Chapter 14

A Closed-tube Loop-Mediated Isothermal Amplification Assay for the Visual Endpoint Detection of *Brucella* spp. and *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

LAMP (loop-mediated isothermal amplification) is an isothermal nucleic acid amplification technique that is characterized by its efficiency, rapidity, high yield of final product, robustness, sensitivity, and specificity, with the blueprint that it can be implemented in laboratories of low technological complexity. Despite the conceptual complexity underlying the mechanistic basis for the nucleic acid amplification, the technique is simple to use and the amplification and detection can be carried out in just one step. In this chapter, we present a protocol based on LAMP for the rapid identification of isolates of *Brucella* spp. and *Mycobacterium avium* subsp. *paratuberculosis*, two major bacterial pathogens in veterinary medicine.

Key words Lamp, Isothermal amplification, Molecular detection, *Brucella*, *Mycobacterium avium* subsp. *paratuberculosis*, Brucellosis, Paratuberculosis

1 Introduction

Brucella spp. and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are pathogenic microorganisms of veterinary concern. *Brucella* spp. are the etiological agents of brucellosis, leading to abortion in cattle, sheep, pigs, and goats, while MAP is the causal agent of Johne's disease in cattle, a chronic diarrhea and wasting disease [1, 2]. The traditional methods for detecting these pathogens are largely based on phenotypic traits, and the diagnosis typically involves bacteriological culture, histopathology, and serological tests [3, 4]; however, the isolation by culture is considered the gold standard. This process is time consuming for MAP, which requires up to 2 months' growth in culture media. Nucleic acid amplification has allowed the sensitive diagnosis of different bacteria, minimizing the requirement of biosafety conditions and often replacing time-consuming techniques. In addition to contributing to the diagnosis, nucleic acid amplification provides an accurate

Kimberly A. Bishop-Lilly (ed.), *Diagnostic Bacteriology: Methods and Protocols*, Methods in Molecular Biology, vol. 1616, DOI 10.1007/978-1-4939-7037-7_14, © Springer Science+Business Media LLC 2017

molecular tool for identification at the species or subspecies level. The loop-mediated isothermal amplification (LAMP) technique is characterized by its simplicity because the entire process of amplification and detection can be performed in a single step [5, 6]. The *Bst* polymerase plays a key role in the LAMP reaction process; in addition to its polymerization activity, its displacement activity can separate the non-template strand from the template DNA under isothermal condition, and so this technology requires less specialized equipment than conventional PCR. Compared to PCR, the LAMP assay displays an equivalent sensitivity and specificity, but the reaction time shorter. Thus, LAMP is an interesting and promising option for rapid pathogen identification and diagnosis.

In previously published work, we developed two protocols based on conventional targets *bscp*31 and IS*900* to identify *Brucella* spp. and MAP, respectively. Guidelines are presented herein to help ensure the simple, rapid, and specific detection of *Brucella* spp. and MAP with the same sensitivity as conventional PCR.

2 Materials

2.1 General Considerations

All buffers and solutions should be prepared with ultrapure, molecular biology grade water. Plasticware used must be RNase/DNase-free certified. Disposable filter tips must be used for all pipetting. In order to prevent contamination with target DNA, the preparation of the reagents and the steps involving DNA manipulation must be performed in separate rooms, and at the end of the amplification reaction it is mandatory to avoid opening tubes in the area where the reaction is set up. Separate sets of pipettors and tips should be used specifically for manipulations involving DNA or DNA-free manipulations in order to preclude contaminating reagents. LAMP reaction mixtures should be prepared on ice.

2.2 Equipment

1. Biological safety cabinet.
2. Dry block heater or water bath.
3. Benchtop microcentrifuge.
4. Disposable plasticware: sterile 0.2-, 0.5-, and 1.5-mL microtubes and racks; RNase/DNase-free sterile filter tips (P10, P20, P200, P1000); sterilized inoculating loops (for 10 μL inoculation).
5. Two sets of pipettes covering the volumes 200–1000; 20–200; 2–20; 1–10 μL.

2.3 Reagents and Solutions

1. Ultrapure, molecular biology grade water.
2. Oligos Buffer: 10 mM Tris–HCl pH 8, 1 mM EDTA.
3. LAMP primers according the pathogen to test (*see* Table 1 and **Note 1**).

Table 1
List of LAMP primers

Organism (target)	Temperature	Primer name	Sequence (5′–3′)	Reference
Brucella spp. (*bcsp*31)	60 °C	F3-Bru	CAGACGTTGCCTATTGGGC	[7]
		B3-Bru	GGCTCATCCAGCGAAACG	
		FIP-Bru	CGGGTAAAGCGTCGCCAGAAGTTTT-GCACCGGCCTTTATGATGG	
		BIP-Bru	ACGATCCATATCGTTGCGCGTTTTT-GCTTGCCTTTCAGGTCTGC	
		LF-Bru	CGCAAATCTTCCACCTTGCC	
		LR-Bru	GGATGCAAACATCAAATCGGTC	
M. avium subsp. *paratuberculosis* (IS*900*)	65 °C	F3-MAP	CGCAACGCCGATACCGT	[7]
		B3-MAP	CCCAGGATGACGCCGAA	
		FIP-MAP	CATCACCTCCTTGGCCAGGC-CCGCTAACGCCCAACAC	
		BIP-MAP	GCGACACCGACGCGATGAT-TCCGGGCATGCTCAGGA	
		LF-MAP	AGTGGCCGCCAGTTGTTG	
		LR-MAP	ACCGCCACGCCGAAATC	

4. 1 M and 10 mM Tris–HCl, pH 8.
5. 25 mM NaOH.
6. SYBR Green I nucleic acid stain, 10,000× concentrate in DMSO. To prepare the working solution (1:10 in water), mix 50 μL of commercially available 10,000× SYBR Green I nucleic acid stain with 450 μL of water. Make aliquots of 50 μL and keep it at −20 °C protected from light until use (*see* **Note 2**).
7. 100 mM $MgSO_4$. Store at −20 °C.
8. 100 mM dNTPs set. Store at −20 °C.
9. 5 M betaine solution (Sigma-Aldrich) (*see* **Note 3**). Store at 4 °C.
10. *Bst* DNA polymerase, large fragment (8 U/μL) (New England Biolabs). Store at −20 °C.
11. 10× ThermoPol buffer: 100 mM $(NH4)_2SO_4$, 100 mM KCl, 200 mM Tris–HCl, 1% Triton X-100, 20 mM $MgSO_4$, pH 8.8 (New England Biolabs). Store at −20 °C.
12. Positive control DNA sample (*see* **Note 4**). Store at −20 °C.

3 Methods

The main steps to perform LAMP for the detection of MAP or *Brucella* spp. are summarized sequentially in Fig. 1.

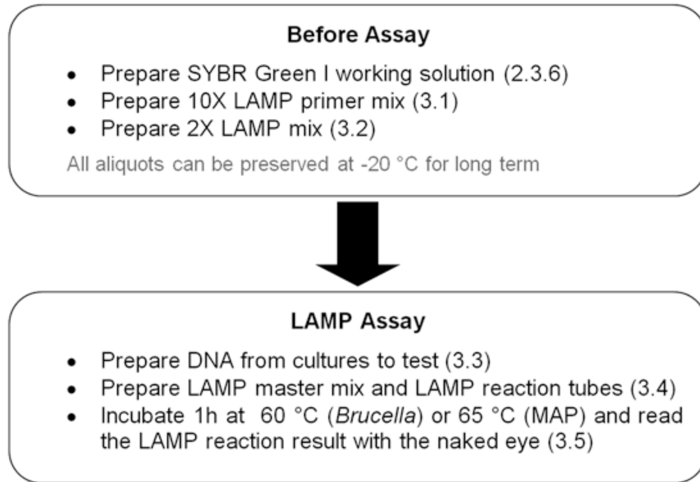

Fig. 1 Sequential steps to perform LAMP to test *Brucella* spp. or MAP from cultures

Table 2
Preparation of 10× LAMP primers mix

Reagent	Stock solution (μM)	[a]Volume (μL)	10× LAMP primers mix (μM)
Oligos buffer	–	48.80	–
Primer F3	100	1.60	1.60
Primer B3	100	1.60	1.60
Primer FIP	100	16.00	16.00
Primer BIP	100	16.00	16.00
Primer LF	100	8.00	8.00
Primer LR	100	8.00	8.00

[a]Volumes listed are for 100 μL of 10× LAMP primers mix

3.1 Preparation of 10× LAMP Primers Mix

1. Reconstitute each primer in Oligos Buffer to get 100 μM stock solutions (*see* **Note 5**).
2. Mix the tubes by inversion and rotation ten times.
3. Spin the tubes for 3 s in a benchtop microcentrifuge and put the tubes on ice.
4. Use the 100 μM primers stock and Oligos Buffer volume indicated in Table 2 to prepare 100 μL of 10× LAMP primers mix (*see* **Note 6**).
5. Mix by pipetting or tapping ten times.
6. Store the 10× LAMP primers mix tubes at −20 °C.

Table 3
Preparation of 2× LAMP mix. List of reagents, initial and final concentrations

Reagent	Stock solution	[a]Volume (μL)	2× LAMP mix
Water		248	
Betaine	5 M	320	1.6 M
dATP	100 mM	28	2.8 mM
dTTP	100 mM	28	2.8 mM
dCTP	100 mM	28	2.8 mM
dGTP	100 mM	28	2.8 mM
$MgSO_4$	100 mM	120	12 mM
ThermoPol buffer	10×	200	2×

[a]Volumes indicated are for 1 mL of 2× LAMP mix

3.2 Preparation of 2× LAMP Mix

1. Thaw all LAMP reagents (except *Bst* DNA polymerase and primers, *see* Table 3) at room temperature and keep on ice.
2. Prepare 2× LAMP mix using the reagents volume indicated in Table 3 (*see* **Note 7**).
3. Make 100 μL aliquots to minimize the number of freeze/thaw cycles.
4. Store the aliquots at −20 °C until use (*see* **Note 8**).

3.3 DNA Extraction from Culture

1. Pick one single colony to test from the corresponding selective media and resuspend in a 1.5 mL microtube containing 50 μL of 25 mM NaOH (*see* **Note 9**).
2. Inactivate the bacterial suspension in a water bath for 5 min at 98–100 °C for *Brucella* spp. or 10 min at 100 °C for MAP (*see* **Note 10**).
3. Add 4 μL of Tris–HCl buffer (1 M, pH 8.0) to neutralize the bacterial suspension.
4. Centrifuge to pellet the unbroken cells at 10,000 × *g* for 5 min at room temperature.
5. Transfer supernatant into a new 1.5 mL microtube for use as DNA template. Add 2 μL to the reaction tube for the LAMP assay (*see* **Note 11**).

3.4 LAMP Operating Procedure

Each isothermal amplification reaction is prepared to a final volume of 25 μL, including the addition of 2 μL of template (*see* **Note 12**).

1. Prepare a master reaction mixture for all the DNA samples to test, including the positive and negative (molecular biology

Table 4
Master Mix reagents volumes for a single LAMP reaction

Reagent	[a]Volume (μL)
Water	7
2× LAMP mix	12.5
10× LAMP primers mix	2.5
Bst polymerase	1
DNA (*see* **Note 15**)	2

[a]Volumes required for 25 μL LAMP reactions

grade water) controls. Set up LAMP master reaction mixture according to Table 4.

2. Add all the reagents except DNA (*see* **Note 13**).
3. Dispense 23 μL of the master mix into each reaction tube (0.2 mL microtubes).
4. Carefully add 1 μL of SYBR Green I working solution inside the cap of each tube (*see* **Note 14** and Fig. 2a).
6. Add 2 μL of each template DNA to be tested, and the same volume of a related template DNA (positive control) or molecular biology grade water (negative control) and gently close the tubes (*see* **Note 15**).

3.5 LAMP Reaction and End Point Detection

1. Incubate in a water bath or a block heater for 60 min at the temperature corresponding to the microorganism to be detected (*see* Table 1).
2. Inactivate the polymerase to stop the reaction at 80 °C for 5 min (*see* **Note 16**).
3. Rehydrate the SYBR Green I which remained in the cap of each reaction tube mixing thoroughly by inversion (*see* Fig. 2b and **Note 17**).
4. Visualize (with the naked eye) the end point of the reaction. The reaction mixture will turn green in the presence of LAMP products, while it will remain orange in their absence (*see* Fig. 2c and **Note 18**).

4 Notes

1. While PCR requires only a pair of primers to amplify target gene sequences, LAMP is quite complex since four to six primers are needed. F3 and B3 are the external primers necessary for initial steps; FIP and BIP are hybrid primers and essential

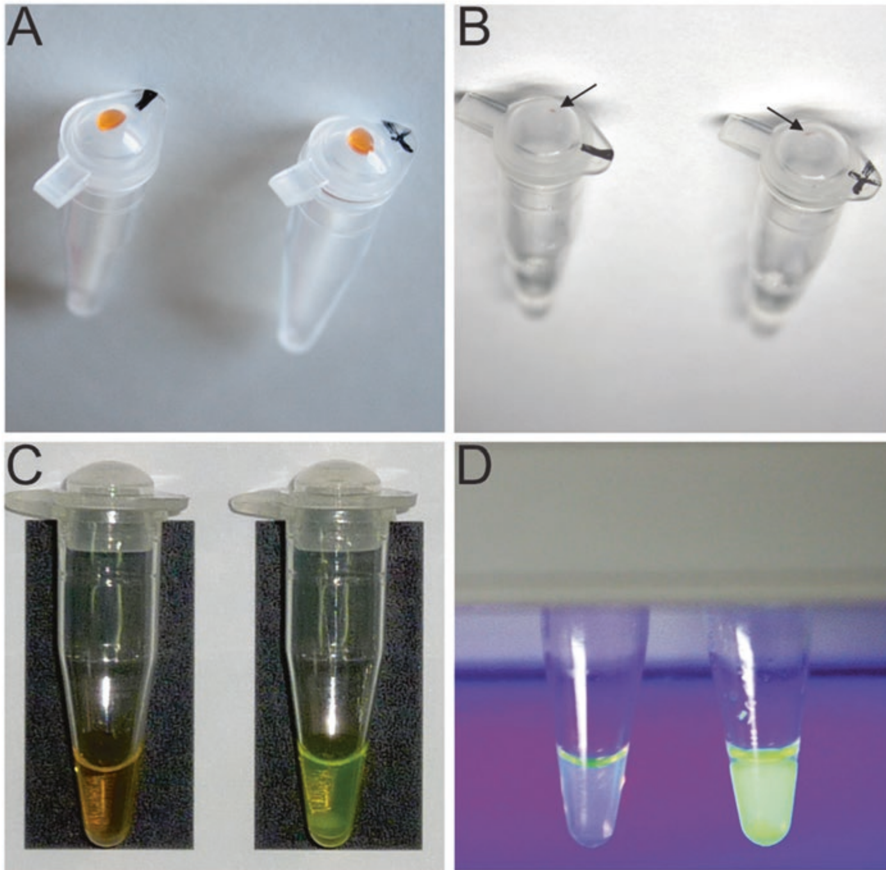

Fig. 2 SYBR Green I for visualization of LAMP reaction results. (**a**) SYBR Green I is added into the cap during the preparation of the LAMP reaction tubes. (**b**) The same tube as (**a**), post-LAMP reaction. The drop has dehydrated and is barely seen (indicated by *arrowheads*). (**c**) Visualization of tubes with the naked eye after rehydration of SYBR Green I. A positive test reaction (*green*) is clearly differentiated from a negative test (*orange*). (**d**) Fluorescence of a positive sample when a UV-transilluminator is used

in all reactions; LF and LR are optional and help in the reaction. The primer design is the bottleneck of the assay and can be carried out with bioinformatics support. In the present chapter, the Bru-LAMP and MAP-LAMP primers were designed with the publicly available software https://primerexplorer.jp/e/v4_manual/index.html.

2. SYBR Green I is commercially available as a concentrated solution in DMSO at 10,000×, used usually in real-time amplifications. In LAMP protocols, a concentration higher than that described for real-time PCR is used. However, the enzyme used for LAMP is also inhibited at high concentrations of SYBR Green I, and therefore the addition of the dye to the reaction mixture is usually performed post-amplification or must be physically separately until amplification reaction is finished,

as we propose here. Another dye that could be used is SYBR Safe. In our experience, the SYBR Safe performance is suitable for visualization with UV-transilluminator but not with the naked eye.

3. According to our results, the use of commercially available 5 M betaine solution leads to better performance than the prepared solution from the solid drug. If you choose this last option betaine chlorhydrate cannot be used as a substitute, just use betaine.
4. A reference strain or a well-characterized field strain must serve as a positive control. Process this strain as explained below in Subheading 3.3.
5. The outer and loop primers can be ordered as desalted. However, it is recommended to use HPLC-purified FIP and BIP primers. Depending on the inner primers and the target, these primers may work as desalted; however, they may not be as efficient as HPLC purified. It is strongly recommended to spin the tubes before primer reconstitution to prevent loss of lyophilized primer mass.
6. Some authors suggest keeping the stock primers at −70 °C to avoid primer degradation. In this protocol, Oligos Buffer is used rather than water; hence, you could store the primers at −20 °C. However, for long-term preservation it is recommended to store each 100 μM LAMP primer stock tube at −70 °C.
7. 2× LAMP mix is a concentrated solution containing all the components required for LAMP reaction, except primers, *Bst* polymerase, and DNA. This mix strongly reduces pipetting steps, increasing throughput and reproducibility, while reducing the risk of contamination, mainly for routine testing.
8. Aliquots of 2× LAMP mix retain functional properties over 2 years preserved at −20 °C.
9. For confluent growth take a loopful. Broth cultures can also be used as a starting point for DNA extraction. For *Brucella* use tryptose agar plates or tryptic soy broth (Difco, BD, USA) and for MAP use conventional fecal culture media (Herrold's egg yolk agar containing mycobactin) or 7H9 liquid medium (Difco, BD, USA) supplemented with 0.2% mycobactin J (Allied Monitor, Fayette, MO, USA).
10. Up to this step you need a biosafety cabinet. Once the sample is inactivated, you could work on a conventional laboratory bench top. If liquid culture medium is used, wash the cells with water as follows. Centrifuge a volume of culture (100–1000 μL depending on growth phase) at 10,000 × *g* for 2 min and remove the supernatant. Then, add 100–200 μL of water

and vortex briefly for washing. Repeat the step twice and resuspend the pellet in 100 μL of ultrapure water or 10 mM Tris–HCl pH 8.

11. The cellular lysate can be repeatedly freeze/thawed.
12. The final reaction volume can be reduced, thus reducing the cost of the reaction. According to our experience, satisfactory results were obtained by using a final volume of 10 μL (with up to 3 μL of template DNA).
13. Similar to PCR, this step, and the following steps, must be performed on ice to avoid nonspecific amplifications.
14. This step must be performed carefully to avoid contact with the master mix. As mentioned above, high concentration of SYBR Green I has an inhibitory effect on *Bst* polymerase.
15. Consider the total number of samples to test, including the positive and negative controls, to prepare the volume of master mix needed. Dispense the volume of master mix, without DNA, in each reaction tube to further add the template DNA. Perform this last step in a separated room with a different pipette to avoid contamination of reagents with DNA.
16. The inactivation step is important to avoid false-positive results. However, this step can be omitted if the visual assessment (Subheading 3.5, **step 3**) is performed quickly after the amplification due to the SYBR Green I inhibitory effect.
17. This step does not require opening the tube, and strongly reduces cross-contamination.
18. The positive and negative controls must be visualized green and orange respectively. A UV-transilluminator or even a domestic use currency reader may be used to increase the fluorescence intensity (*see* Fig. 2d).

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