 5 and 6: samples with no Wright titer.

DISCUSSION

Brucellosis has not yet been eradicated in some countries including Iran. Rapid and accurate diagnosis of brucellosis, plays an important role in improving the disease in humans. Brucellosis diagnostic methods based on serological tests and *Brucella* culture are time-consuming and costly (8). Molecular techniques based on the amplification of nucleic acids, such as PCR can be a suitable alternative method for rapid and more accurate diagnosis of slow-growing pathogens, like *Brucella* (22). Although PCR tests are more expensive for the diagnosis of human diseases than conventional diagnostic methods, the correct and accurate diagnosis of brucellosis and de-

creasing the hazards for laboratory staffs by molecular tests could be used as a complementary method. Previously, some researchers have offered that standard agglutination test titers of 1/160 are not emblematic of effective infection of *Brucella* in endemic regions, therefore the results of these titers should not be considered without further follow-ups (14, 23, 24). In Iran, beside the appropriate clinical presentation, all cases of human brucellosis are usually diagnosed through serological testing in clinical laboratories. Adherence to the necessary standards in performing these tests is of particular importance. Otherwise, it will lead to false-positive and false-negative results. In this study, we investigated the usefulness of PCR assay for the diagnosis of *Brucella* DNA in people who had wright serological titers ≥ 1/20. The PCR method establishes the detection of brucellosis in a short time, and moreover minimizes the risk of exposure to bacterial isolates in *Brucella* cultures (6).

Different researchers examined molecular methods for identifying *Brucella* in different samples. According to published studies, PCR-based methods are more useful and practical than conventional methods for detecting *Brucella* at the species level and even at the biovars level (18, 23, 24). Besides, the extraction of DNA from clinical specimens, authorizes isolates genetic fingerprinting in epidemiological studies of brucellosis for its prevention and treatment. Also, the PCR method does not have the disadvantages of serological diagnostic methods such as little sensitivity, and the presence of cross-reactive antibodies and inefficiency in the diagnosis of chronic disease (17-19, 22).

Here in this study, three separate PCR evaluations were performed: One PCR reaction for the *Brucella* genus-specific detection, and two PCR reactions for the species-specific identification of *B. abortus* and *B. melitensis* species.

As mentioned before, among the 145 cases that had Wright titers ≥1/20, 63 (43.44%) samples were positive by *Brucella*-genus detection with BCSP31-PCR, and the other 82 (56.66%) samples were negative based on our PCR method. Among these samples, we oserved some cases with wright positive titers that were negative in PCR assay. Our results were in agreement with Garshasbi et al. that had some false-negative results for Wright-positive samples. We also observed 3 cases with serological titer =1/80 which were negative for PCR of *Brucella* genus DNA among 18 people who had wright titers ≥1/80 (16), and assume that these serological results arouse from antibodies that produced against other bacteria that cross-react with *Brucella* antigen. Also, Wright positive sera without clinical signs are often seen in people with repeated exposure to the *Brucella* antigen. Therefore, the results of serum tests in occupational brucellosis are of limited value. On the other hands, we saw 36 and 9 positive PCR results in people who had wright titers equal to 1/20 and 1/40 respectively, which is not clinically reliable for Iranian physicians. In these cases, we assumed that may be there are some deficiencies in the serological kit that we used for identification of *Brucella* antibodies in this study or may be due the inaccurate diagnosis of our research team. Our results are in agreement with the results of researchers that mentioned the results of standard agglutination tests with low titers should not be disregarded without further follow ups (24).

By the way, these results focus attention on the noteworthiness of utilizing more than one type of detective technique for the diagnosis of human brucellosis besides the clinical signs of suspected patients, especially with epidemiological intention. In western countries which brucellosis is an occupational disease and infection with *B. melitensis* is not common, the valuable titer for treatment is 1/160. But in an area of endemicity like Iran, titers of 1/80 and above are more valuable for treatment (25). Therefore, while the use of standard laboratory methods is recommended in the diagnosis of brucellosis, it is necessary to consider clinical, and laboratory information with epidemiological data, which in this study is of our shortcomings.

Finally In this study, owing to the specificity of selected genes for the diagnosis of *Brucella* in the genus and species levels, the PCR technique was evaluated as a promising method in the rapid and safe detection of Brucellosis besides the serological tests for more accurate detection of brucellosis especially in cases that are not definitive. Although we think if we wanted to select, we choose that the speed, accuracy, specificity, and sensitivity of PCR assay in the diagnosis of brucellosis are much higher than those of serology test.

ACKNOWLEDGEMENTS

This research was supported by Hamadan University of Medical Sciences, Deputy of Research and

Technology (Grant No.970225985). We thank our colleagues from Famenin health center and the core facilities of Hamadan University of Medical Sciences for supporting and their assistance in this research. We especially thank Mrs. Maryam Fathi for her excellent assistance especially in collecting the samples from urban and sub-urbane parts of Famenin.

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