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A Novel PCR Assay for Detecting *Brucella abortus* and *Brucella melitensis*

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Objectives: Brucellosis is a major zoonotic disease that poses a significant public health threat worldwide. The classical bacteriological detection process used to identify *Brucella* spp. is difficult and time-consuming. This study aimed to develop a novel molecular assay for detecting brucellosis.

Methods: All complete sequences of chromosome 1 with 2.1-Mbp lengths were compared among all available *Brucella* sequences. A unique repeat sequence (URS) locus on chromosome 1 could differentiate *Brucella abortus* from *Brucella melitensis*. A primer set was designed to flank the unique locus. A total of 136 lymph nodes and blood samples were evaluated and classified by the URS-polymerase chain reaction (PCR) method in 2013–2014.

Results: Biochemical tests and bacteriophage typing as the golden standard indicated that all *Brucella* spp. isolates were *B. melitensis* biovar 1 and *B. abortus* biovar 3. The PCR results were the same as the bacteriological method for detecting *Brucella* spp. The sensitivity and specificity of the URS-PCR method make it suitable for detecting *B. abortus* and *B. melitensis*.

Conclusion: Quick detection of *B. abortus* and *B. melitensis* can provide the most effective strategies for control of these bacteria. The advantage of this method over other presented methods is that both *B. abortus* and *B. melitensis* are detectable in a single test tube. Furthermore, this method covered 100% of all *B. melitensis* and *B. abortus* biotypes. The development of this URS-PCR method is the first step toward the development of a novel kit for the molecular identification of *B. abortus* and *B. melitensis*.

Key Words: brucellosis, *Brucella abortus*, *Brucella melitensis*, polymerase chain reaction

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INTRODUCTION

Brucella is a genus of aerobic nonmotile gram-negative coccobacilli [1]. *Brucella melitensis* and *Brucella abortus* are causative agents of brucellosis in small ruminant animals and cattle, respectively [2]. The most significant clinical signs of brucellosis in animals are abortion, reproductive disorders, and placental retention in females and orchitis and epididymitis in males [3]. Outbreaks of bovine brucellosis generally occur with abortion in the last 3 months of pregnancy and result in weak calves and bovine infertility [4]. Four species including *Brucella canis*,

Brucella suis, *B. abortus*, and *B. melitensis* are human pathogens [5]. Human brucellosis is primarily a consequence of the contact with infected animals or consumption of unpasteurized dairy products. People in the Mediterranean, Middle East, and Latin American areas are at high risk of brucellosis [6–8]. The bacterium causes febrile septicemia or localized infection in the bone, tissues, and other organs in humans. Various reports from the endemic regions of *B. melitensis* showed an increased abortion incidence in pregnant women without any clinical signs [9].

Diagnosis of *Brucella* strains in suspected samples is usually based on culture and serology tests. Identification of *Brucella* isolates at the species and biovar levels using classical bacterial methods is the gold standard, but is time-consuming and requires long incubation times and multiple phenotypical tests [10]. Different genes are candidates for the detection of brucellosis and identification of *Brucella* species using polymerase chain reaction (PCR) [11–15].

Molecular methods for brucellosis detection are faster and more sensitive than traditional methods, but the sensitivity and specificity of PCR tests may be vary among laboratories [16].

Many previously reported molecular methods for the isolation and differentiation of *Brucella* species are invalid and not applicable because of the deposit of many new genomic sequences of *Brucella* isolates since 2009. Thus, developing new molecular methods to differentiate species is crucial.

The prevalence of brucellosis in Iran is caused by traditional husbandry of ruminant animals and poor sanitary equipment in rural areas. In humans and animals, the molecular detection of brucellosis is critical to meet epidemiological and preventive objectives. This study aimed to evaluate a novel PCR method for the identification and differentiation of *B. melitensis* and *B. abortus*. Due to some disadvantages of traditional brucellosis detection assays, development of new molecular methods that are more useful for detection, epidemiological, and surveillance studies is needed. Thus, this study focused on developing a novel PCR-based method for the identification and discrimination of two prevalent species of *Brucella*.

MATERIALS AND METHODS

1. Bacterial samples and growth conditions

The present work was a molecular experimental study. The bacterial field strains used in this study are shown in Table 1. These strains include reference strains of *Brucella* species and bacterial strains that are serologically related to *Brucella* spp. In this study, 136 blood and lymph node samples were divided into two groups. Group 1 included a total of 48 human blood samples received from the Ministry of Health and Medical Education, while Group 2 included 88 bovine blood and lymph node samples from the Iranian Veterinary Organization during 2013–2014. Ethical approval was granted by the Razi Institute Agreement Committee in 2000 (no. Razi-1388). These samples were evaluated by bacteriological and PCR methods (Table 2).

Clinical samples were received from different provinces of Iran. First, 10 mL of each human blood sample was cultured on Castaneda medium and incubated at 37°C for 21 days. Then, grown colonies were transferred to *Brucella*-specific agar and incubated at 37°C for 5–7 days. Bovine samples were cultured directly on *Brucella*-specific agar and incubated at 37°C for 21 days [17].

2. *Brucella* species phage typing

Phage typing was done according to the method recommended by the World Health Organization. To initiate the growth, 10% CO_2 was supplied; the H_2S production was evaluated with lead acetate indicator. Acriflavin and crystal violet tests were used to discriminate between the smooth and rough *Brucella* strain colonies. Standard strains contain *B. abortus* biotype 1 (strain 544) and *B. melitensis* biotype 1 (strain 16 M) were used as control cultures. A dye sensitivity assay was performed in recommended solution as follows: thionin: 1/25,000, 1/50,000, 1/100,000; and basic fuchsin: 1/50,000, 1/100,000. We used Tb phages in the routine test dilution (RTD) and RTD × 10. *Brucella* cell wall antigens (A and M) were evaluated using monospecific anti-A and anti-M sera agglutination tests [17].

Table 1. Bacterial strains used in specificity assay

Bacterial strain	Origin	No.
Brucella abortus biovar 1 (544)	ATCC 23448	1
Brucella abortus biovar 2	Field isolate	1
Brucella abortus biovar 3	Field isolate	1
Brucella abortus biovar 5	Field isolate	1
Brucella abortus biovar 9	Field isolate	1
Brucella melitensis biovar 1 (16M)	ATCC 23456	1
Brucella melitensis biovar 2	Field isolate	1
Brucella melitensis biovar 3	Field isolate	1
Brucella suis biovar 1 (1330)	ATCC 23444	1
Escherichia coli O:157	Human	1
Vibrio cholera O:1	Human	1
Yersinia enterocolitica O:9	Human	1

Table 2. Bacteriological test results

Year	Host	Sample	No	Ring test	RBT	Bacterial isolation	Phage typing
2013	Bovine	Lymph node	+30			+	*B. abortus* biotype 3
2013	Bovine	Lymph node	–8			–	
2013	Bovine	Blood	+9		+	+	*B. abortus* biotype 3
2013	Bovine	Blood	–31		–	–	
2013	Bovine	Milk	+6	+		+	*B. abortus* biotype 3
2013	Bovine	Milk	–13	–		–	
2013	Human	Blood	+27		+	+	*B. melitensis* biotype 1
2014	Bovine	Lymph node	+28			+	*B. abortus* biotype 3
2014	Bovine	Lymph node	–2			–	
2014	Bovine	Blood	+8		+	+	*B. abortus* biotype 3
2014	Bovine	Blood	–6		–	–	
2014	Bovine	Milk	+7	+		+	*B. abortus* biotype 3
2014	Bovine	Milk	–9	–		–	
2014	Human	Blood	+21		+	+	*B. melitensis* biotype 1

RBT, Rose Bengal Test; *B.*, *Brucella*.

3. DNA extraction

Bacterial cultured plates were washed with 5 mL phosphate buffered saline, and 100 μL of the bacterial suspension was centrifuged at 8,000 RPM at 4°C for 5 minutes and the supernatant was discarded. The DNA of all strains was extracted using Roche kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. The concentration of extracted DNA was determined by an ND-1000 spectrophotometer (Nano Drop, Wilmington, DE, USA).

4. Comparative genome analysis and primer design

Nucleotide sequences of chromosome 1 with 2.1 Mbp lengths were compared among all of the *Brucella* species whole genome sequences from GenBank by online software such as Basic Local Alignment Search Tool. Primers were designed by in flanking of polymorphic locus with 100% coverage for all *B. abortus* and *B. melitensis* bacteria.

5. Unique repeat sequence (URS)-PCR assay

In this study, UF1 and UR1 primers were used to detect and discriminate between *B. melitensis* and *B. abortus*. The PCR mixture used to detect *B. abortus* and *B. melitensis* included 10 pmol UF1 and UR1 primers, 50 ng DNA, 2.5 mM $MgCl_2$, 1.25 units Taq DNA polymerase, 200 μM dNTP, 2.5 μL 10X PCR buffer, and up to 25 μL distilled water. The thermal cycle program was designed with one initial denaturation at 95°C for 4 minutes followed by 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 45 seconds. The final extension step was performed at 72°C for 5 minutes. PCR products were visualized in 10% acrylamide gel electrophoresis and stained with silver nitrate.

Table 3. Frequencies of *Brucella* species isolated from bovine and human samples in 2013 and 2014 were estimated by GenALEx 6.41 software

Year	Samples	No. of *Brucella*-positive samples	Species frequencies	
			B. abortus 3	*B. melitensis* 1
2013	Bovine	45	0.978	0.022
	Human	27	0.00	1.00
2014	Bovine	43	1.00	0.00
	Human	21	0.00	1.00

B., *Brucella*.

6. Sensitivity assay

Well-purified genomic DNA of reference strains, *B. abortus* 544 and *B. melitensis* 16 M, were prepared and 10-fold serial dilutions were made as follows: 500 ng/μL, 50 ng/μL, 5 ng/μL, 0.5 ng/μL, and 0.05 ng/μL. One microliter of each dilution was used as a template in the PCR assays.

7. Specificity assay

To determine PCR specificity, a group of well-characterized *Brucella* and non-*Brucella* strains were evaluated (Table 1).

8. Statistical analysis

All statistical analyses were performed by Gen ALEX 6.41 software (Table 3).

RESULTS

1. Comparative genome analysis and URS-PCR

Only one novel locus was found in chromosome 1 at nucleotide positions 1048645–1048562 of *B. melitensis* 16M. A comparative sequence study showed two repeat sequences (TCT TTG GGG GT) in all *B. abortus* strains, while only one repeat was observed in all of the *B. melitensis* strains. This locus had the capacity to design appropriate primers to differentiate between *B. melitensis* and *B. abortus* based on the full genome sequences of *Brucella* deposited before 2014 in GenBank. This locus is a URS included 15 nucleotide variations between *B. abortus* and *B. melitensis*. In this study, a primer set comprising forward UF1 (5′-GGC TAT CGG CTG GGA AAG G-3′) and reverse UR1 (5′-CCT TCC GAA GAA AAT ACC CCT-3′) was designed to flank the polymorphic repeat sequence region. Two specific amplicons (84 bp and 99 bp long) were produced for the detection of *B. melitensis* and *B. abortus*, respectively. These primers covered all intraspecies biovars based on available sequences in nucleotide databases.

A total of 136 *Brucella* (48 human and 88 bovine) isolates were evaluated (Tables 2 and 3). The *Brucella* strains were typed by biochemical and standard phage typing methods using the Tb phage as described by Alton et al [17]. The bacteriological typing results indicated that all the human isolates were categorized into *B. melitensis* biovar 1 and *B. abortus* biovar 3 in samples received from 2014 (Tables 2 and 3). Genomic DNA of *B. melitensis* and *B. abortus* were amplified by the UF1 and UR1 primers. The amplicons were 84 bp and 99 bp long for *B. melitensis* and *B. abortus*, respectively, and visualized in 10% acrylamide gel electrophoresis using the silver staining method (Figure 1).

This PCR assay detected all *B. abortus* reference and field strains that were classified using the bacteriological method as the gold standard; thus, the sensitivity was 100% for *B. abortus* 544 and the limit of detection was 0.5 ng of genomic DNA. *B. melitensis* (reference and field strains) were detected by the URS-PCR method, whose results were in agreement with those of the conventional bacteriological method. Furthermore, the limit of detection was 0.65 ng of genomic DNA (Figure 2).

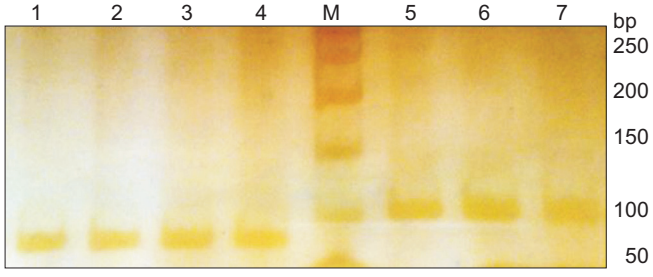

Figure 1. Differentiation of *Brucella abortus* and *Brucella melitensis* visualized in 10% acrylamide gel electrophoresis using the silver staining method. Lane M: 50-bp DNA ladder (Fermentase); lane 1: *B. abortus* 544 (99 bp); lanes 2–4: *B. abortus* biovar 3 strains; lane 5: *B. melitensis* 16M (84 bp); lanes 6–7: *B. melitensis* biovar 1 isolates.

DISCUSSION

Acute febrile illness (AFI) is an important clinical syndrome that requires supportive treatment. *Brucella*, the causative pathogens of AFI, is considered a critical issue in public health in developing countries such as Iran. Most people are threatened by brucellosis because of their traditional lifestyles [18]. The incidence of human brucellosis is directly related to the prevalence of animal brucellosis in specific regions, while the actual rate of human brucellosis is estimated to be 10–25 times higher than those reported [19-21]. Brucellosis remains endemic in most Mediterranean and Middle East countries despite all preventative procedures in recent decades. The detection of native *Brucella* species biovars in infected animals and humans is critical for establishing

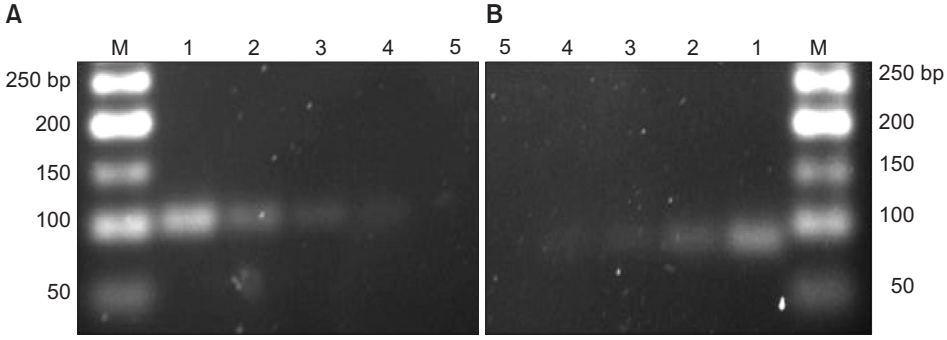

Figure 2. (A) Sensitivity of polymerase chain reaction assay for the detection of *Brucella abortus* 544 DNA. Lane M: 50-bp DNA ladder; lanes 2–5: 10-fold serial dilution of template DNA as follows: 500 ng/μL, 50 ng/μL, 5 ng/μL, 0.5 ng/μL, 0.05 ng/μL. (B) Sensitivity of the polymerase chain reaction assay for the detection of *Brucella melitensis* 16M DNA. Lane M: 50-bp DNA ladder (Fermentase); lanes 1–5: 10-fold serial dilution of template DNA as follows: 650 ng/μL, 65 ng/μL, 6.5 ng/μL, 0.065 ng/μL.

preventive factors and controlling the disease.

The first isolate of *Brucella* was identified in a bovine fetus (*B. abortus* biovar 3) in Iran in 1944 [22–24]. In an epidemiological study, 3,031 *Brucella* isolates were characterized by the standard phage typing method. All Iranian *B. abortus* isolates were grouped into seven biovars (1–6 and 9). A dominant strain was *B. abortus* biovar 3 in Iran. In Turkey, *B. abortus* biovar 3 is dominant in dairy farms as well [25].

According to epidemiological studies, species and isolates are similar in this region. However, the first *B. melitensis* isolate (biovar 1) was identified in Iran 6 years after the first *B. abortus* isolation in 1950 [26,27]. *B. melitensis* biovars 1–3 were identified in Iran, but biovar 1 was dominant in human brucellosis. Previous studies by Zowghi et al [28] and Khosravi et al [29] showed that *B. melitensis* biovar 1 was dominant in human brucellosis in Iran. The gold standard for the diagnosis of brucellosis in humans and animals is based on the isolation of *Brucella* bacteria [30]. Due to some limitations in the isolation of *Brucella* bacteria, such as the need for high biosafety level facilities, personnel skill, and risk of laboratory infection, several molecular methods to improve sensitivity and specificity, decrease cost, offer the rapid brucellosis detection, identify and differentiate *Brucella* species have been developed [31].

In previous studies, different primers were designed for the detection of all intraspecies biovars of *B. melitensis* and *B. abortus* [11–15], but they had insufficient efficacy against all intraspecies biovars based on new deposited whole genome sequences. In this study, two novel primers were designed to flank a unique locus on chromosome 1, while a single URS-PCR was developed to simultaneously identify and differentiate between *B. abortus* and *B. melitensis* at the species level. The URS-PCR results showed 100% agreement with those of the conventional phage typing method. This technique covered all biovars.

Brucellosis is worldwide zoonotic disease that causes several economic and public health problems. Control and eradication of this disease is dependent upon its rapid detection and monitoring. As such, access to a fast and accurate method of identifying the causative agent is important. Note that bacteriological methods are time-consuming and require special equipment and conditions for the detection of *Brucella* strains. Because of the high similarity among species within the *Brucella* genus, discrimination is problematic.

Based on the results of an in silico study on *Brucella* chromosomes, we found repeat sequences that can be used for *Brucella* intraspecies detection. Thus, we suggest that this novel URS-PCR method that was designed based on a URS in chromosome 1 be used for the rapid detection of *B. abortus* and *B. melitensis*. The advantage of this method over other presented methods is that both *B. abortus* and *B. melitensis* are detectable in a single test tube. Unlike methods in previous studies, this method covered 100% of all *B. melitensis* and *B. abortus* biotypes.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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