


Evaluation of an in-house loop-mediated isothermal amplification for *Mycobacterium tuberculosis* detection in a remote reference laboratory, Thailand

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) is a simple and efficient nucleic acid amplification method for the rapid diagnosis of infectious diseases. This study assessed the performance of an in-house LAMP for tuberculosis (TB) diagnosis at a remote reference laboratory in the endemic setting of Thailand. As part of the routine service, 1,882 sputum samples were processed for mycobacterial culture in Lowenstein-Jensen and MGIT media. The DNA was extracted from the remaining decontaminated samples after the culture procedure for real-time polymerase chain reaction (PCR) analysis using Anyplex plus MTB/NTM detection kit. 785 (40.28%) were positive by mycobacterial culture. Of these, 222 DNA remnants were available and subjected to LAMP analysis. Based on culture as reference (*Mycobacterium tuberculosis*; MTB= 209/ non-tuberculous mycobacteria; NTM= 13), the overall sensitivity of LAMP and Anyplex plus assays for MTB detection were 89.95% (188/209; 95% confidential interval [CI]: 85.05-93.67%) and 96.65% (202/209; 95% CI: 93.22-98.64%), and the accuracy values were 88.74% (197/222; 95% CI: 83.83-92.58) and 96.40% (214/222; 93.02-98.43%), respectively. The sensitivity and accuracy of the in-house LAMP and the Anyplex plus real-time PCR assay were high in comparison to culture results. The high sensitivity and accuracy suggested that this in-house LAMP was promising and might be useful for early TB diagnosis.

KEYWORDS: LAMP. Tuberculosis. Anyplex real-time PCR. Culture. Reference laboratory.

INTRODUCTION

Tuberculosis (TB) is one of the most significant causes of death and remains an important public health problem worldwide. Recently, there were an estimated 10 million new TB cases and 1.4 million TB deaths globally¹. Thailand is a TB endemic country with an incidence of 150 cases per 100,000 and over 105,000 cases and 9,600 deaths in 2020¹. Although microscopic examination for acid-fast bacilli is easy, fast, and inexpensive, this method has limited sensitivity and cannot distinguish between *Mycobacterium tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM)². To detect MTB rapidly and increase diagnostic accuracy, molecular techniques have been developed and employed. In 2010, the World Health Organization (WHO) endorsed Xpert MTB/RIF (Cepheid, Sunnyvale, CA), a fully automated real-time polymerase chain reaction (PCR) for early TB diagnosis³. This test can simultaneously detect MTB and its resistance to rifampicin, one of the most effective TB drugs. Subsequently, the Xpert MTB/RIF Ultra assay was released

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to overcome the limited sensitivity⁴. However, the Xpert MTB/RIF requires a specific machine that limits its use only in some places and Xpert cartridges are quite expensive. Furthermore, it cannot detect NTM. Recently, an Anyplex plus MTB/NTM assay (Seegene, South Korea) has been introduced and is a Conformance Européenne (CE) marked, which can be used for *in vitro* diagnostic purposes⁵. This multiplex real-time PCR system has been used widely in Thailand due to its potential to detect and differentiate MTB and NTM directly from clinical specimens at the same time and the semi-automated platform also offers the ability to detect both isoniazid and rifampicin in the same tube of the next step⁶. The loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification test (NAAT) reported for the first time in the year 2000⁷. Since then, LAMP assays have been developed to detect various pathogens including MTB. It provides high sensitivity and specificity at a low cost and no need for sophisticated equipment⁸. In 2016, WHO recommended the LoopampTM MTBC kit (TB-LAMP) as a simple NAAT for rapid detection of MTB⁹. Previously, Pandey *et al.*¹⁰ reported the high sensitivity and specificity of the in-house LAMP for the detection of MTB directly from processed sputum specimens. We applied our LAMP assay and compared the results with those of culture as a reference test. The assay showed a high sensitivity (94.7%) and specificity (100%) for MTB detection, in comparison to the Xpert MTB/RIF¹¹. In addition, its sensitivity was comparable with the conventional PCR and mycobacterial culture¹², and its simplicity and cost-effectiveness was attractive for clinical laboratories. Apparently, the simple, economical, and reliable molecular methods for rapid TB diagnosis are still required in resource-limited and high TB burden countries. Since in-house assays have usually been developed to suit local conditions, the in-house LAMP may be feasible as an additional test. In this study, we assessed the potential of use and the performance of the in-house LAMP for MTB detection by comparing it to those of routine tests, Anyplex plus real-time PCR, and mycobacterial culture. This study was carried out at the 12th Regional Reference TB Laboratory in Yala, the southernmost province of Thailand.

MATERIALS AND METHODS

Specimens and mycobacteriology

The sputum samples were collected from suspected TB patients who visited TB clinics at hospitals in the 12th healthcare area, Southern region, Thailand. After microscopic examination for acid-fast bacilli (AFB) at the hospitals, the specimens were shipped to the regional

reference TB laboratory for mycobacterial culture, molecular and drug sensitivity testing. The samples were processed according to the routine mycobacterial diagnostic procedures. Molecular tests were conducted on sputum by the Xpert MTB/RIF or Anyplex plus MTB/NTM PCR according to the requests from hospitals.

The sputum was decontaminated by the sodium hydroxide-N-acetyl-L-cysteine-Sodium hydroxide NALC-NaOH method¹³. Briefly, each sample and 4% NALC-NaOH were transferred into a 50 ml sterile centrifuge tube in an equal proportion, mixed by vortexing for 30 seconds and left at room temperature (20-25 °C) for 15 min. Then, phosphate buffer saline (pH 6.8) was added to a final volume of 50 ml followed by centrifugation at 3,000 g, 4 °C for 20 min. The supernatants were discarded and the pellets were re-suspended in a final volume of 1 mL phosphate buffer saline (pH 6.8) for subsequent culture and further analysis.

The mycobacterial culture was conducted in both solid (Lowenstein-Jensen; LJ) and MGIT liquid medium (Becton Dickinson, San Diego, CA). After the inoculation with decontaminated samples, the culture media were incubated at 35-37 °C for 8 weeks and examined for growth weekly. Microscopic examination by Ziehl-Neelsen staining for AFB was performed in positive cultures and MTB was confirmed by a rapid immunochromatographic identification test using SD BIOLINE TB Ag MPT64 Rapid kit (Standard Diagnostics, South Korea). The samples were considered “culture-positive” if growth was detected in either LJ or MGIT culture, regardless of the smear status.

Processing of specimens for Anyplex plus MTB/NTM assay

The DNA was extracted from the remaining decontaminated sediments by the Anyplex DNA extraction protocol according to the package insert. Finally, 5 µL of each DNA supernatant was added to 15 µL of the real-time PCR solution. The PCR reaction was run on a CFX96TM real-time PCR system (Bio-Rad). The amplified results were detected via fluorescent signals and interpreted automatically with the instrument’s software according to the threshold and cutoff values recommended by the manufacturer. The target for MTB detection was the IS6110 and MPB64 genes, while NTM detection targeted a portion of the 16S rRNA gene. Positive and negative controls provided in the kits were included in all runs.

In-house LAMP assay

The DNA samples that remained from a process of Anyplex DNA extraction were submitted to a posteriori

LAMP analysis. The stored remnant DNA was retrieved for the first time and further analyzed by the in-house LAMP test. The LAMP test was performed using a set of LAMP primers, the reaction mixture, and the condition as described previously^{11,12}. Briefly, the reaction mixture was prepared using 12 µL of LAMP master mixture (2 µL 10X buffer, 4 µL 2mM dNTP each, 3.2 µL 5M betaine, 1.2 µL 100 mM MgSO₄, 1.6 µL primer mixture), 1 µL *Bst* DNA polymerase enzyme (New England Biolabs, Ipswich MA, USA), and 1 µL Calcein fluorescent detection reagent¹⁴. Finally, 6 µL of each DNA sample was added to the LAMP mixture resulting in a final volume of 20 µL. Amplification of reaction mixture was carried out in the heating blocks at 65 °C for 60 min and then examined for color change directly through visual observation. The samples were processed with positive and negative controls (with and without *M. tuberculosis* genetic materials) in each run. The LAMP assay would be considered “positive” if the color of the reaction mixture changed from orange to green, either observed by the naked eye or if there was fluorescence detected under UV light. The test would be considered “negative” if the color of the mixture remained unchanged or there was no fluorescence (Figure 1).

Data analysis

The sensitivity, specificity, and accuracy of LAMP in comparison to Anyplex plus MTB/NTM PCR were calculated based on culture, as the gold standard with the 95% confidence interval (CI).

RESULTS

A total of 1,882 sputum samples were received from hospitals and processed as part of the routine laboratory

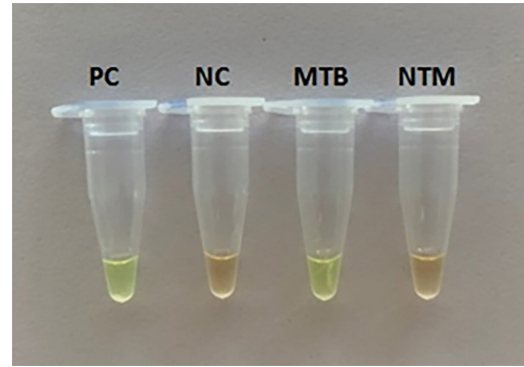


Figure 1 - Detection results of LAMP. PC = Positive control; NC = Negative control; MTB = *M. tuberculosis*; NTM = non-tuberculous mycobacteria.

diagnosis for TB. Of all the samples, 785 (40.28%) were positive by mycobacterial culture. Among these positive samples, 222 samples had the remnant DNA extracts from Anyplex plus real-time PCR analyses that could be further analyzed by the LAMP test. The detected results were obtained as shown in Figures 1 and 2, and in Table 1.

The in-house LAMP could be established and validated by testing with the remnant DNA extracts after the real-time PCR analysis and storage at -80 °C. Of the 222 available samples, 209 were MTB and 13 were NTM detected based on mycobacterial culture and identification results. For 209 MTB, Anyplex plus MTB/NTM assay was positive and negative for MTB in 202 and 7, respectively. Using LAMP analysis, 188 of 209 samples were positive and 21 of 209 were negative for MTB. For 13 NTM, Anyplex plus MTB/NTM tested positive 1 and 12 samples were negative for MTB, respectively, while LAMP detected 4 false positives from NTM strains. The overall diagnostic sensitivity and specificity of LAMP and Anyplex plus MTB/NTM test for MTB detection when compared to MTB culture were 89.95% (95% confidential interval [CI]: 85.05-93.67.0%) and 96.65%

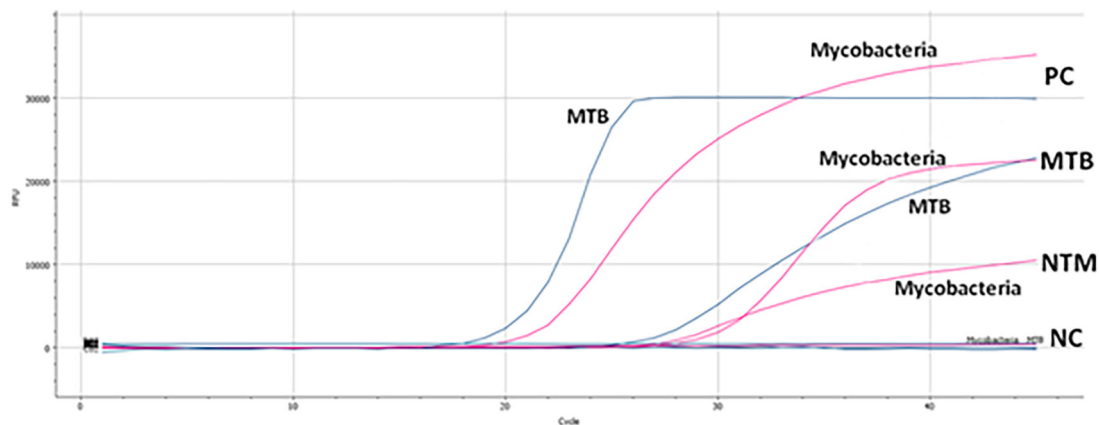


Figure 2 - Detection results of Anyplex plus MTB/NTM real-time PCR (in blue = MTB detected via FAM; in red = mycobacteria detected via Cal Red 610).

Table 1 - The sensitivity, specificity and accuracy of the in-house LAMP and Anyplex plus real-time PCR for MTB detection in comparison to culture.

Method		Culture (222)		Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)
		MTB (209)	NTM (13)			
TB-LAMP	+	188	4	89.95 (85.05-93.67)	69.23 (38.57-90.91)	88.74 (83.83-92.58)
	-	21	9			
Real-time PCR	+	202	1	96.65 (93.22-98.64)	92.31 (63.97-99.81)	96.40 (93.02-98.43)
	-	7	12			

(95% CI: 93.22-198.63%), respectively. The specificity of LAMP and Anyplex plus MTB/NTM for MTB detection were 69.23% (9/13; 95% CI: 38.57-90.91%) and 92.31% (12/13; 95% CI: 63.97-99.80%), respectively. When compared to MTB culture, the accuracy of LAMP and Anyplex plus MTB/NTM assay were 88.74 (95% CI: 83.83-92.58%), and 96.40 (95% CI: 93.02-98.43%), respectively. The summary data of sensitivity, specificity, and accuracy analyses are shown in [Table 1](#).

DISCUSSION

Early and accurate TB diagnosis is extremely important for effective TB control. LAMP is a practical, inexpensive, and effective nucleic acid amplification. This study successfully demonstrated the potential for use of the in-house LAMP and its performance compared to the Anyplex plus real-time PCR and culture for TB diagnosis. Real-time PCR assays were known as a rapid and sensitive molecular tool for rapid TB diagnosis which was superior to smear microscopy for insensitivity and culture on a lengthy turnaround time^{15,16}. However, the complexity, high costs, and the need for well-trained personnel caused obstacles for testing numerous samples using real-time PCR in high-burden settings. Since the LAMP assay is simple, laboratory technicians can perform testing with accuracy after a short period of training; thus facilitating its use. The findings have shown the high sensitivity and accuracy of the in-house LAMP when sputum sediments were applied in DNA preparation following Anyplex plus DNA extraction protocol. It could share the processed samples from routine procedures and could be performed in the TB laboratory.

According to the literature, the sensitivity of this in-house LAMP reported in the present study was similar to the majority of the previous studies in comparison to the conventional PCR, Xpert MTB/RIF and culture¹⁰⁻¹², and similar to that reported in the study carried out in the community hospital (88.8%)¹⁷. Whereas, the sensitivities of Anyplex plus MTB/NTM PCR using culture as a reference standard were reported at 89.0%⁵, and 87.5%¹⁸. Our

findings demonstrated the great sensitivity of Anyplex plus MTB/NTM at 96.65% while the in-house LAMP sensitivity for MTB detection was 89.95%. Nonetheless, we proposed that the in-house LAMP was feasible to be used for early TB diagnosis based on its performance and the characteristics of the test. Furthermore, the overall sensitivity of our in-house LAMP was found to be in the range of the sensitivity of Loopamp™, a commercialized TB-LAMP recommended by WHO. According to the recent meta-analysis, several studies reported the sensitivity of TB-LAMP (Eiken, Japan) as a replacement test for smear microscopy ranging from 66 to 91%¹⁹. The LAMP assay would be the potential molecular test to increase case detection before culture. Even though accurate commercial tests are available, more tests with low cost and high efficiency are better.

According to the accuracy of the in-house LAMP, this study showed the fair concordance between the in-house LAMP (88.74%) and the real-time PCR (96.40%) for MTB detection. The in-house LAMP was reliable even with lower sensitivity and accuracy than the real-time PCR including the Xpert MTB/RIF¹¹. Also, it was developed and modified for potential use, in addition to the low cost at around US\$ 2-3 compared to approximately US\$ 6 for the commercial TB-LAMP according to the Global Drugs Facility Catalogue. Considering the time taken for the TB culture, the LAMP assay may help to screen TB instead of routine testing of several samples with complicated assays. It may be more applicable according to its ease, affordability, and effectiveness.

There were some acknowledged limitations to this study. First, the number of non-TB samples tested as negative samples were very low, which might affect the specificity value. In addition, the culture-negative samples for MTB/NTM were not included. Second, there were no data on acid-fast staining and quality of sputum samples which were associated with the positivity of tests. Thus, the evidence to support the usefulness of the LAMP test in the detection of MTB in smear-negative samples was not available from this study. Third, the study was conducted retrospectively using DNA remnants extracted

from processed sputum samples and did not compare to the Xpert MTB/RIF, a molecular test recommended by WHO. The explanation for this event is that DNA and sputum remnants are usually not available from the Xpert MTB/RIF and using remnant samples from the routine process is practical and saves costs and time. To evaluate the in-house LAMP by comparing it to the Xpert MTB/RIF and the commercial TB-LAMP, more sputum samples are needed. It should be noted that this LAMP was previously evaluated in comparison to the Xpert MTB/RIF. By using processed sputum and comparing it to culture results, the sensitivities of the in-house LAMP and Xpert MTB/RIF were 82% and 86.9%, respectively¹¹. The direct comparison to the Xpert MTB/RIF showed 94.7% sensitivity¹¹. Furthermore, the evaluation of the in-house LAMP for the direct detection of MTB in sputum should be conducted and compared to the commercial LAMP and should be broadly and prospectively tested in future studies.

Overall, we described an efficient LAMP assay for the detection of MTB in a high TB incidence setting. The assay was sensitive and accurate among clinical samples compared to culture and real-time PCR. It could be used outside of a central laboratory suitable for settings with resource limitations or difficulties to implement real-time PCR assays or Xpert MTB/RIF and for cost-saving. The potential use of LAMP as a simple molecular test in peripheral laboratories could be feasible with any validated DNA extraction methods that are safe and can be directly used with sputum samples for early TB diagnosis.

CONCLUSION

The test showed high sensitivity and accuracy for the TB diagnosis when performed in a reference laboratory. Further evaluation should be performed.

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CONFLICT OF INTERESTS

None to declare.

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