

**Use of Loop-Mediated Isothermal Amplification of DNA for the rapid detection of
Mycobacterium tuberculosis in clinical specimens.**

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Abstract

Loop-mediated isothermal amplification (LAMP) is a recently developed molecular method that has successfully been implemented in the detection of *Mycobacterium tuberculosis* in clinical specimens. LAMP has several advantages such as rapidity, high sensitivity, ease of application and cost-effectiveness. As a result, it is anticipated that its use for the detection of *M. tuberculosis* is likely to become widespread, especially in low-resource countries. The present review aimed to present this method and all available information on its implementation in the detection of *M. tuberculosis* in clinical specimens.

Key words: Loop-mediated isothermal amplification; LAMP; *Mycobacterium tuberculosis*; molecular diagnostics.

Tuberculosis (TB) remains a major public health issue worldwide. Approximately one third of the world population, mainly in developing countries, is exposed to *Mycobacterium tuberculosis* at some stage and TB is the leading cause of death due to a single infectious agent in adults [1]. The need for rapid and sensitive detection of *M. tuberculosis* resulted in the introduction of various molecular methods in the routine workflow of laboratories, such as PCR-based detection, nucleic acid sequence-based amplification and strand displacement amplification [2-4]. However, such methods require expertise and highly sophisticated settings that are not cost-effective for the laboratory in low-resource countries. The loop-mediated isothermal amplification method is a recently developed method that has several advantages, including rapidity, high sensitivity, ease of application and low cost. The present review aims to present the available data on the implementation of this method in the detection of *M. tuberculosis* in clinical samples.

In 2000, Notomi et al. presented a novel molecular method that had the ability to accurately amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions with great accuracy [5]. This method was termed loop-mediated isothermal amplification method and abbreviated as (LAMP). According to the authors' description, this method is dependent on i) auto-cycling strand displacement DNA synthesis which is performed by a DNA polymerase with high-strand displacement activity and on ii) a set of four specially designed primers that identify a total of six distinct sequences in the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This single-stranded DNA serves as a template for DNA synthesis primed by the second inner and outer primers which hybridize to the other end of the target, thereby producing

a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA as well as a new one with a stem that is double the length. The final products are stem-loop DNAs with a number of inverted repeats of the target, and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand [5]. Eiken Chemical Company, Japan exhibit a detailed presentation and a helpful animation of the method online [6,7].

In the experiments reported by Notomi et al, heat denaturation and rapid cooling on ice were applied to a DNA sample containing the target sequence (M13mp18) and the four specific primers [5]. The LAMP reaction was then initiated by addition of the *Bst* DNA polymerase large fragment (New England Biolabs) and carried out at 65°C for 1 h. The products were separated by agarose gel electrophoresis and identified by restriction enzyme digestion and Southern blot hybridization with appropriate probes. Great caution was taken in the experimental design and the concentrations of the primers used to achieve melting temperatures (T_m) within certain ranges and to ensure that synthesis occurred earlier from the inner primers than from the outer ones. The efficacy of the method was greatly dependent on the size of the target DNA and the authors concluded that it should be set to less than 300 bp. The selection of the appropriate DNA polymerase was of great significance. Moreover, it was noted that chemicals destabilizing the DNA helix markedly elevated the amplification efficacies of the method. LAMP was found to be highly sensitive, with an ability to detect DNA in as few as six copies in the reaction mixture. Additionally, the method was less prone to the presence of irrelevant DNA as compared to PCR [5].

Findings from the same research centre (Eiken Chemical Company) showed that when nucleic acid is amplified by the LAMP method a precipitate of magnesium pyrophosphate is formed [8]. This formation occurs due to the fact that pyrophosphate ion, which is released from dNTP as a byproduct during DNA polymerization, reacts with magnesium ion in the reaction buffer, yielding a precipitate [3]. The generation reaction of the precipitate is infrequently affected by the presence of DNA or organic compounds in reaction buffer (e.g. surfactant). The turbidity derived from the precipitate is produced based on the progress of the reaction. The DNA yields and the turbidity are in a linear relationship and turbidity reflects the DNA yield in the LAMP reaction [8]. Thus, the presence of the template DNA in testing a specimen can be judged by amplification using the LAMP method, followed by the measurement of turbidity for the reaction mixture. The accumulated precipitate can easily be confirmed by spectrophotometry or even with visual inspection following centrifugation. Furthermore, when the precipitate, previously dissolved in hydrochloric acid, is treated with Titan Yellow, the color of solution changes from yellow to red [8,9].

Further developments were made and substantial improvement of the method was achieved. Nagamine et al. demonstrated that the LAMP reaction does not require a denatured DNA template [10]. These authors were able to accelerate the LAMP reaction by using additional primers, termed loop primers [11]. These loop primers hybridize to the stem-loops, with the exception of loops that are hybridized by the inner primers, and prime strand. Following the addition of the loop primers the reaction time was reduced to less than half that of the original LAMP method [11]. In a subsequent study, Tomita et al. used calcein, whose fluorescence is quenched by the binding of manganese ions for more effective endpoint detection, to make the method even more applicable for resource-limited laboratories [12].

As anticipated, the advantages of the method led to the early application of LAMP for the rapid detection of *M. tuberculosis* in clinical samples, as in the case of Iwamoto et al. who applied LAMP for the detection of genus *Mycobacterium* (MYC), *M. tuberculosis* complex (MTBC), *Mycobacterium avium* (MAV) and *Mycobacterium intracellulare* (MIN) directly from sputum specimens, as well as for the detection of culture isolates grown in a liquid or on a solid medium [13]. For comparison reasons, these authors utilized the LAMP method concomitantly with a commercial system [Amplicor assay (Roche Diagnostics GmbH)], whose specimen preparation kit was used for the extraction of DNA from the sputum specimens. Further details on the application of the method are shown in Table 1. In order to increase the rate of identification through visual inspection, SYBR-Green I (Molecular Probes Inc.) was added to the LAMP reaction solution. The addition of SYBR Green I results in a LAMP reaction mixture that contains amplified fragments turning green, whereas a solution with no amplicons retains the original orange color of SYBR Green I. Consequently, the detection limit of LAMP was enhanced so as to approximate that of electrophoresis [13]. It should be noted, however, that the opening of the tube to add SYBR Green I involves a high risk of workspace contamination with amplicons [14]. The LAMP reaction is a highly sensitive one; thus, in order to reduce the chances of contamination the opening of the amplification vessel should be performed in a different setting from where reagents and reaction mixtures are prepared [14].

To evaluate the species specificity of the primers used, the authors tested 35 mycobacterial strains and 7 non-mycobacterial species grown on solid media [13]. Significant amplification of the DNAs isolated from the targeted organisms was observed following a 35-min incubation. In contrast, non-targeted strains were not amplified even after 60 min of incubation. The species-specific primers correctly amplified 5/5 (100%) MTBC, 5/5 (100%)

MAV and 5/5 (100%) MIN [13]. On the other hand, no positive signals were obtained from the aforementioned primers when used for 20/20 (100%) mycobacteria of different species and 7/7 (100%) of non-mycobacterial strains. Universal primers for the genus *Mycobacterium* (MYC) were also used to confirm extraction of the proper DNAs. The primers amplified all of the mycobacteria after 35 min of incubation. Apart from the mycobacteria, four actinomycetes were also amplified with these universal primers [13].

The study by Iwamoto et al showed that the LAMP method allows for the mycobacterial species to be identified in 35 min from a solid-medium culture, whereas a 60-min incubation time period is required for identification from a liquid-medium culture or sputum specimen after DNA extraction. This longer incubation time period required for liquid than for solid media was attributed to the small number of cells in the specimens. The detection limit of the LAMP reaction with a 60-min incubation and visual inspection was found to be 5 - 50 genomes per test for the three mycobacterial species, whereas the sensitivity of the method was decreased 10 - 100 times when the reaction time was reduced to 35 min [13]. These authors concluded that the LAMP-based assay allows for the rapid and accurate identification of clinically significant mycobacteria in both culture isolates and sputum specimens. However, it was noted that the lack of an internal amplification control designed to detect inhibition in the processed samples may present a problem for the use of the method in routine diagnosis [13].

Eiken Chemical Company transformed the technique into a more convenient kit format using oligonucleotide primers that were slightly modified from those designed by Iwamoto et al. [13,15] Using this kit, the raw sputum is treated with two extraction solutions and following heating for 10 min at 100°C, the sputum is briefly centrifuged at 2,000 g. Two hundred fifty microliters of supernatant are aspirated across a detachable filter membrane on the tip of a

syringe and washed twice with 1.0 ml of buffer. The filtration tip is placed directly into the reaction tube in which the lyophilized reaction mix, including *Bst* DNA polymerase, has been reconstituted with 30 microliters of buffer. Amplification is carried out at 67°C for 40 min and the reaction is terminated automatically by inactivating the polymerase at 80°C for 2 min.

In 2007, Boehme et al performed the first clinical evaluation of this LAMP-based kit for the detection of pulmonary TB in peripheral microscopy centers in the countries of Peru, Bangladesh and Tanzania [15]. Their study aimed to determine the operational applicability of this technique in resource-limited laboratories. Calcein, a chelating reagent that is fluorescent unless combined with manganese, was included in the reaction mixture (Table 1). The binding of manganese with pyrophosphate, produced in abundance as a by-product of the LAMP reaction, releases free calcein, resulting in UV fluorescence. The combination of turbidity from manganese pyrophosphate and calcein fluorescence allows for the determination of reaction results via visual inspection [15].

The assessment of LAMP was performed using 725 sputum specimens from 380 patients with suspected TB. Ziehl-Neelsen microscopy and Lowenstein-Jensen culture were used as comparators. LAMP was carried out by six technicians, who had no prior experience in nucleic acid amplification technology, after 1 week of training. These technicians were strictly blinded to the smear results. Simple rooms without biosafety cabinets were used. The results obtained in that study were: (a) The sensitivity of LAMP for pulmonary TB in smear-positive, culture-positive specimens was 97.7% [173/177 specimens; confidence interval (CI), 95.5 to 99.9]; (b) in the small number of smear-negative, culture-positive specimens, the sensitivity of LAMP was 48.8% (21/43 specimens; CI, 33.9% to 63.7%); (c) the overall sensitivity of LAMP in the 220 culture-positive specimens was 88.2% (CI, 83.9 to 92.5), and (d) the specificity of LAMP in

culture-negative samples was 99.0% (500/505 specimens; 95% CI, 98.1 to 99.9) [15].

Conclusions drawn from that study included the fact that: (a) the frank blood (when present in the sample) did not have any significant inhibitory effect on the amplification or fluorescence detection; (b) the hands-on time (active working time required to test six samples and two controls, without the 40-min amplification time) was found to be 54 min; (c) the interreader variability (differences during independent interpretation of LAMP results by two readers) was negligible (0.4%); (d) there was no need to open the amplification vessel, which minimized the risk of workspace contamination with amplicon; (e) the assay was exempted from the N-acetyl-L-cysteine–NaOH processing method, a prerequisite of other techniques; and (f) based on the answers of all study teams on relevant questionnaires, the complexity of the LAMP assay was rated comparable to that of culture for the sample-processing steps but LAMP was achieved more readily and rapidly than culture and microscopy for result interpretation [15]. The authors concluded that the assay is a strong straightforward assay that can be deployed in developing countries outside of reference laboratories [15].

Since 2008, an increasing number of studies have been published with regards to the implementation of LAMP for the detection of *M. tuberculosis* [14,16-18]. Using the Primer Explorer V3 software (Eiken Chemical Ltd) [19], Pandey et al designed a set of six primers that identified eight distinct regions of the 16S rRNA gene [17]. The specificity of the primer set was examined by LAMP reaction on the extracted DNA from various mycobacterial and non-mycobacterial strains. The *Bst* DNA polymerase was used and the mixture was incubated at 64°C for 60 min in a Loopamp real-time turbidimeter (LA-200; Teramecs). Efficient DNA amplification was observed in the *M. tuberculosis* complex, including *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*, whereas no DNA amplification was noted in the remaining

bacterial species evaluated [17]. Sensitivity of the LAMP assay was evaluated by examining 100 acid-fast bacilli (AFB)-positive sputum samples obtained from Nepalese pulmonary TB patients, and 100 AFB-negative sputum samples obtained from Nepalese patients presenting with chest pain, cough and fever. The freezing and boiling methods were used to extract mycobacterial DNA from sputum samples [20]. In culture-positive (C^+) samples, *M. tuberculosis* was detected by LAMP in all 90 smear-positive (S^+) and 6 smear-negative (S^-) samples, and the overall sensitivity of the LAMP assay in C^+ specimens was 100% (96/96). The LAMP specificity in C^- samples was 94.2% (98/104; 95% CI 90.5–97.9 %), whereas the positive and negative predictive values were determined to be 94.1 and 100 %, respectively. The minimum detectable number of live *M. tuberculosis* species in a sputum sample was determined to be as low as 10. Subsequently, Pandey et al concluded that the LAMP method may prove to be a powerful and useful tool for the early diagnosis of TB [17].

In 2009, Zhu et al. reported the successful implementation of the LAMP assay for the detection of *M. tuberculosis* and *M. bovis*, not only in pure bacterial culture, but also in clinical samples of sputum, pleural fluid and blood in South China [14]. Four primers were designed based on the *rimM* (encoding 16S rRNA-processing protein) gene sequence. *Bst* DNA polymerase large fragment was used and the visual LAMP reaction was performed by adding the reagents calcein and manganese chloride to the conventional LAMP reaction mixture, allowing the results to be read by simple visual observation of color change in a closed-tube system. The optimum temperature was set to 65°C for 1 h and the reaction was terminated by increasing the temperature to 80°C for 4 min. The raw sputum samples were initially digested and decontaminated with 2-volume 4% NaOH treatment. The DNA from sputum, pleural fluid and blood samples was extracted with the QIAGEN QIAamp DNA mini kit (QIAGEN), as

previously described [21]. Using the pure bacterial cultures, the visual *rimM* LAMP correctly detected all 84 *M. tuberculosis*, 3 *M. bovis* and 1 *M. bovis* BCG strains, whereas no amplification products were detected from any of the non-tuberculous mycobacterial strains, other bacterial species and negative (no template) reaction control. All of the eight LAMP reactions with identified clinical samples from patients with TB yielded positive results, whereas none of the five LAMP reactions with clinical samples from patients without TB was positive. The sensitivity of the visual *rimM* LAMP was extremely high, with as little as 1 pg of genomic DNA being detected, which was 10-fold more sensitive than that of the corresponding conventional PCR. Zhu et al therefore concluded that the visual *rimM* LAMP assay was simple and economical to perform and more advantageous than the PCR method for the detection of *M. tuberculosis* [14].

Additionally, Lee et al, reported a method for the rapid detection of viable *M. tuberculosis* from clinical samples in Taiwan by combining reverse transcription, loop-mediated isothermal amplification and enzyme-linked immunosorbent-hybridization (RT-LAMP-ELISA) [16]. 16S rRNA was selected as the target for reverse transcription. The RT-LAMP amplicons were obtained from the LAMP using specific primers to amplify the 16S rRNA region specific to *M. tuberculosis*. The RT and LAMP were developed in a single tube using ELISA plates. Amplification was then carried out using specific internal primers for the RT-LAMP products used as templates. Liquid hybridization was conducted in the same tube using smart probe specific to *M. tuberculosis*. ELISA was used for the colorimetric assay to detect amplified *M. tuberculosis* DNA. The six LAMP primers were designed using the Net Laboratory LAMP primer design support software program [19]. The RT-LAMP ELISA hybridization assay was evaluated using 150 decontaminated sputum samples from suspected TB patients. When

compared to positive cultures the sensitivity, specificity, positive predictive value, and negative predictive value of the method for *M. tuberculosis* diagnosis were 94.1%, 94%, 82.1% and 98.2%, respectively. The cost of the method was estimated to be \$10/per reaction and the overall time for the procedures was 5 h [16].

Recently, Aryan et al. described a new LAMP-based assay for the detection of MTBC, including *M. tuberculosis*, *M. bovis* (including *M. bovis* BCG), *M. africanum*, *M. microti*, *M. canettii*, *M. caprae* and *M. pinnipedii* in clinical samples obtained from Iranian patients [18]. The assay was based on the amplification of the Insertion Sequence 6110 (IS6110) and a set of six new primers was designed using the online Primer Explorer V4 software [19]. To confirm the specificity of these primers, genomic DNA isolated from 35 mycobacterial reference strains and 10 non-mycobacterial species were examined. The assay correctly detected all the MTBC strains, whereas it did not yield positive results for strains not related to MTBC bacteria. The IS6110-LAMP assay was able to detect as little as 5 fg of purified DNA per reaction equivalent to one copy of the MTB genome. This high sensitivity was achieved with an initial heat-denaturation of template DNA. In the absence of such a step a 200-fold reduction of the sensitivity of the assay was noted. The increase of sensitivity of the LAMP reaction by an initial heat-denaturation step was also recently verified by Geojith et al. [22]. Sensitivity of the IS6110-LAMP was much higher than that reported from LAMP assays with different targets (Table 1). Aryan et al attributed this discrepancy to the fact that IS6110 is a repetitive sequence and not a single copy gene as in the case of other targets.

The assay was also applied to 15 sputum specimens from TB patients confirmed by positive culture. The boiling method was used to extract DNA from the clinical samples [23]. The LAMP assay detected *M. tuberculosis* in all 15 sputum specimens of the TB patients. It was

therefore concluded that the IS6110-LAMP assay was a cost-effective (estimated cost of \$1/reaction), rapid and extremely accurate technique for the detection of MTBC strains in clinical samples. However, since the number of samples tested was limited, additional studies with a much larger number of clinical samples was required to confirm the preliminary results [18].

In conclusion, LAMP is a molecular method that has been successfully implemented in the detection of *M. tuberculosis* in clinical samples. The method offers various advantages, summarized as follows: (a) rapid and efficient amplification of DNA under isothermal conditions without any significant influence by of the co-presence of non-target DNA; (b) high specificity of the target sequence, since multiple independent sequences are identified by the primers; (c) the method is simple, straightforward to use and cost-effective as it requires only a few primers, a DNA polymerase and a regular laboratory water bath or heat block for reaction; (d) it can be combined with reverse transcription and amplify RNA sequences; (e) DNA amplification is readily detected by visual endpoint judgment of turbidity; (f) real-time turbidity analysis of LAMP allows for the quantification of the initial amount of template DNA in the samples; (g) a reduced risk of workspace contamination with amplicons was noted; as well as (h) exemption from the N-acetyl-L-cysteine–NaOH processing method [5,8,13-18,24,25].

Due to the aforementioned advantages LAMP is anticipated to become the dominant and most widely used molecular method of choice for the rapid detection of *M. tuberculosis* in clinical samples, especially in resource-limited countries.

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Table 1. Studies on the LAMP-based detection of *M. tuberculosis* from clinical samples.

Source	Mycobacterium species	Specimen type	Target DNA	DNA polymerase	Tm	Detection method	Incubation time	Detection limit
Iwamoto et al., 2003	MT, MB, MV, MI, MY	SP, SC, LC	<i>gyrB</i> ; 16S rDNA	<i>Bst</i>	63°C	SYBR Green I	SC: 35 min SP, LC: 1 h	5 copies of DNA
Boehme et al., 2007	MT	SP	<i>gyrB</i>	<i>Bst</i>	67°C	calcein/UV fluorescence	40 min	-
Pandey et al., 2008	MT, MB, MA, MM	SP	16S rDNA	<i>Bst</i>	64°C	real-time turbidimetry	60 min	10 copies of DNA
Zhu et al., 2009	MT, MB	SP, SC, PF, BL	<i>rimM</i>	<i>Bst</i>	65°C	calcein/manganese chloride	60 min	1 pg DNA
Lee et al., 2009	MT	SP	16S rRNA	<i>Bst</i>	63 °C	colorimetric	60 min	<10 ⁻⁹ ng RNA
Aryan et al., 2010	MT, MB, MA, MM, MC, MR, MP, SC	SP, SC	IS6110	<i>Bst</i>	65°C	SYBR Green I	90 min	1 copy of DNA

MT: *M. tuberculosis*; MB: *M. bovis*; MV: *M. avium*; MI: *M. intracellulare*; MY: genus *Mycobacterium*; MA: *M. africanum*; MM: *M. microti*; MC: *M. canettii*; MR: *M. caprae*; MP: *M. pinnipedii*; SP: sputum; SC: solid culture; LC: liquid culture; PF: pleural fluid; BL: blood

