Comparison of Loop-Mediated Isothermal Amplification and conventional PCR for Diagnosis of common *Brucella* species

Ali Moeini-Zanjani
Babol University of Medical Science Faculty of Medicine
Abazar Pournajaf
Babol University of Medical Science Faculty of Medicine
Elaheh Ferdosi-Shahandashti
Babol University of Medical Science Faculty of Medicine
Mehrdad Gholami
Mazandaran University of Medical Sciences
Faramarz Masjedian
Tehran University of Medical Sciences
Soraya Khafri
Babol University of Medical Science Faculty of Medicine
Ramazan Rajabnia (ramazan69@yahoo.com)
Babol University of Medical Science Faculty of Medicine

Research note

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Abstract

Objective: Rapid, reliable, and affordable detection of *Brucella* species via the molecular methods remains a challenge. In recent years, loop-mediated isothermal amplification (LAMP) is a functional nucleic acid amplification technique offering a substitute to polymerase chain reaction (PCR). So, we compared the LAMP assay with the conventional PCR for the identification of common *Brucella* species in Iran. In this study, LAMP assay was comprehensively evaluated against the common PCR method. A group of specific LAMP primers were used to amplify a highly specific fragment from the sequence of the *Brucella abortus BCSP31* gene. Sensitivity and specificity values of tests were done with a set of 78 (50 *Brucella* and 28 non-*Brucella*) strains.

Results: A dilution series of *Brucella abortus* DNA indicated that the LAMP reaction could reliably detect 10 (fg/µl) DNA target copies per reaction within 36 minute, which is 10 times greater than the PCR assay. In summary, we conclude that LAMP assay provide accurate and fast test results to identify of common *Brucella* species in low-complexity labs, mainly in low and lower middle income countries.

Introduction

Brucella species are small, coccobacilli, gram-negative, absolute aerobic and non-moving bacteria which causes undulant fever in humans and leads to abortion and infertility in animals (1). *Brucella* can be transmitted to humans through direct contact with animals or their products that are contaminated with these bacteria (2). *Brucella* genus has six species that cannot be distinguished from each other due to the close phenotypic and antigenic interactions with conventional microbiological methods (3, 4). From these six species, [*B. abortus*, *B. melitensis*, *B. suis* and *B. canis*] generally causes human infection (5). About half a million cases of human brucellosis are reported annually which is estimated to be 10–25% less than the real number of the 1997 world health organization (WHO) report (6). The centers for disease control and prevention strategic planning group as listed *B. abortus*, *B. melitensis*, *B. suis* as category B biothreat agents (5, 7). There are currently three major methods for diagnosis of brucellosis (8). The microbiological method is performed through blood or marrow culture, however this method is considered as the gold standard for laboratory diagnosis, but it usually takes about 10 days or more (9, 10). and has a low sensitivity in the range of 15–75% depending on the bacterial species and infectious phase (11). On the other hand, this method due to class III pathogenicity of *Brucella* is very dangerous and pollutant for laboratory personnel (12, 13). Although serologic methods are easy, their results are not satisfactory; they have low sensitivity, due to the structural similarity of *Brucella* lipopolysaccharide (LPS) with other Gram-negative bacteria (3, 14). The development of diagnostic methods based on the DNA such as PCR, because of their sensitivity and specificity increases (15, 16, 17). These molecular methods also have their own dis-advantages; for example; most molecular techniques require a thermocycler machine, which is not feasible for laboratories in deprived areas such as rural laboratories (18). The LAMP system has been developed as a new diagnostic technique which could proliferate the target DNA without the need for a thermocycler. In this method with unique *Bst* DNA polymerase large fragment characteristics such as auto-claving and strand displacement, it became possible to eliminate the thermocycler and replaced it with thermal block or bain-marie, it was also possible to see the results by visual inspection because of turbidity generated in positive samples (19, 20). This technique has already been evaluated and tested to identify and detect different bacteria and virusesand its high

sensitivity and specificity have been proven (21–24). So, we compared the LAMP with the conventional PCR method for the identification of common *Brucella* species in Iran.

Methods

Bacterial strains and sample processing

In this study, to standardize the LAMP protocols, 78 bacterial stains including 50 *Brucella* and 28 non-*Brucella* strains were analyzed (details mentioned in Table 1). *Brucella* strains was cultured in a BSL2 laboratory on 5% sheep blood agar medium (Merck, Germany) and on *Brucella* agar medium with 5% sheep blood (Merck, Germany) under about 5% CO_2 in an-anaerobic jar for 36 hours at 37 ºC. Other non-*Brucella* strains were cultured in trypticase soy broth (Merck, Germany) and 5% sheep blood agar medium for 18 hours at 37 ºC.

Table 1
Bacterial strains used in this study and the results of PCR and LAMP amplification

Strains	Species (biovar)	No. of strains	Source	LAMP results	PCR results
B. abortus	1	1	S99 (Reference)	+	+
B. abortus	1	1	S19 (vaccine strain)	+	+
B. abortus	2	1	clinical isolate	+	+
B. abortus	3	18	clinical isolate	+	+
B. abortus	3	7	animal isolate	+	+
B. melitensis	1	1	16M (ATCC23456)	+	+
B. melitensis	1	13	clinical isolate	+	+
B. melitensis	1	8	animal isolate	+	+
Escherichia coli	O157:H7	4	clinical isolate	-	-
Staphylococcus aureus		4	clinical isolate	-	-
Vibrio cholerae	O1	4	clinical isolate	-	-
Klebsiella pneumoniae		4	clinical isolate	-	-
Acinetobacter baumannii		4	clinical isolate	-	-
Pseudomonas		4	clinical isolate	-	-
Shigella flexineri		4	clinical isolate	-	-

Extraction Of Bacterial DNA

For the starins that were cultured on blood agar or *Brucella* agar medium, colonies were suspended in 5 ml phosphate buffered saline (PBS) until its opacity reaches #2 McFarland standard turbidity. For the strains cultured in Trypticase Soy Broth after centrifuging the bacteria with the culture medium, the medium discarded and bacterial pellet diluted with PBS until its turbidity reaches to #2 McFarlane standard turbidity. The genomic DNAs of strains were prepared according to the manufacturer's instructions [High Pure PCR Template Preparation Kit (Roche, Germany)].

Primers

We tried to detect the same gene for each of the two techniques to minimize the variables so the *BCSP31* gene selected for the detection in both techniques. The primers sequences used for LAMP and PCR in this study are listed in Table 2.

Table 2
Sequences of primers used for LAMP and PCR assay

assay	primer	Sequence	Amplicon size (bp)	Reference
LAMP	F3	5'-GCTTTACGCAGTCAGACGT-3'	189	(25)
	B3	5'-GCTCATCCAGCGAAACGC-3'		
	FIP	5'-AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG-3'		
	BIP	5'-GGCGACGCTTTACCCGGAAATTCAGGTCTGCGACCGAT-3'		
	LF	5'-CCTTGCCATCATAAAGGCC-3'		
	LB	5'-CGTAAGGATGCAAACATCAA-3'		
PCR	B4	5'-TGGCTCGGTTGCCAATATCAA-3'	223	(15)
	B5	5'-CGCGCTTGCCTTTCAGGTCTG-3'		

Conventional PCR

This method is a modification of the method described by Baily et al (15). Amplification targeting *BCSP31* gene was performed in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany) according to the conditions mentioned in Table 3.

Table 3.

PCR condition for *bcsp31* template

Feature	Temperature (°C)	Time
Gene (*bcsp31*)		
Initial Denaturation	95	5 min
Denaturation	95	60 s
Annealing	65	30 s
Extension	72	60 s
Final Extension	72	6 min
Cycle	35	-

LAMP reaction optimization

We used six primers for LAMP assay in this study, LAMP outer primers (F3 and B3), forward inner primer (FIP) and backward inner primer (BIP), which identify four different fragments on the DNA target sequence, and two loop primers (LF and LB) to increase proliferation speed. LAMP assay targeting the *BCSP31* gene were optimized by modifying concentration of reaction components and conditions such as reaction time (20–50 min), amplification temperatures (61–67° C), concentration of dNTPs (0 to 2 mM), MgSO4 (0–6.4 mM) and Bst polymerase (2–12 Unit). The optimized reaction mixture is as follows: 5 pmol l^{-1} each of outer primers (F3 and B3), 40 pmol l^{-1} each of inner primers (FIP and BIP), 20 pmol l^{-1} each of loop primers (LF and LB), 1.4 mmol l^{-1} each deoxynucleoside triphosphates, 0.8 mol l^{-1}betain (Sigma, B0300,St. Louis, USA), 20 mmol l^{-1}Tris–HCl, 10 mmol l^{-1} (NH4)2SO4, 10 mmol l^{-1}KCl, 8 mmol l^{-1} MgSO4, 0.1 % Triton X-100, 8 units of Bst polymerase (New EnglandBiolabs, M0275S, Beverly, USA) and 2 µl of genomic DNA. before adding the Bst DNA polymerase. Each mixture in a Micro-tube was heated up to 95 ºC for 5 minutes for easing the separation of DNA strands then microtubes containing master mixes were placed on ice for 10 minutes to Provide conditions for adding Bst DNA polymerase. incubated was performed at 63 ºC for 35 min with Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany) and finally, again micro tubes heated at 95 ºC for 2 min for stopping the reaction. Five microliters of the product were subjected to 2% agarose gel electrophoresis.

Results

All the 50 strains of 4 serotypes of *Brucella* were shown to be positive by LAMP.

Sensitivity Of Lamp Reaction

After adjusting the best conditions for the LAMP reaction, the lower detection limit of the LAMP was measured using 10-fold serial dilution (initial concentration of 10 ng) of the DNA samples for *BCSP31* gene. Ladder-like pattern on 2% agarose gel was identified by target DNA of as low as 10 fg.

Naked-eye detection of positive *BCSP31* LAMP amplification

Under situations where detection and urgent care requires rapid response, it is greatly favorable for specify all positive samples as soon as possible. This is one of the advantages of the LAMP assay against PCR. To achieve this important, the products inside the 0.2 ml microtubeswere placed under white light and the turbidity caused by magnesium pyrophosphate ($Mg_2P_2O_7$) in positive samples was observed with the naked

eye. Products were also observed by adding 1 to 100 diluted fluorescent detection reagent (SYBR Green I, S9430, Sigma-Aldrich, Germany) under normal and UV light, there was no difference between the LAMP results detected by fluorescence and turbidity.

Assay Specificity Through Several Types Of Bacterial Spp

To assess the specificity of the LAMP assay, all 78 bacterial strains including four kinds of *Brucella* species (50 strains) and 7 kinds of other species (28 strains)were tested by *BCSP31*gene-based LAMP assay.The results of this assay are shown in Table 1; the test result for non-*Brucella* species was negative. Our results showed that the P-1 primer set successfully and specifically amplified *BCSP31* gene of *Brucella* species in vaccine strains, clinical isolates and animal isolates. Whereas other non-*Brucella* strains did not show any turbidity, fluorescent and any bands on the agarose gel electrophoresis under equal conditions.

Discussion

This is the primary description on the employment of LAMP to the identification of common species of *Brucella* based on the *BCSP31* gene in Iran. Early diagnosis in conjugation with effective therapy is importance for initiating adequate preventive and control measures of brucellosis. Culturing of *Brucella* and sera-conversion are time-consuming (26). The Rose Bengal test (RBT) as a easy method for the identification of specific antibodies against *Brucella*. However, their fruitfulness is restricted via elevated frequencies of *Brucella*-specific antibody titer in high- brucellosis burden countries and low sensitivity at acute phase of disease (27, 28). Hence, the growth of *Brucella spp*. is time consuming and complex, subsequently bacteriological culture and microscopic examination are cumbersome and difficult (29). Our major purpose of this work was to assess and compare the diagnostic capabilities of two different molecular detection methods, i.e. LAMP and PCR techniques and to find out whether the LAMP technique is a good alternative to PCR. We used direct culture test as the “Gold standard” For the preparation of fresh DNA and the uniformization of the terms of the techniques. The LAMP assay is advantageous in compare to PCR because of its feasible, easy construction, quick answer and visual recognition. Its simplicity and using low-cost equipment, including laboratory water bath that prepare a stable heat of 63 °C is acceptable for the test, and contrast to PCR, the result is straightly observable with the unaided eye negating the necessity for electrophoretic investigation (19). Moreover, the LAMP assay unlike other molecular techniques does not require special and expensive devices such as thermal cycler and can be performed inlow-budget labs in deprived areas, which there is a probability of an outbreak or expose to brucellosis. Characteristics evaluated for the comparison of two techniques in order to overcome one over another in the studiesIncludes the duration of the amplification, the sensitivity, specificity of the technique and the limit of detection. The PCR technique lasted approximately 90 minutes and 100 fg of *Brucella* DNA was successfully completed. In addition, non-specific responses to multiple negative controls were not observed. In the LAMP assay, 10 fg of *Brucella* DNA was favorably amplified during 35 minutes and, the primers used did not have any non-specific reaction to bacterial DNA that was used as negative controls. In comparison of these 2 assays, PCR took about 90 min, while the *Brucella* LAMP can be finished within 35 min also according to the limit of detection in two assays the sensitivity of the LAMP assay was 10 times higher to that of PCR. About the specificity of the two techniques, each of the two techniques does not have any nonspecific reaction with multiple negative controls and their specificity was almost equal. The *Brucella* LAMP technique examined in the work is a quick,

highly specific and sensitive method that can be substituted for PCR assays in the low-budget labs of Iran and are able to identify native strains of *Brucella* in Iran. This is a suitable system for peripheral laboratories to diagnosis and investigation of human brucellosis in endemic setting.

Limitations

The assay should be evaluated using blood or other clinical specimens from infected patients.

Abbreviations

LAMP; loop-mediated isothermal amplification, PCR; polymerase chain reaction, WHO; world health organization, PBS; phosphate buffered saline, LPS; lipopolysaccharide. RBT; Rose Bengal test

Declarations

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Author's contributions

Ramazan Rajabnia: Conceptualization and design of the study. **Ali Moeini-Zanjani:** Collected the data, cultured the samples, and performed the experimental tests in collaboration with **Abazar Pournajaf**, **Elaheh Ferdosi-Shahandashti:** Advisor in the study and contributed to the analysis of the data, **Mehrdad Gholami**: Contributed to the analysis of the data and wrote /revised of the manuscript in collaboration with **Abazar Pournajaf, faramarz Masjedian** Assisted in sample collection **and Soraya Khafri:** Performing the statistical analysis of data.

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Ethics approval and consent to participate

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

There is no limit to the publication.

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