

Full Length Research Paper

Evaluation of loop mediated isothermal amplification for diagnosis of *Mycobacterium tuberculosis* complex in clinical samples

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Tuberculosis (TB) remains an important global public health problem. The lack of rapid and accurate diagnostic testing is an important impediment to global tuberculosis control. Loop mediated isothermal amplification (LAMP) is a rapid method for nucleic acid amplification. In this study, we assessed the performance of an in-house LAMP assay for the detection of tuberculosis. Six oligonucleotide primers specific for *Mycobacterium tuberculosis* complex were designed corresponding to *IS6110* gene sequence. Optimization of LAMP reaction was performed. A total of 133 clinical sputum samples and 80 bacterial cultures were studied by LAMP method. Sensitivity of this assay for detection of genomic DNA was 5 fg. This assay successfully detected *M. tuberculosis* complex not only in the bacterial cultures but also in the clinical sputum samples from patients with TB. The sensitivity of LAMP in culture-positive samples was 100% (60/60) and the specificity in culture-negative samples was 95.9% (70/73, 95% confidence interval 91.3 to 98.7%). Thus, LAMP is a rapid, highly sensitive and specific DNA amplification technique for early diagnosis of TB.

Key words: Loop mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), *IS6110* gene, *Mycobacterium tuberculosis*, diagnosis.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex. While both preventable and curable, TB remains a major public health problem. Every year, about 9 million people develop TB and over 1.7 million die as a consequence (WHO, 2008). Early diagnosis of *M. tuberculosis* (MTB) in clinical samples becomes important in the control of tuberculosis both for the treatment of patients and for the curbing of the disease transmission to others in the community (Brodie and Schluger, 2005).

Laboratory methods play a crucial role in establishing

the TB diagnosis. The conventional methods that were discovered have limitation of speed, specificity and sensitivity. Bacteriological testing for TB in the majority of laboratories is restricted to microscopic examination of the acid-fast stain sputum smear (Greenaway et al., 2002). Sputum smear microscopy is the least expensive, simple and relatively easy to perform but has both the problems of sensitivity and specificity (Tiwari et al., 2007). Culture is more sensitive and is presently the yardstick for diagnosis, but the time required and frequent negative results in paucibacillary specimens are important limitation (Katoch, 2003; Haldar et al., 2007). In the last few years, many investigators have described various molecular methods of amplification, especially by polymerase chain reaction (PCR), and promising results have been obtained (Cheng et al., 2005). Nevertheless, although simplified

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Table 1. Sequences of LAMP primers.

Primer	Sequence (5'→3')	Length
F3	CCTAACCGGCTGTGGGTA	18
B3	CGAGTACGCCTTCTTGTTGG	20
FIP (F1c + F2)	GACGTAGGCGTCGGTGACAAAGGCAGACCTCACCTATGTGTC	42
BIP (B1c + B2)	GTCGCTTCCACGATGGCCACGGTCCAGATGGCTTGCTC	38
FLP	TAGGCGAACCCCTGCCCA	17
BLP	TGGTCTCGACGCGATC	17

procedures were investigated, all of these methods remained too complex, long and not reliable enough for use in routine clinical practice (Katoch, 2003).

Recently, loop mediated isothermal amplification (LAMP) assay has been developed as a novel technique for nucleic acid amplification (Notomi et al., 2000). LAMP can amplify DNA with high specificity and efficiency under isothermal conditions using six sets of primers that recognize eight distinct regions on the target sequence. Unlike PCR, LAMP reaction does not require a denatured DNA template and relies on auto cycling strand displacement DNA synthesis by a *Bst* DNA polymerase (Nagamine et al., 2001). The large amount of DNA generated in less than an hour and positive LAMP reaction can be visualized with the naked eye, without need for gel electrophoresis (Mori et al., 2001).

Here, we reported the development of a MTB-specific LAMP assay based on the amplification of the Insertion Sequence 6110 (IS 6110), which has been applied successfully to clinical specimens for diagnosis of *M. tuberculosis* complex. Furthermore, we evaluated the sensitivity and specificity of *M. tuberculosis* complex detection by LAMP, comparing it with PCR in clinical specimens.

MATERIALS AND METHODS

A total of 213 samples were collected which included 133 clinical sputum specimens from patients suspected to have pulmonary TB referred to TB Control and Research Department, Enqelab Health Center, Shiraz, Iran and 80 specimens isolated from culture of *M. tuberculosis*. Informed consent was obtained from each patient before the study.

Acid-fast microscopy and culture

Sputum specimens were digested and decontaminated by the Petroff's method (Bartelt, 2000). Smears were prepared, stained with Ziehl-Neelsen stain and examined for acid-fast bacilli (AFB) by microscopy. All clinical specimens were inoculated onto Lowenstein-Jensen (LJ) media and incubated at 37°C for 6 to 8 weeks.

DNA extraction

The raw sputum samples were digested and decontaminated with 2-volume 4% NaOH treatment, then neutralized with phosphate buffer and subsequently concentrated by centrifugation at 6000 rpm

for 1 min. DNA was extracted from homogenized sputum using a DNA extraction kit (DNG kit, CinnaGen, Iran) in the following process: Tubes containing 100 µl of homogenized sputum samples were placed at 95°C in water bath for 20 min. Then, 400 µl lysis buffer was added to each tube and subsequently, 300 µl precipitate buffer (isopropanol) was also added. The samples were centrifuged at 12000 rpm for 10 min. After decanting, 1 ml of 70% ethanol was used for washing which was then centrifuged at 12000 rpm for 5 min. Finally, ethanol was completely poured off and dried, DNA pellet was dissolved in 30 µl distilled water. Also, *M. tuberculosis* DNA was extracted from fresh culture by boiling method (McFadden, 1990).

Primer design for LAMP

The gene sequence of IS6110 (accession no. X17348) were obtained from GenBank. A set of six primers comprising two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FLP and BLP) recognizing eight distinct regions on the target sequence were designed using online Primer Explorer V4 software (Eiken Genome; <http://primerexplorer.jp/>). The FIP primer contains F2 and complementary sequence of F1 (F1c). The BIP primer contained B2 and complementary sequence of B1 (B1c). The sequences of these primers are shown in Table 1.

LAMP assay

LAMP was carried out in a total volume of 25 µl, containing 0.2 µM each of F3 and B3, 1.6 µM each of FIP and BIP, 0.8 µM each of FLP and BLP, 20 mM Tris-HCl (pH: 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 9 mM MgSO₄, 1.4 mM dNTP, 0.8 M Betaine (Sigma-Aldrich), 8 U *Bst* DNA polymerase (New England Biolabs, USA) and 5 µl DNA sample. To find the optimum time and temperature for LAMP assay, the reactions were carried out at 60 to 68°C for 35 to 90 min. A positive and negative control was included in each run. LAMP testing was blinded as compared to smear microscopy, culture and PCR.

Detection of LAMP products

LAMP amplicons were directly detected with the naked eye by adding 0.1% SYBR Green I (Invitrogen lot: 49743A, USA) to the tube and observing the color of the solution under UV light. The solution turned green in the presence of a LAMP amplicon, while it remained orange with no amplification. To confirm the structure of the LAMP products, the amplicons were analyzed by gel electrophoresis in 2% agarose gel.

PCR amplification and detection

To compare the results obtained by LAMP assay, PCR was also

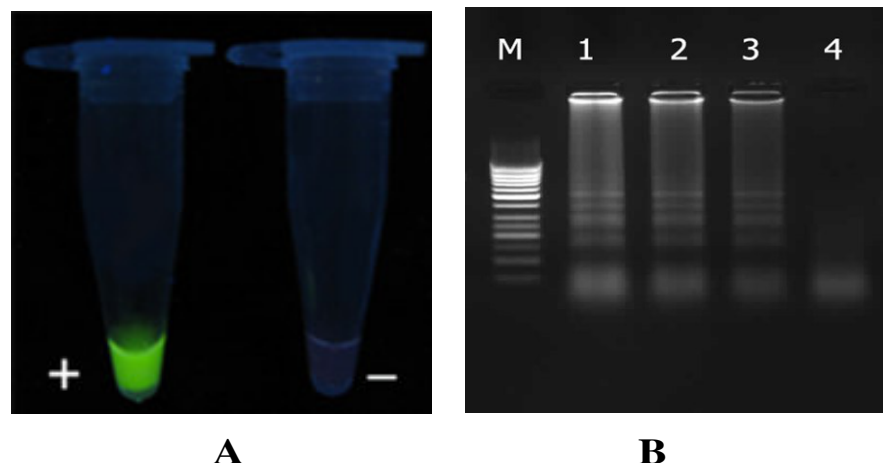


Figure 1. Monitoring of LAMP amplification. (A) Visual detection of LAMP reaction under UV light. (B) Electrophoretic analysis of LAMP reaction. Lane M: 50 bp DNA Ladder Plus, Lane 1: LAMP product of positive control, Lanes 2 and 3: positive samples, Lane 4: negative control.

performed to amplify 245 bp sequence unique to *M. tuberculosis* complex insertion sequence (IS6110) as described by Rohani et al. (2009) with slight modifications. The primer pair used was MTB-F 5' CGT GAG GGC ATC GAG GTG GC 3' and MTB-R 5' GCG TAG GCG TCG GTG ACA AA 3'. Amplification was carried out in a final volume of 25 μ l. Each PCR mixture contained 0.4 μ M of each primer, 200 μ M of each dNTP, 2.5 U Taq polymerase (BioFlux, Japan), 0.5 mM MgCl₂, 1X PCR buffer, and 5 μ l template DNA. Reaction conditions included initial denaturing at 94°C for 5 min, followed by 40 cycles of 45 s at 94°C and 1 min at 72°C; and ending with a final extension for 7 min at 72°C. The PCR products were electrophoresed and visualized in an ethidium bromide stained with 2% agarose.

Cloning of PCR product as a positive control

The PCR product was cloned in pTZ57R vector by using T/A cloning kit (Fermentas, cat: K1214, USA).

Comparison of specificity and sensitivity between LAMP and PCR

To evaluate the species specificity, 10 mycobacterial strains, including *Mycobacterium chelonae*, *Mycobacterium avium*, *Mycobacterium fortuitum*, *Mycobacterium xenopi*, *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium bovis* BCG, *Mycobacterium intracellulare*, *Mycobacterium goodii*, *M. tuberculosis* H37Rv and 7 non-mycobacterial species such as *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Streptococcus pneumonia* and *Actinomyces pyogenes* were used for the test. The DNA of mycobacterial strains was prepared from the Swedish mycobacterial strain collection at Swedish Institute for Infectious Disease Control (SMI). Non-mycobacterial species were clinical isolates which were provided by the Department of Microbiology, Shiraz University of Medical Science, Iran. The sensitivity was examined using 10-fold serial dilutions of 100 ng μ l⁻¹ target DNA from *M. tuberculosis* H37Rv. Extra dilutions of 100 fg μ l⁻¹ DNA, that is, 50, 25 and 5 fg μ l⁻¹, were also included. Detection limit of the LAMP was determined by denatured and non-denatured DNA. The

genome copy number of each dilution was calculated based on the fact that 5 fg of DNA is equivalent to a single genome of MTB (Aryan et al., 2009; Pandey et al., 2008). The detection limit was defined as the last positive dilution and the reactions were performed three times to examine the reproducibility of the test.

RESULTS

Evaluation of LAMP reaction

A successful LAMP reaction with newly designed primers was carried out under optimal conditions. The best results were obtained when the reaction was done at 66°C for 60 min. The positive reaction turned green after the addition of SYBR Green I whereas, the negative reaction with no amplicons remained the original orange color of SYBR Green I under UV light (Figure 1A). Electrophoresis of LAMP amplified products showed typical ladder-like patterns and produced many bands of different sizes (Figure 1B). A total of 80 specimens isolated from the culture of *M. tuberculosis* were LAMP positive.

Specificity and sensitivity

To evaluate the specificity of the newly designed primers, we tested various mycobacterial and non-mycobacterial species. LAMP and PCR correctly detected *M. tuberculosis* and *M. bovis* BCG; and the specificity of these tests for detection of *M. tuberculosis* complex was 100%, while no amplification products were detected from any of atypical strain and other bacterial species. For determination of sensitivity, serial dilutions of DNA from *M. tuberculosis* H37Rv were provided. The detection limit of LAMP assay was 25 fg of DNA per reaction but after initial heat-denaturation of the DNA at 95°C for 3 min, detection limit

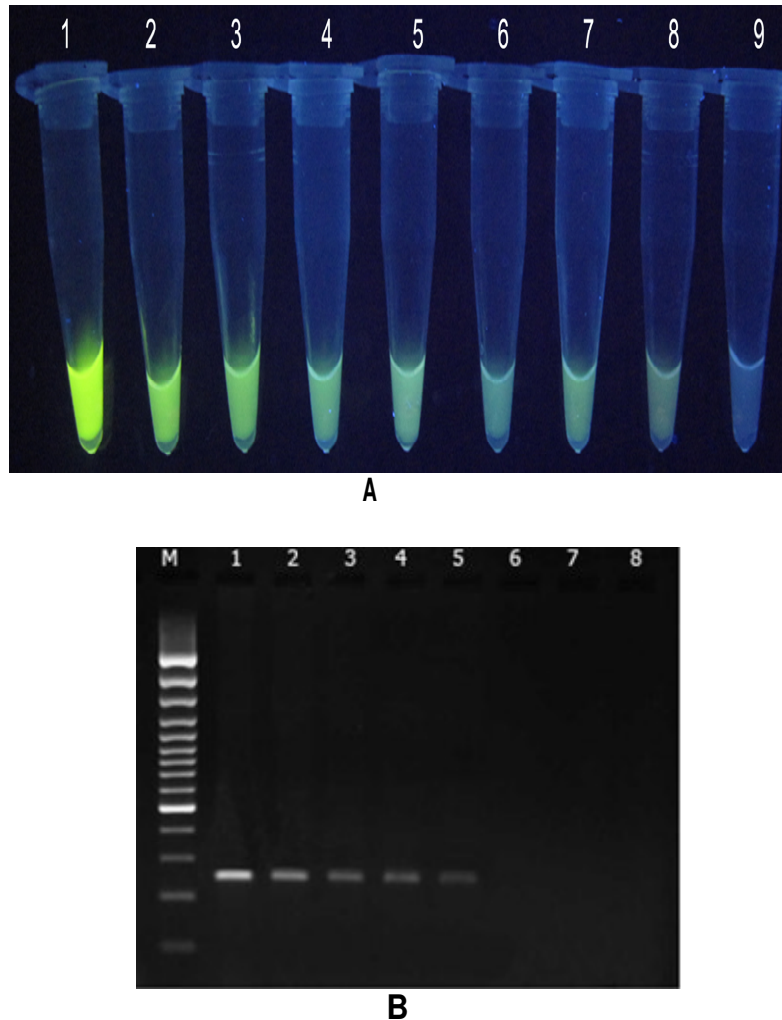


Figure 2. Comparison of LAMP and PCR sensitivity. (A) Sensitivity of LAMP reaction: detection limit of the LAMP was 5 fg. Tubes 1 to 9 contained 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 50 fg, 25 fg, 5 fg and 0 fg of DNA as a negative control, respectively. (B) Sensitivity of PCR: detection limit of the PCR was 100 fg. Lane M, 100 bp DNA ladder and Lanes 1 to 8 contained 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 50 fg, 25 fg and 0 fg of DNA as a negative control, respectively.

was 5 fg of DNA (Figure 2A). This result suggested that as little as one copy of MTB, DNA can be detected using our designed primers. This detection sensitivity was greater than that for conventional PCR, which can detect 100 fg (20 copies) of DNA per reaction (Figure 2B).

Clinical samples

A total of 133 clinical specimens were studied by smear, culture, PCR and LAMP methods. Results are shown in Table 2. All the clinical samples from patients with TB had positive results by LAMP method. In cultured positive specimens, LAMP detected *M. tuberculosis* in 48 smear positive/PCR positive, 7 smear negative/PCR positive and 5 smear negative/PCR negative samples. In cultured

negative samples, LAMP was positive in 3 smear negative/PCR positive samples. As shown in Table 3, the sensitivity and specificity of LAMP test in comparison with culture were 100 and 95.9% (70/73, 95% confidence interval 91.3 to 98.7%), respectively. The sensitivity of smear versus that of culture was 80% (48/60, 95% confidence interval 73 to 87%) and PCR sensitivity was 91.8% (55/60, 95% confidence interval 87-97%). The positive and negative predictive values of LAMP were 95.2 and 100%, respectively.

DISCUSSION

Despite continuous effort in monitoring and treatment of TB, the disease remains a major public health problem

Table 2. Results of different tests for detection of MTB.

Number of specimen	Smear	Culture	PCR	LAMP
48	Pos	Pos	Pos	Pos
7	Neg	Pos	Pos	Pos
3	Neg	Neg	Pos	Pos
5	Neg	Pos	Neg	Pos
70	Neg	Neg	Neg	Neg

Table 3. Results obtained by smear, PCR and LAMP in comparison with culture.

Method	Sensitivity (%)	Specificity (%)	^a NPV (%)	^b PPV (%)
Smear	80	100	85.8	100
PCR	91.6	95.9	93.3	94.8
LAMP	100	95.9	100	95.2

^aPPV, Positive predictive value; ^bNPV, negative predictive value.

Table 4. Summary of studies on detection of *M. tuberculosis* by LAMP method.

Study	Number of specimen	Target gene	Detection limit (copy of DNA)	Sensitivity (%)	Specificity (%)
Iwamoto et al. (2003)	66	gyrB	5-50	-	-
Boehme et al. (2007)	725	gyrB	-	88.2	99
Pandey et al. (2008)	200	16S rRNA	10	100	94.2
Zhu et al. (2009)	30	rimM	200	-	-
Aryan et al. (2009)	10	IS6110	1	-	-
Kohan et al. (2011)	133	IS6110	1	100	95.9

and is the first cause of death due to a single infectious agent in adult (Neonakis et al., 2008). One of the important reasons for failure to control TB is the lack of affordable simple diagnostic methods that have better sensitivity and specificity than conventional methods commonly used in clinical mycobacteriology laboratories (Cheng et al., 2005; Neonakis et al., 2008). Nucleic acid amplification (NAA) is one of the most valuable tools for diagnosis of infectious diseases. NAA tests were particularly attractive for the diagnosis of TB because of the slow growth of TB, and these tests have advantages such as greater sensitivity and specificity and faster results than conventional laboratory diagnostics tests. Among the NAA tests, the PCR has been most widely used for the detection of *M. tuberculosis* in clinical specimens (Mori et al., 2009). However, expensive laboratory infrastructure and sophisticated technical skill needed to conduct this test make it unsuitable for routine use in most diagnostic laboratories (Aryan et al., 2009). Thus, there is an urgent need to develop a simple and low cost technique for rapid detection of TB. In this study, we evaluated LAMP assay for the detection of *M. tuberculosis* in clinical specimens. LAMP has several advantages in comparison with PCR. LAMP amplifies DNA

under isothermal conditions, requiring only a regular water bath or heating block for maintaining the temperature at 66°C, and make it more economical and practical than PCR. In addition, LAMP is more effective and rapid than conventional PCR. In PCR, nearly 3 h is required for the detection and post PCR analysis, while LAMP assay require less than 1 h. LAMP produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube. The visual detection eliminates the need for time-consuming electrophoresis and costly specialized equipment (Cho, 2007; Zhu et al., 2009).

Several investigators, including our group, evaluated LAMP assay for the detection of *M. tuberculosis* (Table 4). Our results showed that LAMP reaction was able to detect 5 fg μl^{-1} of DNA (one copy of *M. tuberculosis* DNA). This detection sensitivity was greater than that for PCR, which can detect 100 fg μl^{-1} DNA (20 copies of *M. tuberculosis* DNA). A number of previous studies have described LAMP assay targeting *gyrB* (Iwamoto et al. 2003, Boehme et al. 2007) and *rrs* (Pandey et al. 2008) for detection and diagnosis of MTB infections. LAMP based assay targeting IS6110 also has recently been

described by Aryan et al. (2009); this assay was performed on 15 clinical specimens. Its detection limit with non-denatured target DNA was reported to be 200 copies per reaction, whereas after initial heat-denaturation of target DNA, the detection limit was one copy (Aryan et al., 2009). We also tested the LAMP assay with denatured and non-denatured DNA. The higher sensitivity equal to one copy was achieved with heat-denatured template. Although, the assay developed by non-denatured DNA has lower analytical sensitivity, but was able to detect 5 copies of DNA. In addition, the sensitivity of LAMP for pulmonary TB in culture positive specimens was 100%. These observations show that novel LAMP assay targeting the *IS6110* gene is more sensitive than a LAMP method used in a previous study (Boehme et al., 2007), in which the sensitivity of LAMP assay targeting the *gyrB* gene was 88.2% (95% CI, 83.9 to 92.5). Similarly, another study by Pandey et al. (2008) showed the feasibility of LAMP assay targeting the *rrs* gene; the sensitivity of LAMP was 100% in culture positive sputum samples and specificity was 94.2% in culture negative sputum samples. In this study, the specificity of LAMP was 95.9%. So, our study develops the LAMP assay with newly designed primers that showed high specificity and sensitivity in comparison with LAMP methods used in previous studies.

Conclusion

We evaluated a LAMP assay targeting *IS6110* gene for diagnosis of *M. tuberculosis* in clinical samples. Our results indicated that this assay has high sensitivity and specificity. Thus, LAMP is a rapid, simple and low cost method that could be suitable for routine use in detection of *M. tuberculosis*.

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