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The performance of an in-house loop-mediated isothermal amplification for the rapid detection of *Mycobacterium tuberculosis* in sputum samples in comparison with Xpert MTB/RIF, microscopy and culture

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ABSTRACT

Simple, low-cost and effective diagnostic tests for tuberculosis (TB) are needed especially in TB-high burden settings. The present study evaluated the performance of an in-house loop-mediated isothermal amplification (LAMP) for diagnosing TB by comparing it to Xpert MTB/RIF, microscopy and culture. In Thailand, a total of 204 excess sputum samples volume after the processing of cultures were used for *Mycobacterium tuberculosis* (MTB) detection by Xpert MTB/RIF and LAMP. Based on culture results as the gold standard, the overall sensitivity of LAMP and Xpert MTB/RIF were 82.1% (126/153; 95% confidential interval [CI]: 75.4-88.98%) and 86.9 % (133/153; 95% CI: 80.5-90.8%) respectively, and the specificity of both tests was 100% (51/51; 95% CI: 93.0-100.0%). In comparison with Xpert MTB/RIF, the sensitivity and specificity of LAMP were 94.7% (126/133; 95% CI: 89.5-97.9%), and 100.0% (73/73; 95% CI: 94.9-100.0%), respectively. The average threshold cycle (Ct) of Xpert MTB/RIF detection for positive and negative LAMP results was statistically different, of 18.4 and 27.0, respectively ($p < 0.05$). In comparison with the acid-fast staining technique, and analyzing LAMP and Xpert MTB/RIF in smear-negative/culture-positive specimens, there was an increase of the detection rate by 47.7% (21/44) and 54.6% (24/44). The diagnostic sensitivity and specificity of LAMP appeared to be comparable to those of Xpert MTB/RIF. We claim that this LAMP has potential to provide a sensitive diagnostic test for the rapid TB diagnosis. It allowed a fast detection of MTB before the cultures and it could be used in resource-limited laboratory settings.

KEYWORDS: LAMP. Tuberculosis. Diagnosis. Sputum.

INTRODUCTION

Tuberculosis (TB) remains a global health problem. Recently, in 2018, there were an estimated 10 million new TB cases and 1.4 million deaths worldwide[1]. Rapid and accurate diagnostic tests are required to notify TB cases. Even though microscopy for acid-fast bacilli is easy, fast and inexpensive to perform, this method has a low sensitivity and cannot distinguish between *Mycobacterium tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM)[2]. For this reason, several tools based on molecular techniques to detect MTB nucleic acids directly from clinical specimens were developed. In 2010, the World Health Organization (WHO) endorsed Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), an automated real-time PCR[3,4]. This molecular technique can simultaneously detect MTB DNA and

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the resistance to rifampicin, the most effective drug used for TB therapy, based on the detection of the *rpoB* gene and its mutations, which confer resistance to rifampicin. GeneXpert machines and cartridges have been distributed and available in many regions in both, high and low-income countries. It has rapidly become widespread as a molecular point-of-care test (POCT) and provided a new approach for the rapid TB diagnosis. However, the economical technique is still required in many resource-limited countries with a high-TB burden like Thailand, and others, since GeneXpert instruments require high maintenance service charges and cartridges are expensive for low-income areas. A quite effective, but inexpensive screening test for MTB detection might reduce the costs from using Xpert MTB/RIF tests in many TB cases. Loop-mediated isothermal amplification (LAMP), a novel nucleic acid amplification test was developed[5,6] and has several advantages for the rapid MTB detection. It provides a highly sensitive and specific detection at low costs and no need of sophisticated machines. A commercial product to detect MTB based on the LAMP technique has been developed by Eiken Chemical Company (Tokyo, Japan)[7] and now is available in more countries. Previously, Pandey *et al.*[8] proposed the use of an in-house LAMP to detect MTB directly from processed sputum samples. In comparison with a gold standard culture technique, this in-house LAMP showed high sensitivity (100.0%) and specificity (94.2%). This could be applied for the rapid confirmation of MTB growth in culture media and we reported its high sensitivity and specificity[9]. However, the diagnostic performance data of this in-house LAMP in this setting is limited. Here, we evaluated the performance of an in-house LAMP for the rapid detection of MTB in processed sputum samples by comparing it to Xpert MTB/RIF and mycobacterial culture. The study was carried out in Thailand, a TB-endemic setting, where the use of a LAMP technique would be appropriate.

MATERIALS AND METHODS

Clinical specimens and sample processing

This study was approved by the Ethics Committee of Mae Sot Hospital. From April to September 2018, sputum specimens were consecutively obtained from patients with symptoms compatible with TB. The clinical specimens were processed according to the routine mycobacteria laboratory procedure at Mae Sot Hospital, Tak province. After receipt, sputum samples were checked and decontaminated using the sodium hydroxide-N-acetyl-L-cysteine (NaOH-NALC) method[10]. Briefly, an equal volume of the decontamination solution together with digestion agents were added to each sputum sample in a 50-mL centrifuge tube. After remaining at room temperature for 15 min, the mixture was vortexed and subsequently neutralized with a phosphate-buffered saline (PBS; pH 6.8). Then, the mixture was centrifuged at 3,000 × g for 15 min at 4 °C and the supernatant was discarded. The pellets were resuspended using 2 mL of PBS. The bacterial culture was then conducted in both, solid (Löwenstein–Jensen; LJ) and liquid medium (Mycobacteria Growth Indicator Tube; MGIT; Becton Dickinson, San Diego, CA, USA) by inoculating ~100 µL and 500 µL of the cell suspension to LJ and MGIT culture media, respectively. Mycobacterial growth was observed periodically. Microscopic examination for acid-fast bacilli (AFB) was performed in positive cultures and the growth of bacteria was confirmed for MTB by a rapid immunochromatographic identification test (SD BIOLINE TB Ag MPT64 Rapid kit; Standard Diagnostics, Korea) according to the manufacturer's instruction. Cultures that showed contamination were repeated and excluded from further analysis.

DNA extraction

DNA was extracted using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) with modifications. Briefly, 500 µL of the remaining cell suspension from the process of mycobacterial culture were used. After centrifugation, the sediment was added to 50 µL of buffer FG2/QIAGEN protease and vortexed immediately until the pellet was completely homogenized. The mixture was placed in a heating block or water bath and incubated at 65 °C for 10 min, and then at 95 °C for 10 min to stop the enzymatic activity. Fifty microliters of isopropanol (100%) were added and mixed thoroughly by inversion. Centrifugation at 13,000 × g for 5 min was performed and the supernatant was subsequently decanted. After another centrifugation at 13,000 × g for 5 min followed by discarding the supernatant, DNA was recovered. The sample was air-dried for at least 5 min. Finally, 30 µLof sterile distilled water were added to re-suspend the DNA. The resulting DNA was kept at -20 °C for further experiments.

In-house loop-mediated isothermal amplification assay

A set of LAMP primers, the reaction mixture, and the amplification conditions were as described previously[8,9,11]. Briefly, LAMP reactions were performed in a total volume of 20 µL consisting of 30 pmol of each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 20 pmol each of the loop primers (FLP and BLP), 1.4 mM

deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 8 U *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA) and 1 µL of the fluorescent detection reagent (FDR; Eiken Chemical, Tokyo, Japan) with 6 µL of extracted DNA. Amplification was performed at 65 °C for 60 min in a water bath. Each run contained a positive purified MTB H37Ra DNA (0.1 ng/mL) control and a negative control (distilled water). LAMP results were examined directly by visual observation. The result was considered positive when the color turned from orange to green. For the amplicon confirmation, 5 µL of the LAMP reaction product were analyzed by electrophoresis on 2% agarose gels.

Xpert MTB/RIF assay

The Xpert MTB/RIF assay was performed according to the manufacturer's protocol. Briefly, 500 µL of cell suspension were mixed with 1.5 mL of the sample reagent from the test kit. After being vortexed and standing for 15 min, 2 mL of the mixture were transferred into the Xpert MTB/RIF cartridge which then inserted in the module of the GeneXpert machine. The system operated automatically and interpreted the result of the MTB detection and the resistance to rifampicin by measuring the fluorescent signals. The threshold cycle (Ct) of the Xpert MTB/RIF detection in each sample was recorded and analyzed. Invalid results were excluded from further analysis.

Statistical analysis

Determination of sensitivity, specificity with 95% confidential interval (CI) and comparison of means were performed using the Student's *t*-test (SAS® University Edition[12,13]). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Acid-fast staining and MTB culturing results of clinical specimens

After exclusion of culture contamination and Xpert MTB/RIF invalid results, a total of 204 processed sputum specimens were obtained and analyzed. Of these, 109 were AFB-positive and 95 were AFB-negative by the microscopy of smears. All AFB-positive samples were positive by culture and identified as MTB. For AFB-negative samples, 44 were confirmed as MTB by culture and the other 51 were culture-negative.

Performance of in-house LAMP and Xpert MTB/RIF for MTB detection comparing to acid-fast staining and MTB culturing

All samples were subjected to LAMP and Xpert MTB/RIF analyses. Of the 204 samples tested, 121 were LAMP positive (color change from orange to bright green) and two were considered positive after the analysis on agarose gels. (given the MTB-positive pattern; Figure 1). Twenty-seven samples were negative by LAMP. For Xpert MTB/RIF, 133 from the 204 samples were considered MTB detected and the others were MTB not detected. The overall sensitivity and specificity of LAMP when compared to MTB culture were 82.4% (95% confidential interval [CI]: 75.4-88.0%) and 100.0% (95% CI: 93.0-100.0%), respectively. When compared with MTB culture, Xpert MTB/RIF showed 86.9% of sensitivity (95% CI: 80.5-91.8%) and 100.0% of specificity (95% CI: 93.0-100.0%). Sensitivities of in-house LAMP and Xpert MTB/RIF were 96.3% (105/109) and 100.0% (109/109) in smear-positive/culture-positive specimens, and 47.7% (21/44) and 54.6% (24/44) in smear-negative/culture-positive specimens. The summary data of the sensitivity analyses were shown in Table 1.

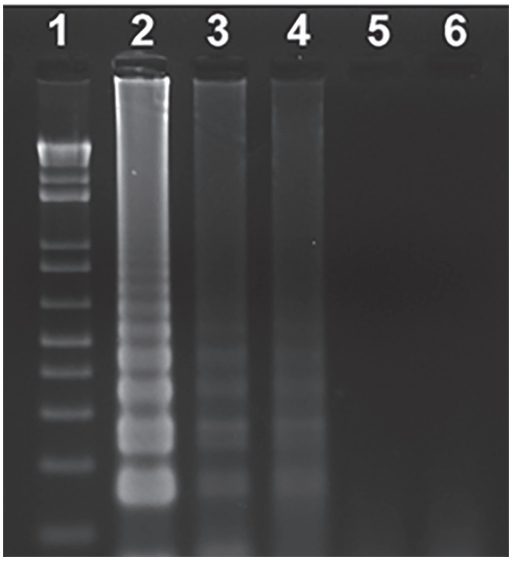

Figure 1 - Gel electrophoresis pattern of the in-house LAMP assay (lane 1- 1 kb DNA ladder; lane 2- positive control; lane 3-4: negative sample by color change, but positive by gel electrophoresis; lane 5; negative sample by color change and gel electrophoresis; and lane 6- negative control).

Comparison of in-house LAMP and Xpert MTB/RIF for MTB detection

For the MTB detection, LAMP showed an overall sensitivity and specificity of 94.7% (95% CI: 89.5 to 97.9) and 100.0% (95% CI: 94.9 to 100.0), respectively, when

Table 1 - Comparison of an in-house LAMP and the Xpert MTB/RIF results with MTB cultures

Test		MTB culture		% sensitivity (95% CI)	% specificity (95% CI)
		Positive	Negative		
LAMP	Positive	126[1]	0	82.4 (75.4 to 88.0)	100.0 (93.0 to 100.0)
	Negative	27[2]	51		
Xpert MTB/RIF	MTB detected	133[3]	0	86.9 (80.5 to 91.8)	100.0 (93.0 to 100.0)
	MTB not detected	20[4]	51		

[1]105 were AFB-positive and 21 were negative; [2]4 were AFB-positive and 23 were negative; [3]109 were AFB-positive and 24 were negative; [4]20 were AFB-negative. MTB = *Mycobacterium tuberculosis*; LAMP = loop-mediated isothermal amplification; CI = confidential interval

Table 2 - Comparison of in-house LAMP result with Xpert MTB/RIF

Test	Result	Xpert MTB/RIF		%sensitivity (95% CI)	%specificity (95% CI)
		MTB detected	MTB not detected		
LAMP	Positive	126	0	94.7 (89.5 to 97.9)	100.0 (94.9 to 100.0)
	Negative	7	71		

MTB = *Mycobacterium tuberculosis*; LAMP = loop-mediated isothermal amplification; CI = confidential interval

compared to Xpert MTB/RIF (Table 2). Seven negative cases by LAMP, but positive by Xpert MTB/RIF showed an average threshold cycle (Ct) of 27.0, while an average of the Ct from the other 126 positive samples was 18.4. Comparing the average Ct detected by Xpert MTB/RIF between groups of LAMP-positive and LAMP-negative, a statistically significant difference ($p < 0.05$) was found (Figure 2).

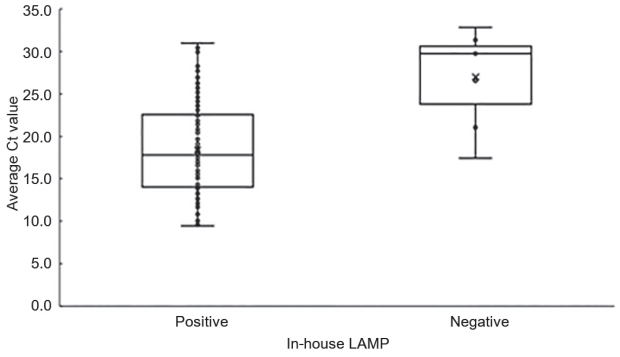

Figure 2 - Distribution of the average Ct values of Xpert MTB/RIF with respect to the in-house LAMP results. Median of average Ct values for LAMP-positive samples (n = 126) and negative samples (n = 7) were 17.9 and 29.8, respectively ($p < 0.05$). LAMP, loop-mediated isothermal amplification.

DISCUSSION

Detection of MTB directly from clinical specimens is a crucial part of TB diagnosis because it helps clinicians to ensure the clinical diagnosis and to reduce the waiting time for the bacterial culture. Acid-fast microscopy remains the main tool for pulmonary tuberculosis diagnosis since it is readily available, rapid and inexpensive. Molecular assays are now selected and used to replace or to be used in combination with the acid-fast staining which exhibits a low sensitivity. In smear-negative cases, the molecular tests have been showing to improve the diagnosis of TB and decrease the time to initiate TB treatment. Xpert MTB/RIF and LAMP are molecular techniques that were recommended by WHO. Because Xpert MTB/RIF is automated, it allows the analysis to be performed in clinics or microscopic centers and it is now widely used in many countries. In contrast, LAMP is a manual cost effective molecular technique proposed to provide an accurate and convenient alternative for TB diagnosis in resource-constrained settings. In the present study, we reported the promising performance of an in-house LAMP targeting MTB *16S rRNA* gene to detect MTB from sputum specimens in comparison with the results of microscopy, culture and Xpert MTB/RIF. The overall sensitivity of the in-house LAMP was 82.4% when compared to the culture, which was lower than in a previous report[8] and slightly lower than the 84.4% of a commercial LAMP used in a multicenter study[14]. However, a recent study in Uganda and Vietnam showed that the sensitivity of a commercial LAMP was only 55.4 and 45.5%, respectively[15,16]. The data from a systematic review and meta-analysis, in 2016, showed that any LAMP assays including both commercially available and an in-house, reached sensitivities around 90%[17]. Indeed, the results of the studies on the performance of techniques can vary greatly according to the prevalence of TB and to the studied populations. Besides differences among LAMP

protocols, specimen types (direct or processed sputum) and DNA extraction protocols were also the main points resulting in variable values of diagnostic sensitivity among studies. When compared to Xpert MTB/RIF alone, the in-house LAMP showed great sensitivity and specificity, of 94.7% and 100.0%, respectively. Several studies reported that LAMP and Xpert MTB/RIF had comparably high levels of sensitivity and specificity for the diagnosis of TB[18-20]. In this comparison, we showed similar sensitivity and specificity of the in-house LAMP and Xpert MTB/RIF. When culture was considered as the gold standard, the sensitivity of LAMP and Xpert were 82.1% and 86.9%, respectively, and the specificity of both tests was 100%. Therefore, the performance of the in-house LAMP was promising. This study has also determined the threshold cycle (Ct) of Xpert MTB/RIF detection in LAMP-positive and LAMP-negative groups. The average Ct value in seven false-negative samples of the in-house LAMP was higher (27.0) compared to that of 18.4 in true-positive cases and the difference was statistically significant. The false-negative results of LAMP in AFB-negative samples might be attributed to the limit of detection of the test. Using culture as the gold standard, the false-negative results were found in 27 samples of the in-house LAMP and in 20 samples of Xpert MTB/RIF which might have occurred because of the lower limit of detection of LAMP and Xpert MTB/RIF compared to that of culture, as described elsewhere[3,8,21].

The LAMP assay showed a high sensitivity and specificity. The method has other advantages such as easy detection, speed, safety and low cost. Even having several advantages, this simple technique has some disadvantages. Firstly, it is not automated, all the processes (DNA extraction, amplification, and result interpretation) are manual. Secondly, the evaluation in a clinical setting is still limited. Importantly, a major weakness of LAMP compared to Xpert MTB/RIF is that it cannot determine the resistance to rifampicin, hence the use of LAMP in a high rifampicin-resistant burden area is not recommended. In addition, the cost-effectiveness between these two molecular assays also needs to be investigated. Furthermore, the colorimetric determination of the LAMP reaction was easy but might be subjective when determined by visual observation alone[22], as we found two samples that were negative by visual observation but were positive by agarose gel electrophoresis. However, in general, theses aspects may not be restrictive for the use of LAMP in the rapid TB diagnosis.

One limitation of our study was that the specimens used for DNA extraction and the in-house LAMP and Xpert MTB/RIF analysis were remaining sediments from the decontamination process of the mycobacterial culture. By this approach, we could compare the molecular results with those of culture which remains the gold standard for MTB diagnosis. We introduced the in-house LAMP into the routine practice by using remaining samples. In general, the diagnosis of TB in the hospital is mainly based on smear microscopy, X-ray, and culture confirmation. Xpert MTB/RIF has been recently incorporated into the routine procedures of TB testing in Thailand. However, all specimens cannot be tested by Xpert MTB/RIF due to the high number of cases versus the high cost and the limited supply of cartridges. Also, Xpert MTB/RIF is available only in large hospitals and medical centers or reference laboratories but not in district hospitals. LAMP is an affordable assay for low resource-settings without the need of expensive instruments. Our in-house LAMP can be performed at a much lower cost, around US$ 1-2, an amount that is similar to the one from a previous report[23], and compared to the US$ 9.98 negotiated prices of the Xpert MTB/RIF[24]. In addition, several samples could be handled at the same time and it could provide a shorter turnaround time to results, within 2-3 h. It can be used in hospitals and it is also suitable for use in mycobacterial culture centers in which it would allow the rapid detection of MTB before bacterial cultures. In conclusion, the performance of this in-house LAMP showed a high sensitivity and specificity for rapid MTB detection when compared to culture and Xpert MTB/RIF. It has potential to be provided a rapid, early diagnosis of TB and can be useful for the effective patient management.

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