

Original Article

Evaluation of in-house loop-mediated isothermal amplification for tuberculosis diagnosis compared with Xpert MTB/RIF

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Abstract

Introduction: To evaluate the diagnostic performances of an in-house loop-mediated isothermal amplification (LAMP) kit and the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in a resource-limited setting, this study was performed at the University Teaching Hospital, Ministry of Health, the Republic of Zambia.

Methodology: Two hundred sputum specimens obtained from new tuberculosis (TB) suspects were used for the evaluation of the diagnostic performance of an in-house LAMP kit in comparison with the Xpert MTB/RIF kit.

Results: The sensitivity of in-house LAMP and Xpert MTB/RIF was 96.9% and 95.4% in smear-positive samples, 96.8% and 100% in smear-positive/culture-positive samples, and 39.1% and 73.9% in smear-negative/culture-positive samples, respectively. The specificity of in-house LAMP and MTB/RIF kits with culture was 96.5% and 94.5%, respectively. This indicated the superiority of the Xpert MTB/RIF kit; however, mechanical errors during sample processing and the insufficient quantity of samples by Xpert MTB/RIF kit occurred at 2.0% and 19.7%, respectively, comparing to the 100% accessibility of in-house LAMP.

Conclusions: Considering the results obtained in this study together with the easy setup with much simpler equipment, such as an aluminum heat block or water bath, in in-house LAMP compared with real-time polymerase chain reaction equipment in Xpert MTB/RIF kit, the applicability of in-house LAMP for the screening of tuberculosis directly from sputum in resource-limited setting seemed to be high.

Key words: in-house LAMP; Xpert MTB/RIF; tuberculosis.

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Introduction

Tuberculosis (TB) is a serious global human health burden [1]. Seventy-five percent of TB patients who are co-infected with human immunodeficiency virus (HIV) live in sub-Saharan Africa [2]. Zambia, located in this region, is estimated to have 427 TB cases per 100,000 individuals and is ranked as having the 13th highest incident rate in the 22 countries with a high TB burden.

Rapid diagnosis and commencement of the appropriate anti-TB treatment are the most important elements in the conquering of the disease. Although smear microscopy is the main diagnostic method for tuberculosis in developing countries, it has moderate sensitivity and lacks the specificity to differentiate *Mycobacterium tuberculosis* complex (MTC; *M.*

tuberculosis, *M. bovis*, *M. africanum*, *M. microti*, and *M. caprae*) from mycobacteria other than tuberculosis (MOTT) [3]. A culture system for growing tubercle bacilli from a specimen, such as Mycobacterium Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, USA), has high sensitivity, but it takes 1–6 weeks for the growth to be observed and determined. In addition, the lack of specificity similarly to smear microscopy and the requirement for a different identification test are shortcomings of this method.

Nucleic acid amplification tests (NAATs) have a high degree of sensitivity and specificity for the detection and identification of medically important mycobacterial species. A number of NAATs have been developed and practically used. For instance, Xpert

MTB/RIF (Cepheid, Sunnyvale, USA) based on a real-time polymerase chain reaction (PCR) platform, which is one of the NAATs to identify MTC and rifampicin drug resistance, requires specialized equipment with periodic maintenance. A meta-analysis of the Xpert MTB/RIF kit has shown 90.4% sensitivity and 98.4% specificity in pulmonary TB for the identification of MTC [4]. The World Health Organization (WHO) recently endorsed the Xpert MTB/RIF kit [5,6]. The Loopamp MTC detection kit (Eiken Chemical Co., Tokyo, Japan) is also a NAAT based on the loop-mediated isothermal amplification (LAMP) platform [7]; the target gene amplification can be accomplished at a constant temperature within one hour. The result can be detected with the naked eye. Recently, this commercial kit was reported to show 88.2% sensitivity and 93.9% specificity in pulmonary TB [8]. An in-house LAMP assay for TB diagnosis, which can be performed with low cost, was developed [9]. The weak point of the in-house LAMP assay was the requirement of the standard treatment of clinical specimen using N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) followed by the collection of tuberculosis bacilli by centrifuge. This hampered the easy accessibility of this method. Boehme *et al.* first demonstrated the use of DNA capture filter for trapping DNA for LAMP assay [10]. Then, we developed a method for the purification of DNA by capturing nucleic acid and magnesium complexes on non-woven fabric filters under alkaline conditions for the gene diagnosis of tuberculosis by loop-mediated isothermal amplification [11]. Nevertheless, these methods had a disadvantage of low applicability to samples with high viscosity. Recently, a kit called Procedure for Ultra Rapid Extraction (Loopamp PURE DNA Extraction Kit) has become commercially available from Eiken Chemicals (Tokyo, Japan). It has been used for TB diagnosis in combination with a commercial version of the LAMP kit for TB detection and demonstrated applicability for sputum samples [8].

In this study, we aimed to evaluate the efficacy of in-house LAMP in combination with a PURE kit in Zambia to examine its possible use for the diagnosis of tuberculosis in resource-limited laboratories.

Methodology

Ethics statement and participants

This study was reviewed and approved by the University Teaching Hospital Zambia University ethics review board, and was conducted at the tuberculosis laboratory of the University Teaching Hospital. Written informed consent was obtained from all participants.

The data were analyzed anonymously. The patients were instructed on how to produce sputum by healthcare workers and were asked to submit morning sputum specimens. A total of 249 sputum samples from new TB suspects without any TB treatment in Lusaka, Zambia (May to September 2013), was collected.

Culture, ID test, and smear microscopy

For culture, samples were processed according to the manufacturer's guidelines for MGIT 960 cultures, and 0.5 mL of final suspension was inoculated into 7.0 mL MGIT culture tubes and incubated in BACTEC MGIT 960 instrument (Becton Dickinson, Sparks, USA). The remaining sputum samples were used for smear, in-house LAMP, and Xpert MTB/RIF tests. Identification of mycobacteria in MGIT culture-positive samples was performed using smear microscopy, and identification of *M. tuberculosis* complex was performed with smear-positive MGIT culture using Capilia TB-Neo (TAUNS Laboratories, Inc., Shizuoka, Japan) according to the manufacturer's instructions. Direct smears were prepared from sputum samples and stained by auramine O-phenol method and observed with a fluorescence microscope, Primo Star iLED (Carl Zeiss Microimaging, Oberkochen, Germany), according to the manufacturer's instructions.

In-house LAMP

An in-house LAMP [9] for TB detection was used in this study in combination with the pretreatment of sputum using Loopamp PURE DNA Extraction Kit (Eiken Chemicals Co., LTD, Tokyo, Japan). Briefly, 60 µL of fresh sputum was transferred to a tube containing a lysis solution, using a special device for sputum transfer included in the kit, and was heated at 90°C for 5 minutes. The tube was then connected to an adsorbent tube containing a powder for the removal of amplification inhibitors. After mixing by shaking up and down, the adsorbent tube was attached to an injection cap. Approximately 25 µL of the extracted DNA solution from the tube was dropped into a reaction tube containing dried-down reagents constructed in accordance with the publications of Pandey *et al.* [9] and Hayashida *et al.* [12]. The reconstituted reaction mixture was then incubated at 64°C for 60 minutes. Reaction was stopped by heating at 90°C for 5 minutes and the result was judged by the naked eye under a fluorescent light.

Xpert MTB/RIF test

For the Xpert MTB/RIF test (Cepheid, Sunnyvale, USA), 1 mL of fresh sputum was used according to the manufacturer’s protocol. Briefly, the sputum was mixed with sample reagent (1:2 v/v ratio) and incubated for 10 minutes at room temperature. Then the mixture was mixed again and incubated further at room temperature for 5 minutes. Finally, 2 mL of the mixture was added to the test cartridge. The cartridge was loaded onto the machine (version G3) and the results were read after a 2-hour run.

Statistical analysis

The McNemar Chi-square test for matched pairs of samples with categorical test results was carried out on sensitivity; significant differences were defined as p values below 0.05 [13]. Positive and negative predictive values [14] of the in-house LAMP and Xpert MTB/RIF kit for smear microscopy test and culture results were also determined.

Results

In this study, the diagnostic performances of in-house LAMP, Xpert MTB/RIF, and other conventional laboratory examinations in Zambia were demonstrated.

Of 249 specimens collected, 49 (19.7%) were excluded because of insufficient quantity for Xpert MTB/RIF tests; the final analysis was performed for 200 specimens. Table 1 summarizes the results of smear and culture tests confirmed by Capilia TB Neo, in-house LAMP, and Xpert MTB/RIF. Among 200 sputum specimens, 64 (32.2%) of smear-positive (scanty ~3+) included 63 MGIT-positive and 1 MGIT-negative samples. The latter is thought to be because of the existence of dead TB bacilli, as positive results were

observed by both in-house LAMP and Xpert MTB/RIF tests. Ninety-nine (49.7%) MGIT cultures were positive. There were 23 (17.0%) MTC positives among 135 smear-negatives. Of the positive MGIT cultures, 11 were considered as contamination of bacteria other than mycobacteria (BOM) from negative results of acid-fast staining of the contents, and the MGIT culture contamination rate was calculated to be 5.5% (11/200). All positive cultures were negative for in-house LAMP, and one of them was positive for Xpert MTB/RIF. Two of the smear-negative MGIT-positive samples were considered to be MOTT according to the positive results of smear and negative results of Capilia TB, in-house LAMP, and Xpert MTB/RIF. In addition, a smear-positive MGIT-positives sample considered to be MOTT according to the negative results of Capilia TB showed positive results by in-house LAMP and Xpert MTB/RIF. This phenomenon may be caused by the mixed culture of both MTC and MOTT. This sample might contain both MOTT and MTC, with the majority being MOTT. Of 135 sputum smear negatives, 99 were negative for MGIT and determined to be MTC culture negative. There were 88 cultures positive for acid-fast bacilli, of which 85 were detected as MTC by Capilia TB assay.

Table 2 shows the results of in-house LAMP and Xpert MTB/RIF tests with smear results for 200 sputum specimens according to the MTC determination above. Two out of 64 smear-positive samples were Xpert MTB/RIF positive but in-house LAMP negative. On the other hand, three Xpert MTB/RIF data were not obtained because of the insufficient quantity of clinical specimens. Hence, the overall sensitivity for in-house LAMP and Xpert MTB/RIF comparing to smear test were 63/65 (96.9%) and 62/65 (95.4%), respectively.

Table 1. Summarized results of smear, culture, in-house LAMP, and Xpert.

Smear	Culture	Total	LAMP			Xpert				
			Positive	Negative	Positive ratio (%)	Positive	Negative	ND	Positive ratio (%)	Error rate (%)
Negative	Negative	99	2	97	2.0	3	95	1	3.1	1.0
Negative	BOM	11	0	11	0.0	1	10	0	9.1	0
Negative	MOTT	2	0	2	0.0	0	2	0	0.0	0
Negative	MTC	23	9	14	39.1	17	6	0	73.9	0
Scanty*	MTC	8	7	1	85.7	8	0	0	100	0
Scanty	BOM	1	1	0	100	1	0	0	100	0
1+	MTC	22	21	1	95.5	22	0	0	100	0
2+	MTC	13	13	0	100	12	0	1	100	7.7
2+	Negative	1	1	0	100	1	0	0	100	0
3+	MTC	20	20	0	100	18	0	2	100	10.0

* 1 – 9 bacteria in whole smear ; BOM: bacteria other than mycobacteria as identified by positive-MGIT culture, but AFB negative-smear of MGIT culture; MOTT: mycobacteria other than tuberculosis as identified by positive-MGIT culture, AFB positive-smear of MGIT culture, but negative-Capilia TB assay; MTC: *M. tuberculosis* complex as identified by positive-MGIT culture, AFB positive-smear of MGIT culture, and positive-Capilia TB assay; ND: No data obtained because of the error of Xpert.

Table 2. Comparison of performance of in-house LAMP and Xpert with smear test.

Result	Smear	LAMP		Xpert		ND*
	No.	Positive	Negative	Positive	Negative	
Positive	65	63	2	62	0	3
Negative	135	11	124	21	113	1

*ND: No data was obtained because of the error of Xpert.

Table 3. Comparison of the performance of in-house LAMP and Xpert with culture.

Result	Culture	LAMP		Xpert		ND*
	No.	Positive	Negative	Positive	Negative	
Positive	86	70	16	77	6	3
Negative	114	4	110	6	107	1

*ND: No data was obtained because of the error of Xpert

Sensitivity and specificity of in-house LAMP and Xpert MTB/RIF for culture results are shown in Table 3. The sensitivity was calculated using MTC-positive result as the standard. The overall sensitivity for in-house LAMP and Xpert MTB/RIF comparing to culture test were 70/86 (81.4%) and 76/86 (88.4%), respectively, and the specificity for in-house LAMP and Xpert MTB/RIF were 110/114 (96.5%) and 107/114 (93.9%), respectively. In-house LAMP showed lower sensitivity for smear-negative/MTC culture-positive samples than Xpert MTB/RIF (39.1% vs. 73.9%; $p = 0.0361$).

Discussion

In this study, we evaluated the diagnostic performances of in-house loop-mediated isothermal amplification kit and Xpert MTB/RIF for the diagnosis of pulmonary tuberculosis in a resource-limited setting using 200 sputum specimens obtained from new TB suspects in Zambia. The sensitivity of in-house LAMP and Xpert MTB/RIF were 96.8% and 100% in smear-positive/culture-positive samples, and 39.1% and 73.9% in smear-negative/culture-positive samples, respectively. The specificity of in-house LAMP and Xpert MTB/RIF with culture was 96.5% and 94.5%, respectively. The sensitivity and specificity of Xpert MTB/RIF in this study was comparable to those shown by meta-analysis [4]. The sensitivity of in-house LAMP in this study in smear-positive/culture-positive samples was also comparable to that obtained by recent studies using Loopamp PURE DNA Extraction Kit and Loopamp MTBC detection kit [8,15]. However, the sensitivity in this study, 39.1%, in smear-negative/culture-positive samples, was lower than that in recent studies (53.6% [8] and 53.8% [15]). This indicated the lower fitness of our in-house LAMP with Loopamp PURE DNA extraction kit and the

requirement for better DNA extraction methods suitable for our in-house LAMP kit.

Comparison of data indicated the superiority of Xpert MTB/RIF to in-house LAMP in diagnosing TB. The Xpert MTB/RIF system also has the advantage of determining drug susceptibility in addition to diagnosing TB. However, there were mechanical errors during sample processing and the insufficient quantity of samples by Xpert MTB/RIF in this study. Namely, four results (2.0%) were not obtained due to mechanical errors during sample processing of Xpert MTB/RIF. Such error results have also been reported in a previous study [16]. In contrast, results from 100% of samples by our in-house LAMP method were obtained. In addition, it was not easy to collect more sputum samples to repeat the test each time to encounter errors in Xpert MTB/RIF. TB suspects cannot sometimes produce enough sputum for the minimum requirement of 1 mL, and insufficient sputum quantity was also a major problem in this study. Of 249 specimens, 49 (19.7%) were not analyzed because of their insufficient quantity for Xpert MTB/RIF, and this was a big disadvantage of this system in our study. On the contrary, our in-house LAMP test in combination with pretreatment of sputum using Loopamp PURE DNA Extraction kit requires only 60 μ L, which was available from all TB suspects. The Xpert MTB/RIF system also requires a relatively stable, clean, uninterrupted electric supply for at least 2 hours. Since there are frequent electric failures in Zambia, it is hard to set up this system at peripheral TB laboratories. Our in-house LAMP test is easily set up and can be performed under isothermal conditions within 1 hour with much simpler equipment, such as an aluminum heat block driven by a portable battery. Additionally, our in-house LAMP can be performed at much lower cost, around US\$ 1, comparing to Xpert MTB/RIF, and might be beneficial in resource-limited

countries with high HIV prevalence where the major requirement of clinicians is bacteriological confirmation of whether smear-positive cases have tuberculosis.

Conclusions

In-house LAMP in combination with a commercially available PURE kit and Xpert MTB/RIF was evaluated with clinical specimens in Zambia. The in-house LAMP kit performance was intermediate between smear microscopy and Xpert MTB/RIF. Since the in-house LAMP kit is a simple, rapid, and easy to use for laboratory workers and has a lower running cost compared to Xpert MTB/RIF, it is favorable as a primary test of TB diagnosis in resource-limited laboratories, especially in developing countries such as Zambia. The use of Xpert MTB/RIF for the detection of RIF resistance of TB-positive samples by our in-house LAMP kit might reduce the total cost of testing.

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