Title

Direct detection of *Mycobacterium tuberculosis* in clinical samples by a dry methyl green loop-mediated isothermal amplification (LAMP) method

Running title: Dry methyl green MTB-LAMP method

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Abbreviations: MTB-LAMP, *Mycobacterium tuberculosis*-loop mediated isothermal amplification; Tuberculosis, TB; MeG, Methyl green; POC, Point of care.

ABSTRACT

The purpose of this study was to develop a simple visual methyl green (MeG) based dry loop-mediated isothermal amplification (LAMP) method for early detection of *Mycobacterium tuberculosis* (MTB) from clinical samples. We identified MeG as an indicator of a positive LAMP reaction, where a positive reaction gave a blue-green color while a negative reaction was colorless. The MeG MTB-LAMP system was further simplified by drying all reagents for ease of use, and was then validated for its ability to diagnose TB directly using Nepalese clinical samples. We evaluated the dry MeG MTB-LAMP with 69 new TB suspected samples from patients that did not have a confirmed history of TB treatment and found the sensitivity in culture positive samples as 92.8% (13/14) and specificity in culture negative samples as 96.3% (53/55). Our LAMP system has the potential to be a point of care test for early diagnosis of active TB in developing countries.

Key words

loop mediated isothermal amplification (LAMP); methyl green; *Mycobacterium tuberculosis*; tuberculosis

1. INTRODUCTION

With an estimated 10 million new cases and 1.6 million deaths from tuberculosis (TB) in 2017, TB is the leading cause of death from an infectious disease globally, particularly in developing countries [1]. The WHO has proposed an 'End TB' strategy that seeks to end the global TB epidemic by reducing new cases by 90% and by decreasing TB deaths by 95% between 2015 and 2035; WHO has strongly emphasized the need for development of accurate and rapid point of care (POC) diagnostic methods as a part of this strategy [2].

The conventional diagnosis of TB comprises clinical examination, chest x-ray and bacteriological examination. Bacteriological examination involves direct observation of acid-fast bacilli in sputum samples and further processing of sputum samples for culture [3]. In spite of the low sensitivity and specificity of sputum microscopy, and the limitation in its ability to differentiate *Mycobacterium tuberculosis* complex (MTBC) from other nontuberculosis mycobacteria (NTM), sputum microscopy is still widely used in developing countries. Bacteriological culture, while considered a gold standard test for TB diagnosis, requires skilled manpower, infrastructure and a 6-8 weeks to get results [4]. In a similar vein, molecular-based rapid diagnostic tools, such as the Genexpert (Cepheid, CA) and Hain line probe assay (Hain life sciences GmbH), might not be convenient for routine diagnosis of TB in developing countries due to their high cost and insufficient laboratory infrastructure.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification assay that can detect DNA or RNA with high specificity, efficiency and rapidity under isothermal amplification conditions [5]. In 2012, WHO recognized the feasibility of molecular detection of TB using LAMP, citing its advantages of being simple and having the potential as a POC diagnostic method [6]. In a policy document released on 2016, WHO advised that a commercial MTB-LAMP (such as Loopamp MTBC detection kit, Eiken chemical company, Japan) may be used as a replacement test for sputum microscopy for the diagnosis of

pulmonary TB in adults with clinical signs and symptoms consistent with TB [7]. Thus, the development a LAMP-based diagnostic test for TB that combines the simplicity and rapidity of sputum microscopy with the reliability of bacterial culture is a clear priority.

Efforts around the research and development of LAMP technology have focused mainly on two areas. First is with the practical application of LAMP to various pathogens in clinical settings, and second is the development of simple and rapid methods for detection of positive LAMP reactions [8]. Several methods have been used to detect positive LAMP reactions: i) by using a real-time turbidimeter that detects turbidity arising from magnesium pyrophosphate formation [9, 10]; ii) by using a real-time PCR machine which detects fluorescence [11]; iii) by visual detection by agarose gel electrophoresis [5, 11]; iv) by visual detection using the commercial Eiken fluorescent detection reagent (FD) which requires a UV illuminator to detect positive LAMP reactions [12]; v) by visual detection using other nucleic acid binding fluorescent dyes such as pico green [12], propidium iodide [14], SYBR green [13, 14]; vi) by non-fluorescent dyes that can visually detect positive LAMP reactions such as hydroxylnaphthol blue (HNB) [15], malachite green (MaG) [16] and leuco crystal violet [17].

In this study, we have identified methyl green (MeG) as a novel dye that can be used to visually detect positive LAMP reactions without the aid of a UV illuminator. We have employed a dry form of MeG based MTB-LAMP to overcome some of the more usual issues with the LAMP reaction such as contamination due to frequent opening of reaction tubes and the need for maintenance of a cold chain for the reagents. Our simple colorimetric, dry-MeG based MTB-LAMP reaction mixture was validated to detect DNA of TB bacteria using clinical samples submitted for TB testing in Nepal.

2. METHODS

2.1. Preliminary testing of dyes

Thirteen leuco dyes were selected to assess their potential as dyes for the detection of a positive LAMP reaction. They were: crystal violet; fuchsin basic; bromophenol blue (Wako, Japan); phenol red sodium salt; basic violet; pararosaniline hydrochloric acid; bromo-cresol green; bromocresol purple sodium salt; comassie brilliant blue; thymol blue; bromothymol blue (all from Tokyo Chemical Industries, Japan); and methyl blue and MeG (both from Sigma-Aldrich, USA). The parameters that were used to assess suitability of the 13 dyes were that tested dyes should not inhibit the LAMP reaction and should be able to visually differentiate between positive and negative LAMP reaction by a change in color. Dyes with proven utility in a LAMP reaction, namely MaG, HNB, SYBR green and Eiken FD (from Eiken Chemical Co. Ltd.) were used as comparators in this study.

2.2. Dilution of dyes

Dyes were dissolved in double-distilled water (DDW) to prepare a stock solution of 1%. The stock solution was further diluted in DDW to prepare different concentrations (0.05%, 0.1%, 0.2% & 0.4%) of working solution to be used for LAMP reactions.

2.3. Screening of dyes for MTB-LAMP reaction

A previously developed and validated MTB-LAMP system that targets the MTB16S rRNA gene [10] was used for the LAMP reaction. The MTB-LAMP reaction was performed in 25 µl of reaction mixture consisting of: 0.2µM of each outer primer (F3 & B3); 1.6 µM each inner primer (FIP & BIP) and 2.2µM of loop primer (FLP & BLP); 1 mM dNTPs; 0.8 M betaine; 20 mM Tris-HCl (pH 8.8); 8 mM KCl; 8mM $(NH_4)_2SO_4$; 0.08% Tween-20; 3 mM $MgSO_4$; 8 units of *Bst* DNA polymerase (New England Biolabs), and 1 µl of dye working

concentrations. *M. bovis* Bacillus Calmette-Guerin (BCG) Tokyo 172 DNA at a concentration of 5 picogram (pg), 500 femtogram (fg) or 50 fg per tube was used for the LAMP reactions. The mixture was incubated at 64°C for 60 minutes in a Loopamp real time turbidimeter (LA-200, Teramecs). The reaction was deemed positive when the turbidity was greater than 0.1 unit within 60 minutes. Subsequently, the change in color and time required for the turbidity to be greater than 0.1 was recorded. The reaction mixture was then heated at 95°C for 5 minutes to terminate the reaction. Each reaction was performed with technical duplicates and repeated in three independent experiments.

2.4. Development of the dry MTB-LAMP method

For the preparation of dry LAMP reagents, 10.7 µl of reaction mixture consisting of: 2 µl of primer mix [consisting of 100 µM of outer primers (F3 & B3), inner primers (FIP & BIP) and loop primers (FLP & BLP) mixed at 1:8:11 ratio]; 1.4 µl of dNTPs (25mM each); 2.5 µl of 2M trehalose; 1 µl of 25× LAMP buffer (500 mM Tris-HCl [pH 8.8], 250 mM KCl); 1.8µl of 100 mM $MgSO_4$; 8 units of *Bst*2.0 warm start DNA polymerase (New England Biolabs) and 1 µl of 0.1% of MeG was used. To dry the mixture, the prepared reaction mixture was placed on the periphery of the inner side of a 0.2 ml tube lid and kept under a flow of clean air on a clean bench for 3 hours (Fig. 1). Then, the tubes were stored in boxes with zeolite molecular sieves. Boxes were wrapped in an opaque plastic bag and stored at room temperature.

For the MTB-LAMP reaction with dried reagents, 23 µl DDW and 2 µl DNA containing *M. bovis* BCG Tokyo 172 DNA at a concentration of 5 pg, 500 fg or 50 fg per tube was added into the bottom of tubes. In negative control tubes, 25 µl DDW was added into the bottom of tubes. The tubes were turned upside down and placed for 3 minutes in the inverted position to allow for the reconstitution of reagents. Then the reaction mixture was

mixed by inverting five times (Fig. 1). The reaction was performed and recorded as described above.

2.5. Validation of dry MTB-LAMP system using clinical TB samples in Nepal

After development of MeG MTB-LAMP method and further simplification of the system by preparing the dry MeG MTB-LAMP method, we validated our dry MTB-LAMP method in Nepal from June to December 2016. Validation of the dry MeG MTB-LAMP method was performed using clinical samples and only simple visual inspection of color development was used to detect positive or negative LAMP reaction.

A total of 69 clinical samples from new TB suspected Nepalese patients who had been referred for TB testing to the National Anti-Tuberculosis Association – German Nepal TB Project (NATA-GENETUP) reference laboratory were evaluated to determine sensitivity and specificity of our developed method. Smear microscopy, decontamination and concentration of the samples were performed as previously described [10, 18]. The processed samples were used for inoculation in culture media as described, and 500 µl of the remaining sample was used for DNA extraction. The DNA was extracted by alternate cycles of boiling (10 minutes at 95°C) and freezing (30 minutes at -20°C) for three times [19]. The extracted DNA was stored at -20°C until analysis.

Freshly dried MeG MTB-LAMP tubes were transported from Japan to Nepal and were stored at room temperature prior to use. For the MTB-LAMP reaction, 20 µl of DDW and 5 µl of extracted DNA were added to the bottom of tubes, whereas for the negative controls only 25µl of DDW was added. The presence or absence of a blue-green color was used to determine positive or negative MTB-LAMP reactions (Fig. 1). The LAMP reaction was always performed by using positive and negative controls to facilitate interpretation of the test results.

In this study, MeG MTB-LAMP was not used for clinical diagnosis of TB. Our study was retrospectively performed after collecting all the samples so as to evaluate the efficacy of our developed method.

3. RESULTS

3.1. Preliminary screening of dyes for MTB-LAMP detection

Our preliminary experiment to assess the suitability of dyes to detect positive MTB-LAMP reactions identified only MeG as being suitable. Using MeG, a positive LAMP reaction developed a blue-green color with an increment of turbidity greater than 0.1 in a Loopamp real time turbidimeter (LA-200, Teramecs) indicating amplification of DNA; meanwhile, the negative reaction mixture was colorless and did not show turbidity, indicating the absence of DNA amplification.

3.2. Comparison of dyes used for detection of MTB-LAMP reaction

We assessed the suitability of MeG in comparison with other proven dyes used for visual detection of LAMP reactions (Table 1). The standard dyes showed the expected utility in the LAMP reaction. We also wanted to compare the relative speed of detecting positive LAMP reactions when these dyes were used. Both MeG and malachite green (MaG) (Nzelu et al, 2014) showed a similar speed in developing a positive LAMP reaction, but the reaction mixture with MeG had a more intense color (Table 2, Fig. 2). For the comparative study, we used the lower concentration [(0.05% stock, final concentration 0.002%) and (0.1% stock, final concentration 0.004%)] of dyes to reduce the likelihood of any inhibition of the reaction and to ensure a minimal effect on turbidity [9].

3.3. Development of dry methyl green MTB-LAMP

We identified a concentration of 0.1% MeG (final concentration of 0.004% in a 25µl reaction volume) to be optimal for the design of dried reagents for LAMP reactions, based on its speed of color development and superior color intensity. (Table 2, Fig. 2). Using this concentration of MeG, the dry MTB-LAMP system successfully detected LAMP reaction using our developed conditions with BCG DNA (Table 3, Fig.1).

3.4. Validation of dry MeG MTB-LAMP system using clinical samples of TB in Nepal

We compared the performance of our dry MeG MTB-LAMP system with microscopy and culture (Table S1). The sensitivity and specificity were evaluated using 69 new TB suspected clinical samples that were submitted for TB testing at GENETUP Nepal. Although we tested 142 clinical samples, we excluded 67 samples from patients that were under treatment, or re-treatment or treatment failure status and also excluded 6 samples with unknown treatment history (Table S1). The sensitivity of our MeG MTB-LAMP system in culture positive samples was 92.8% (13/14) and specificity in culture negative samples was 96.3% (53/55); and the sensitivity and specificity when compared with sputum microscopy was 92.8% (12/13) and 94.6% (53/56) respectively (Table 4) indicating its potential for POC MTB detection method.

4. DISCUSSION

The simplest method to detect a LAMP reaction is by observing the turbidity using the naked eye; however, it is often difficult to distinguish turbidity and it would be unreliable as the basis for a POC test. Although a Loopamp turbidimeter can be used to detect turbidity in real time, using a Loopamp turbidimeter for a POC test is an inconvenient option in developing countries. Thus, developing a simple visual detection system for LAMP is an

attractive option. Some of the reported visual detection systems, such as Eiken fluorescent dye and HNB (Table 1), only show a small color difference between positive and negative reactions (Fig. 2), proving difficult to distinguish with the naked eye.

We identified MeG as a suitable dye to detect MTB-LAMP positive reactions. As with the previously reported MaG [16], MeG could be used for visual detection of LAMP reactions, where the positive reaction was a blue-green color and the negative reaction was colorless. While both MeG and MaG had similar visual detection properties, the color intensity of MeG in positive LAMP reaction was superior to that of MaG (Table 2, Fig. 2), thus making it easier to differentiate between positive and negative reactions. Using the lowest concentration (50fg) of DNA, the MeG-based MTB-LAMP proved more sensitive (detected 4 of 6 reactions, both with 0.05% and 0.1% dye) than the MaG-based MTB-LAMP (1 of 6 with 0.05% dye and 2 of 6 with 0.1% dye) (Table 2). The visual detection of the LAMP reaction using MeG was both reproducible and consistent. Thus, MeG was selected as a novel candidate for developing a simple POC using an MTB-LAMP detection system. We hypothesize that MeG detects changes in DNA concentration, whereby color development is through binding of MeG to the major groove of DNA as previously suggested [20, 21] and in a similar way as previously reported with crystal violet [17].

To simplify the LAMP system as a POC test, we dried the reaction mixture using a vitrification technique as previously described [22]. The preparation of dried reagents was simple and could be easily implemented in developing countries (Fig. 1). Like wet LAMP reagents, dried LAMP reagents successfully yielded LAMP positive reactions (Table 3). These dried LAMP reagents could be stored at room temperature and were stable for up to 4 months (data not shown). Both the wet and dry MTB-LAMP system could detect up to 50fg of TB DNA (Table 2 & 3, Fig. 1 & 2). However, the time detection speed for dry reagents was lower than the wet LAMP system. When we compared the real time detection speed of

both our wet and dry MeG MTB-LAMP (Table 2 & 3) with that of real time turbidity result of the previously reported MTB-LAMP [10], the time detection speed was equivalent suggesting that 0.004% MeG does not interfere with the LAMP reaction. It is estimated that the weight of 4.4 mega base pairs of one MTB bacterial genome is 5 fg. As our LAMP method detected to 50 fg of DNA, this suggest that as few as 10 MTB bacilli could be detected using our method.

Initially, we validated our in-house developed dry MeG MTB-LAMP system by using 142 clinical samples [105 sputum and 37 extra-pulmonary (pus, urine, pleural fluid, biopsy tissue, broncho-alveolar lavage, fine needle aspiration sample, bone marrow)] that were submitted for TB testing at GENETUP Nepal (Table S1). However, to calculate sensitivity and specificity in comparison with the culture results, we excluded 73 samples from patients who were under-treatment, re-treatment or treatment failure, or those samples from patients with unknown treatment history; these samples can provide discrepant results when compared with culture results because of the possibility of the presence of dead MTB bacteria that can be detected by MTB-LAMP but not by culture (Table S1). Thus, for a targeted evaluation of our method, we included only 69 samples from new TB suspected cases (Table S1) and found the sensitivity and specificity when compared with culture results to be 92.8% and 96.3% respectively (Table 4). Our result is superior than the results of a summary of 26 different MTB-LAMP studies that were conducted globally where overall sensitivity and specificity was 89.6% and 94.0% respectively [23]. When we compared the result of MTB-LAMP and smear microscopy of those 69 new suspect TB samples, sensitivity and specificity was 92.3% and 94.6% respectively (Table 4). The advantage of our MTB-LAMP system over smear microscopy is its ability to detect only MTB complex bacteria and to distinguish MTB complex from nontuberculous mycobacteria (NTM). Two retreatment samples that were smear positive/negative, both culture positive and later identified as NTM by a line probe

assay (Hain Lifesciences GmbH) were negative by our MTB-LAMP system (Table S1). From these results, we suggest that our dry MeG MTB-LAMP method could be performed together with smear microscopy as a POC TB diagnosis method.

In this study, we have identified MeG, methyl green, as a novel candidate for simple and visual detection of LAMP reactions. We have simplified and validated our MeG-based MTB-LAMP visual detection system by preparing dried reagents and demonstrated its potential for the diagnosis of TB in developing countries. Further improvement of our dry MeG-based MTB-LAMP system will help to develop it as an effective POC test. Furthermore, MeG is a suitable candidate for simple visual detection of LAMP reactions and can be applied to wide range of other infectious diseases or other diagnostic areas that employ LAMP reactions.

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Conflicts of interest

None to declare

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414 Table 1. Comparison of dyes used for visual detection of LAMP reaction

	Dyes	Detection methods			Reference
		Naked eye	Ultraviolet fluorescence	Inhibit LAMP if added before reaction	
	Propidium Iodide	Yes	Yes (enhanced)	Yes	[13]
	Pico green	Yes	NA	Yes	[11]
*	Hydroxynaphthol blue	Yes	Not required	No	[15]
*	Malachite green (MaG)	Yes	Not required	No	[16]
*	Eiken fluorescent dye	Yes	Yes (enhanced)	No	[12]
*	Methyl green (MeG)	Yes	Not required	No	This study

415 *: Dyes selected for comparison in this study

416 NA: Not available

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Table 2. Time in minutes required for the LAMP solution to exceed the turbidity of 0.1 in real time turbidimeter (LA-200, Teramecs) with different concentrations of dyes and DNA

DNA concentration	0.05%			Eiken
	Methyl green	Malachite green	Hydroxynaphthol blue	fluorescent dye
5 pg	14.4±2.4	14.3±1.4	14.5±1.4	15.4±0.3
500 fg	18.6±2.1	21.9±7.4	18.8±3.7	21.1±4.4
50 fg	37.6±16.5§(4 of 6)	21.6* (1 of 6)	31.6* (1 of 6)	23.7±2.4# (5 of 6)
NC	NA	NA	NA	NA
	0.1%			Eiken
	Methyl green	Malachite green	Hydroxynaphthol blue	fluorescent dye
5 pg	16.5±1.4	15±1.9	17.6±4.1	15.4±0.3
500 fg	20.4±1.3	20.2±1.8	25.3±7.3# (4 of 6)	21.1±4.4
50 fg	32.6±13§ (4 of 6)	25±4.5† (2 of 6)	54.6±3.3† (2 of 6)	23.7±2.4# (5 of 6)
NC	NA	NA	NA	NA

Data is presented as mean ± standard deviation

LAMP reaction was performed in duplicate in three independent experiments. Sample size is 6, unless it is indicated. Information in parenthesis indicate number of times results of LAMP reaction were obtained out of total 6 reactions.

*sample size = 1, † sample size = 2, §sample size = 4, # sample size = 5, these sample size indicate result of positive LAMP reaction.

426 NC= Negative control, DDW

427 NA= not available, inability of turbidity to exceed 0.1 within 60 minutes of reaction.

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Table 3. Time in minutes required for the dried LAMP system to exceed the turbidity of 0.1 in real time turbidimeter (LA-200, Teramecs) with different concentration of DNA

DNA concentration	methyl green 0.1%
5 pg	20.7±1.7
500 fg	27.5±5.6
50 fg	31±4.2[§](4 of 6)
NC	NA

Data is presented as mean ± standard deviation

Sample size is 6, unless otherwise indicated. Information in parenthesis indicates the number of times results of the LAMP reaction were obtained out of a total 6 reactions.

§sample size = 4 as 4 reactions were positive.

NC= Negative control, DDW

NA= not available, inability of turbidity to exceed 0.1 within 60 minutes of reaction

438 Table 4. Comparison of sensitivity and specificity of dry MTB-LAMP, microscopy and culture for diagnosis of TB

		Culture			Sensitivity %	Specificity %
		Positive	Negative	Total		
	Positive	13	2	15		
MTB-LAMP	Negative	1	53	54	92.8	96.3
	Total	14	55	69		
		Microscopy				
		Positive	Negative	Total		
	Positive	12	3	15		
MTB-LAMP	Negative	1	53	54	92.3	94.6
	Total	13	56	69		

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Figure legends

Fig.1. **Flow chart of procedure of methyl green based dry *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP).** The LAMP reagents are dried on the of inner side of a microtube lid. The dried reagents and DNA are reconstituted, mixed and the reaction mixture incubated at 64°C for 60 minutes to perform the LAMP reactions.

Fig.2. **Comparison of colorimetric detection of *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP) reaction by methyl green (MeG), malachite green (MaG), hydroxynaphthol (HNB) and Eiken fluorescent dye.** Both MeG and MaG showed blue-green color with positive LAMP reaction and colorless with negative LAMP reaction, thus offering easy visual detection of LAMP positive reactions with the naked eye. However, HNB and Eiken dye generated smaller color differences between positive and negative LAMP reactions.

S. No	Lab ID	Patient treatment category	Smear	Culture	Dry MTB-LAMP	Sample	Confirmation
1	1207A	Retreatment	Negative	1+	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
2	1260A	New	Negative	Negative	Negative	sputum	
3	1282B	Retreatment	1+	2+	Positive	sputum	
4	1285	Cat I- 6 month	2+	2+	Positive	sputum	
5	1359A	Retreatment	Negative	Negative	Negative	sputum	
6	1382A	New	Negative	Negative	Negative	sputum	
7	1383A	New	Negative	Contamination	Negative	sputum	
8	1448A	New	1+	1+	Positive	sputum	
9	1451A	New	1+	3+	Positive	sputum	
10	1470F	Cat II-3 month	2+	Negative	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
11	1512A	New	1+	1+	Positive	sputum	
12	1538A	Retreatment	1+	4 Colonies	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
13	1498A	Retreatment	2+	1+	Positive	sputum	
14	1549A	New	1+	1+	Positive	sputum	
15	1509B	Retreatment	1+	1+	Positive	sputum	
16	1621A	Retreatment	5AFB	1+	Positive	sputum	
17	1744A	Retreatment	2+	3+	Positive	sputum	
18	1746A	Retreatment	3+	3+	Positive	sputum	
19	1806A	Retreatment	1+	Negative	Positive	sputum	
20	1862A	Retreatment	3+	5 Colonies	Positive	sputum	
21	1863A	Retreatment	2+	Contamination	Positive	sputum	
22	1867B	New	Negative	Negative	Negative	sputum	
23	1871A	New	Negative	Negative	Negative	sputum	
24	1880A	New	Negative	Negative	Negative	sputum	
25	1896A	Retreatment	Negative	1+	Positive	sputum	
26	1900A	New	Negative	Negative	Negative	sputum	
27	1901A	New	Negative	Negative	Negative	sputum	
28	1968	Retreatment	Negative	Negative	Negative	sputum	
29	1841A	New	Negative	Negative	Negative	sputum	
30	1941A	New	1+	2+	Positive	sputum	
31	1959A	Retreatment	1+	1+	Positive	sputum	
32	1966A	New	Negative	Negative	Negative	sputum	
33	1978A	New	Negative	Negative	Negative	sputum	
34	2053A	New	Negative	Negative	Positive	sputum	
35	2108A	New	1+	1+	Positive	sputum	
36	2175B	New	6AFB	1+	Positive	sputum	

37	2233A	New	Negative	Negative	Negative	sputum	
38	2192A	New	Negative	Negative	Negative	sputum	
39	2202A	Retreatment	Negative	Negative	Negative	sputum	
40	1833	Retreatment	Negative	Negative	Positive	sputum	MTB by Genexpert (Cepheid, CA)
41	2526A	Retreatment	1+	2+	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
42	2520A	Retreatment	Negative	Negative	Negative	sputum	
43	2423B	Retreatment	3+	3+	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
44	2404A	New	Negative	Negative	Negative	sputum	
45	2402A	New	Negative	Negative	Negative	sputum	
46	2385A	Retreatment	Negative	Negative	Negative	sputum	
47	2321A	Retreatment	Negative	1+	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
48	2320A	Retreatment	Negative	7 Colonies	Negative	sputum	NTM by Hain LPA (Hain Lifesciences GmBH)
49	2279A	New	Negative	Negative	Negative	sputum	
50	2275A	Retreatment	1+	1+	Positive	sputum	
51	2285	New	Negative	Negative	Negative	sputum	
52	2569A	Retreatment	Negative	Negative	Negative	pleural fluid	
53	2568A	New	Negative	Negative	Negative	pus	
54	2534A	New	Negative	Negative	Negative	urine	
55	2501A	Retreatment	Negative	Negative	Negative	pus	
56	2499A	New	Negative	Negative	Negative	pleural fluid	
57	2380A	New	Negative	Negative	Negative	pleural fluid	
58	2216A	New	Negative	Negative	Negative	urine	
59	2086A	New	Negative	Negative	Negative	pleural fluid	
60	2074A	New	Negative	Negative	Negative	pleural fluid	
61	1446A	New	Negative	Negative	Negative	pus	
62	1855A	Retreatment	Negative	Negative	Positive	pus	
63	1868A	New	Negative	Negative	Negative	pleural fluid	
64	1884A	New	Negative	Negative	Negative	urine	
65	2000A	New	Negative	Negative	Negative	pleural fluid	
66	2001A	New	Negative	Negative	Negative	pleural fluid	
67	2010A	Retreatment	1+	Negative	Positive	pus	
68	2058A	Retreatment	Negative	Negative	Negative	pleural fluid	
69	2061A	New	Negative	Negative	Negative	pleural fluid	
70	1856A	New	Negative	Negative	Negative	bonemarrow	
71	1827A	New	Negative	Negative	Negative	Biopsy tissue	
72	1800A	New	Negative	Negative	Negative	BAL	
73	1795A	Cat I- 1 month	Negative	Negative	Negative	pleural fluid	

74	M23737	Unknown	2+	2+	Positive	sputum	
75	1108F3	Cat II-8 month	Negative	Negative	Negative	sputum	
76	1282B	Retreatment	2+	2+	Positive	sputum	
77	1341A	New	2+	1+	Positive	sputum	
78	1302A	Retreatment	Negative	Negative	Negative	sputum	
79	1341B	New	5 AFB	2+	Positive	sputum	
80	1312B	New	Negative	Negative	Negative	sputum	
81	1415A	Retreatment	2+	3+	Positive	sputum	
82	1512B	New	1 AFB	1+	Positive	sputum	
83	2053B	New	Negative	Negative	Positive	sputum	
84	1498B	Retreatment	2+	1+	Positive	sputum	
85	1806B	Retreatment	4AFB	Negative	Positive	sputum	
86	1790F	Cat II-3 month	1+	Negative	Negative	sputum	MTB by Genexpert (Cepheid, CA)
87	1866A/B	New	Negative	Negative	Negative	sputum	
88	1902A	Retreatment	Negative	1+	Positive	sputum	
89	1958A	Retreatment	3+	1+	Positive	sputum	
90	1833A	New	Negative	Negative	Negative	sputum	
91	1992F	New	Negative	1+	Positive	sputum	
92	2175A	New	2+	Contamination	Negative	sputum	
93	2183A	New	Negative	Negative	Negative	sputum	
94	M24017	Unknown	1+	1+	Positive	sputum	
95	M23650	Unknown	1+	1+	Positive	sputum	
96	M23647	Unknown	3+	3+	Positive	sputum	
97	M23719	Unknown	3AFB	3 Colonies	Positive	sputum	
98	2142A	Retreatment	Negative	1+	Positive	sputum	
99	1367A	Retreatment	Negative	Negative	Negative	sputum	
100	1188A	Retreatment	3+	3+	Negative	sputum	NTM by Hain LPA (Hain Lifesciences GmBH)
101	1359B	Retreatment	Negative	Contamination	Negative	sputum	
102	1372	Cat I-6 month	Negative	Negative	Negative	sputum	
103	1251A	New	Negative	Negative	Negative	sputum	
104	1235B	Retreatment	Negative	Negative	Negative	sputum	
105	1400A	Unknown	1+	Negative	Positive	sputum	
106	1538B	Retreatment	6AFB	Negative	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
107	1964A	New	Negative	Negative	Negative	sputum	
108	1880B	New	Negative	Negative	Negative	sputum	
109	1483F2	Cat I- 2 month	Negative	Negative	Negative	pus	
110	1833	Retreatment	Negative	Negative	Negative	sputum	

111	1435	New	Negative	Negative	Negative	pleural fluid	
112	1483F1	Cat I- 2 month	Negative	Negative	Negative	pus	
113	1387A	New	Negative	Negative	Negative	endometrium tissue	
114	1331F	Cat I- 2 month	Negative	Negative	Negative	sputum	
115	2049A	Cat I- 1 month	Negative	Negative	Negative	sputum	
116	1939	New	Negative	Negative	Negative	urine	
117	1848A	Retreatment	Negative	Contamination	Negative	urine	
118	1427A	Retreatment	Negative	Negative	Negative	FNAC sample	
119	1748F	Cat II-3 month	Negative	Negative	Negative	pleural fluid	
120	1896B	Retreatment	Negative	Negative	Negative	sputum	
121	2141A	New	Negative	Negative	Negative	pleural fluid	
122	1985A	New	Negative	Negative	Negative	pus	
123	1346F2	Cat II-5 month	Negative	Negative	Negative	pleural fluid	
124	2200A	New	Negative	Negative	Negative	bonemarrow	
125	M23738	Cat I- 5 month	3+	Negative	Positive	sputum	
126	1193A	New	Negative	Negative	Negative	sputum	
127	1260	New	Negative	Negative	Negative	sputum	
128	1359A	Retreatment	Negative	Negative	Negative	sputum	
129	1867A	New	Negative	Negative	Negative	sputum	
130	1450F2	Cat I-2 month	1+	Negative	Negative	sputum	MTB by Hain LPA (Hain Lifesciences GmBH)
131	1302	Retreatment	Negative	Negative	Negative	sputum	
132	2510A	New	Negative	Negative	Negative	sputum	
133	2412B	New	4 AFB	1+	Negative	sputum	
134	2367A	Retreatment	1+	Negative	Positive	pleural fluid	MTB by Hain LPA (Hain Lifesciences GmBH)
135	H41	New	Negative	Negative	Negative	urine	
136	H42	New	Negative	Negative	Negative	urine	
137	3288C	Retreatment	9AFB	Positive	Positive	sputum	
138	3392	Retreatment	Negative	Negative	Negative	sputum	
139	3401A	New	Negative	Negative	Negative	sputum	
140	3395A	New	1+	3+	Positive	sputum	
141	2299C	New	Negative	Negative	Negative	sputum	
142	3270C	New	3+	3+	Positive	sputum	

Smear and culture results are interpreated according to WHO guidelines http://www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf

AFB: Acid fast bacilli

MTB: *Mycobacterium tuberculosis*

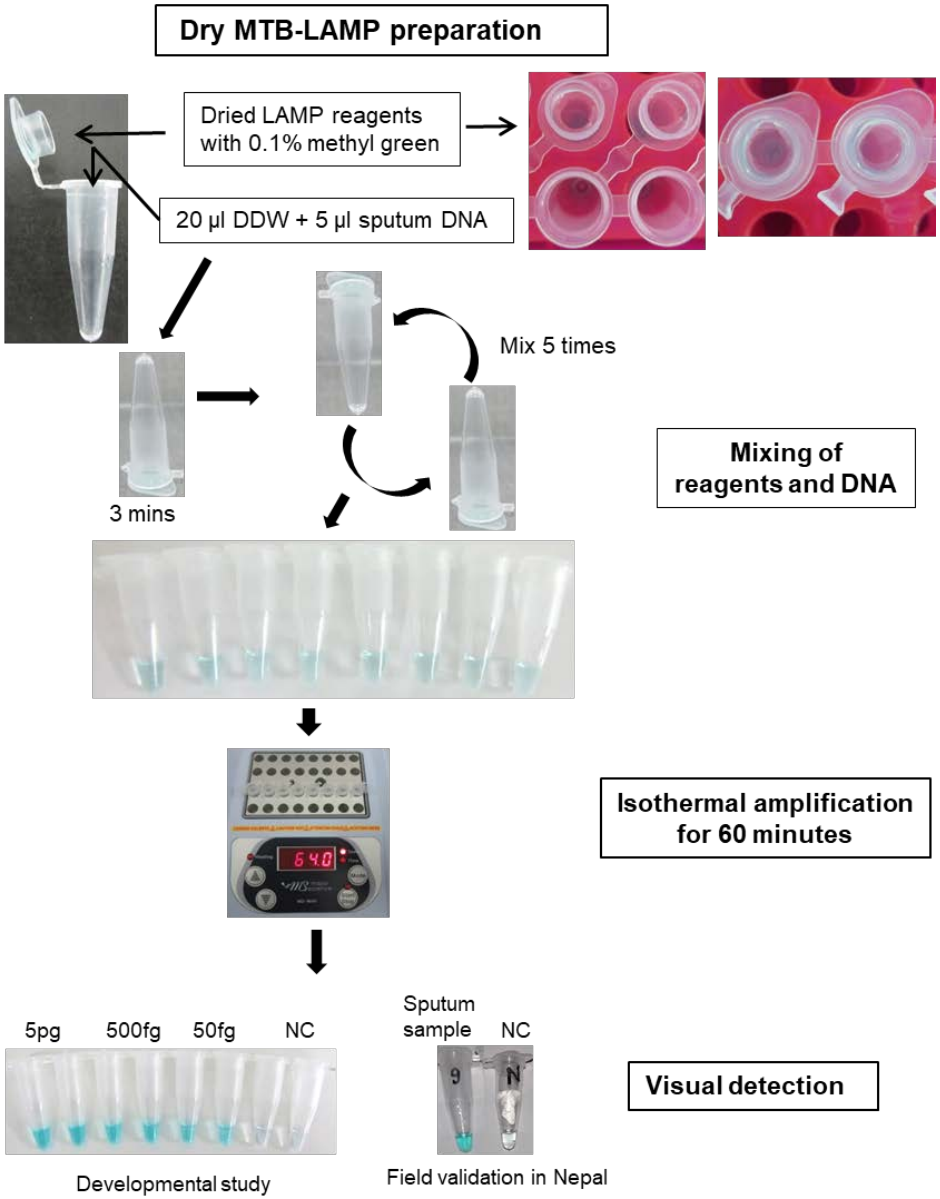
NTM: Nontuberculous mycobacteria

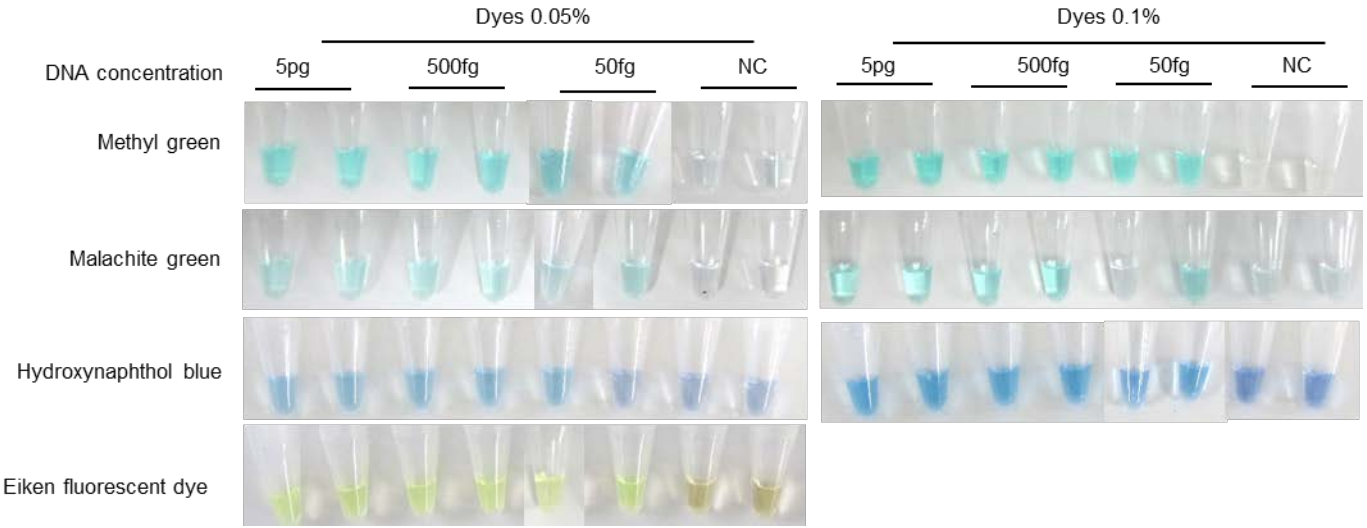
BAL: Bronchoalveolar lavage

FNAC: Fine needle aspiration cytology

S. No	Lab ID	Patient treatment category	Smear	Culture	Dry MTB-LAMP	Sample
1	1260A	New	Negative	Negative	Negative	sputum
2	1382A	New	Negative	Negative	Negative	sputum
3	1448A	New	1+	1+	Positive	sputum
4	1451A	New	1+	3+	Positive	sputum
5	1512A	New	1+	1+	Positive	sputum
6	1549A	New	1+	1+	Positive	sputum
7	1867B	New	Negative	Negative	Negative	sputum
8	1871A	New	Negative	Negative	Negative	sputum
9	1880A	New	Negative	Negative	Negative	sputum
10	1900A	New	Negative	Negative	Negative	sputum
11	1901A	New	Negative	Negative	Negative	sputum
12	1841A	New	Negative	Negative	Negative	sputum
13	1941A	New	1+	2+	Positive	sputum
14	1966A	New	Negative	Negative	Negative	sputum
15	1978A	New	Negative	Negative	Negative	sputum
16	2053A	New	Negative	Negative	Positive	sputum
17	2108A	New	1+	1+	Positive	sputum
18	2175B	New	6AFB	1+	Positive	sputum
19	2233A	New	Negative	Negative	Negative	sputum
20	2192A	New	Negative	Negative	Negative	sputum
21	2404A	New	Negative	Negative	Negative	sputum
22	2402A	New	Negative	Negative	Negative	sputum
23	2279A	New	Negative	Negative	Negative	sputum
24	2285	New	Negative	Negative	Negative	sputum
25	2568A	New	Negative	Negative	Negative	pus
26	2534A	New	Negative	Negative	Negative	urine
27	2499A	New	Negative	Negative	Negative	pleural fluid
28	2380A	New	Negative	Negative	Negative	pleural fluid
29	2216A	New	Negative	Negative	Negative	urine
30	2086A	New	Negative	Negative	Negative	pleural fluid
31	2074A	New	Negative	Negative	Negative	pleural fluid
32	1446A	New	Negative	Negative	Negative	pus
33	1868A	New	Negative	Negative	Negative	pleural fluid
34	1884A	New	Negative	Negative	Negative	urine
35	2000A	New	Negative	Negative	Negative	pleural fluid
36	2001A	New	Negative	Negative	Negative	pleural fluid
37	2061A	New	Negative	Negative	Negative	pleural fluid
38	1856A	New	Negative	Negative	Negative	bonemarrow
39	1827A	New	Negative	Negative	Negative	Biopsy tissue
40	1800A	New	Negative	Negative	Negative	BAL
41	1341A	New	2+	1+	Positive	sputum
42	1341B	New	5 AFB	2+	Positive	sputum
43	1312B	New	Negative	Negative	Negative	sputum
44	1512B	New	1 AFB	1+	Positive	sputum
45	2053B	New	Negative	Negative	Positive	sputum
46	1866A/B	New	Negative	Negative	Negative	sputum
47	1833A	New	Negative	Negative	Negative	sputum
48	1992F	New	Negative	1+	Positive	sputum
49	2183A	New	Negative	Negative	Negative	sputum
50	1251A	New	Negative	Negative	Negative	sputum
51	1964A	New	Negative	Negative	Negative	sputum
52	1880B	New	Negative	Negative	Negative	sputum
53	1435	New	Negative	Negative	Negative	pleural fluid
54	1387A	New	Negative	Negative	Negative	endometrium tissue

55	1939	New	Negative	Negative	Negative	urine
56	2141A	New	Negative	Negative	Negative	pleural fluid
57	1985A	New	Negative	Negative	Negative	pus
58	2200A	New	Negative	Negative	Negative	bonemarrow
59	1193A	New	Negative	Negative	Negative	sputum
60	1260	New	Negative	Negative	Negative	sputum
61	1867A	New	Negative	Negative	Negative	sputum
62	2510A	New	Negative	Negative	Negative	sputum
63	2412B	New	4 AFB	1+	Negative	sputum
64	H41	New	Negative	Negative	Negative	urine
65	H42	New	Negative	Negative	Negative	urine
66	3401A	New	Negative	Negative	Negative	sputum
67	3395A	New	1+	3+	Positive	sputum
68	2299C	New	Negative	Negative	Negative	sputum
69	3270C	New	3+	3+	Positive	sputum


Dry MTB-LAMP preparation
Dried LAMP reagents with 0.1% methyl green
20 µl DDW + 5 µl sputum DNA
Mix 5 times
3 mins
Mixing of reagents and DNA
Isothermal amplification for 60 minutes
5pg
500fg
50fg
NC
Developmental study
Sputum sample
NC
Field validation in Nepal
Visual detection


Dyes 0.05%
Dyes 0.1%
DNA concentration
5pg
500fg
50fg
NC
5pg
500fg
50fg
NC
Methyl green
Malachite green
Hydroxynaphthol blue
Eiken fluorescent dye