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Diagnostics

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

Jeewan Thapa[a,*,1], Bhagwan Maharjan[b,c,d,1], Meena Malla[e], Yukari Fukushima[b], Ajay Poudel[f], Basu Dev Pandey[g], Kyoko Hyashida[h], Stephen V. Gordon[i,j], Chie Nakajima[b,j], Yasuhiko Suzuki[b,j,**]

[a] Department of Medical Laboratory Sciences, Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-ku, Sapporo, Hokkaido, 060-0812, Japan
[b] Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo, Hokkaido, 001-0020, Japan
[c] German Nepal Tuberculosis Project, Kalimati, Kathmandu, Nepal
[d] Healthy Nepal, Balkhu, Kathmandu, Nepal
[e] Shi-Gan International College of Science and Technology, Kathmandu, Nepal
[f] Department of Microbiology, Chitwan Medical College Teaching Hospital, Chitwan, Nepal
[g] Ministry of Health and Population, Kathmandu, Nepal
[h] Division of Collaboration and Education, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo, Hokkaido, 001-0020, Japan
[i] UCD School of Veterinary Medicine, University College Dublin, Dublin, D04 W6F6, Ireland
[j] The Global Station for Zoonosis Control, Hokkaido University Global Institution for Collaborative Research and Education, Kita 20 Nishi 10, Kita-ku, Sapporo, Japan

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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.

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

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

* Corresponding author. Department of Medical Laboratory Sciences, Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-ku, Sapporo, Hokkaido, 060-0812, Japan.

** Corresponding author. Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo, Hokkaido, 001-0020, Japan.

E-mail addresses: jeewan@hs.hokudai.ac.jp (J. Thapa), suzuki@czc.hokudai.ac.jp (Y. Suzuki).

[1] JT and BM equally contributed.

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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: 0.2 µM of each outer primer (F3 & B3); 1.6 µM each inner primer (FIP & BIP) and 2.2 µM of loop primer (FLP & BLP); 1 mM dNTPs; 0.8 M betaine; 20 mM Tris-HCl (pH 8.8); 8 mM KCl; 8 mM $(NH_4)_2SO_4$; 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 pg (pg), 500 fg (fg) or 50 fg per tube was used for the LAMP reactions. The mixture was incubated at 64 °C for 60 min 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 min. 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 min 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 (25 mM each); 2.5 µl of 2M trehalose; 1 µl of 25 × LAMP buffer (500 mM Tris-HCl [pH 8.8], 250 mM KCl); 1.8 µl 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 h (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 min 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 min at 95 °C) and freezing (30 min 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

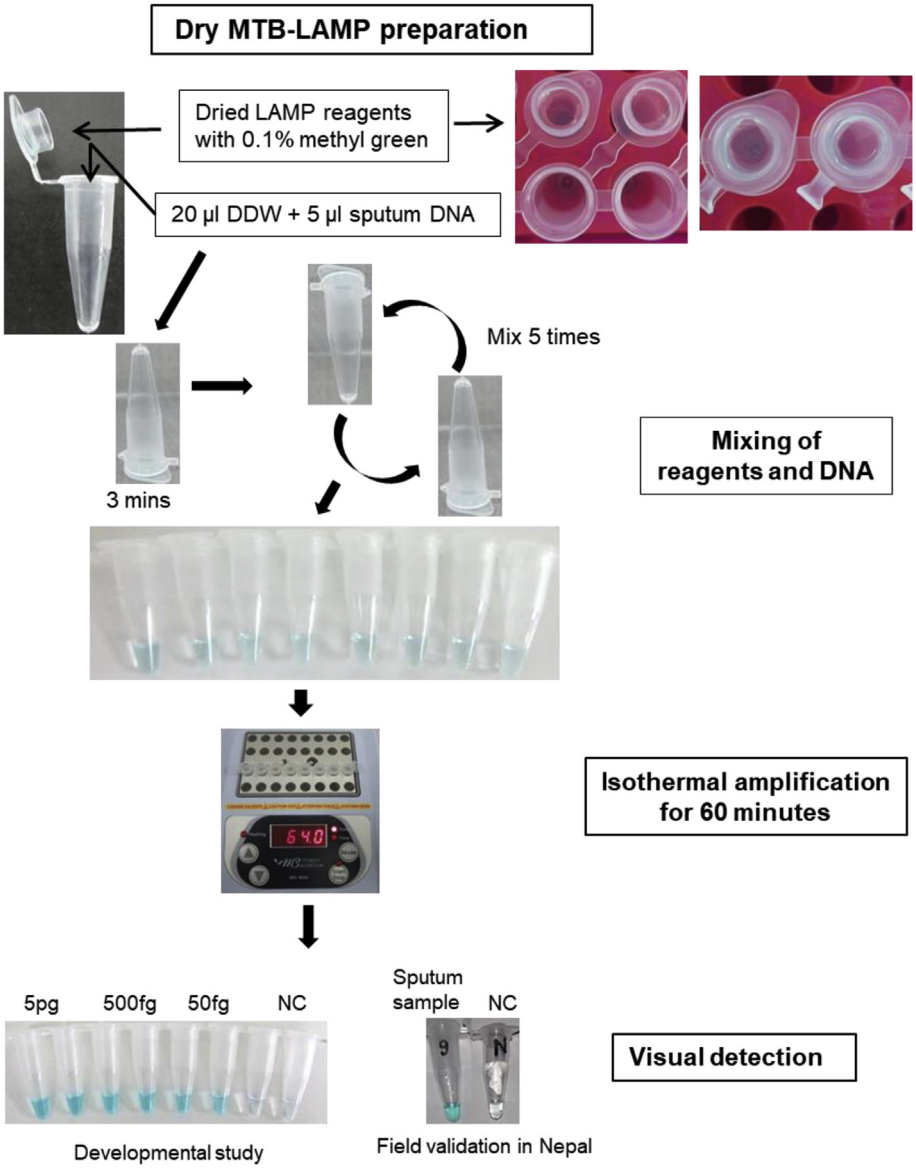

Fig. 1. Flow chart of procedure of methyl green based dry *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP). The LAMP reagents are dried on the of inner side of a microtube lid. The dried reagents and DNA are reconstituted, mixed and the reaction mixture incubated at 64 °C for 60 min to perform the LAMP reactions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 25 µl 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].

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

	Dyes	Detection methods			
		Naked eye	Ultraviolet fluorescence	Inhibit LAMP if added before reaction	Reference
	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

*: Dyes selected for comparison in this study.
NA: Not available.

3.3. Development of dry methyl green MTB-LAMP

We identified a concentration of 0.1% MeG (final concentration of 0.004% in a 25 μl 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 (50 fg) 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

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 fluorescent dye
	Methyl green	Malachite green	Hydroxynaphthol blue	
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[c](4 of 6)	21.6[a] (1 of 6)	31.6[a] (1 of 6)	23.7 ± 2.4[d] (5 of 6)
NC	NA	NA	NA	NA
	0.1%			Eiken fluorescent dye
	Methyl green	Malachite green	Hydroxynaphthol blue	
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[d] (4 of 6)	21.1 ± 4.4
50 fg	32.6 ± 13[c] (4 of 6)	25 ± 4.5[b] (2 of 6)	54.6 ± 3.3[b] (2 of 6)	23.7 ± 2.4[d] (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.
NC = Negative control, DDW.
NA = not available, inability of turbidity to exceed 0.1 within 60 min of reaction.
[a] Sample size = 1.
[b] Sample size = 2.
[c] Sample size = 4.
[d] Sample size = 5, these sample size indicate result of positive LAMP reaction.

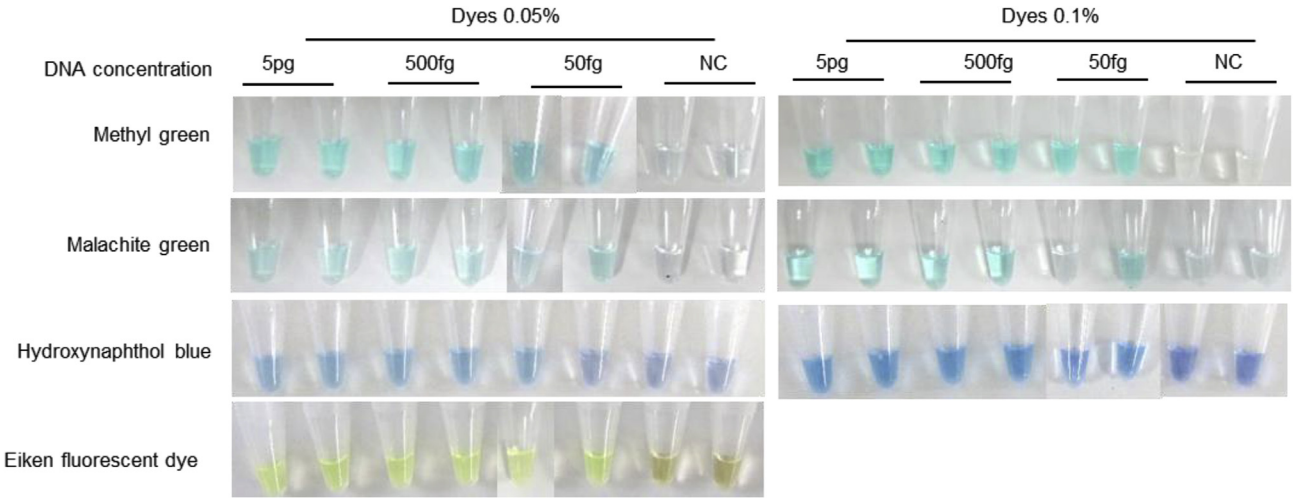

Fig. 2. Comparison of colorimetric detection of *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP) reaction by methyl green (MeG), malachite green (MaG), hydroxynaphthol (HNB) and Eiken fluorescent dye. Both MeG and MaG showed blue-green color with positive LAMP reaction and 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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[a](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.
NC= Negative control, DDW.
NA = not available, inability of turbidity to exceed 0.1 within 60 min of reaction.
[a] Sample size = 4 as 4 reactions were positive.

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 50 fg of TB DNA (Tables 2 and 3, Figs. 1 and 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 (Tables 2 and 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

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

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

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.

Conflicts of interest

None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.05.004.

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