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Original Article

Rapid Identification of *Mycobacterium tuberculosis* in BACTEC MGIT960 Cultures by In-House Loop-Mediated Isothermal Amplification

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SUMMARY: Definitive diagnosis of tuberculosis (TB) by conventional culture, followed by bacterial identification based on biochemical tests is time-consuming and tedious. Simple loop-mediated isothermal amplification (LAMP) specific for *Mycobacterium tuberculosis* complex, targeting the *M. tuberculosis* 16S ribosomal RNA gene, termed TB-LAMP, was evaluated as an alternative for rapid culture confirmation. TB-LAMP was assessed for its ability to detect *M. tuberculosis* complex in BACTEC MGIT 960-positive cultures. Of the 103 cultures evaluated, 100 were identified to contain *M. tuberculosis* complex by TB-LAMP and had concordant results with standard biochemical tests of niacin accumulation, nitrate reductase, lack of heat-stable catalase, and susceptibility to para-nitrobenzoic acid. These results indicate that TB-LAMP in combination with BACTEC MGIT 960 is a specific, reliable, and technically feasible method for rapid and accurate identification of *M. tuberculosis* complex.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a globally prevalent life-threatening disease, accounting for approximately 9.4 million new cases and 1.7 million deaths per year worldwide (1). Among the countries substantially affected by TB, Thailand ranks 18th on the list of 22 ''TB high-burden countries'' in the world, with nearly 70,000 new TB cases estimated to occur annually. The introduction of the Directly Observed Treatment, Short-course (DOTS) method has reduced the number of deaths; however, about 12,000 patients in Thailand still died from TB in 2009 (1).

Rapid and accurate diagnosis, followed by prompt treatment and completed therapy, is essential for the effective control of TB. In most developing countries, a TB diagnosis depends on clinical examination, radiological findings, sputum microscopy, and bacterial culture. Direct microscopic examination of clinical specimens still plays an important role in the early diagnosis of TB, because of its rapidity, simplicity, and cost-effectiveness. However, it has limitations in sensitivity and the ability to differentiate *M. tuberculosis* complex (MTBC) from non-tuberculous mycobacteria (NTM). Culturing is considered the gold standard for diagnosis, with high specificity and sensitivity, although it takes time owing to the low growth rate of mycobacteria (2). Egg-based media are usually used, but liquid culture systems have proven to be better in terms of reduced time and increased sensitivity (3,4). Among broth-based systems, BACTEC MGIT 960, an automated cultivation and detection method based on a fluorescent oxygen sensor, has been widely used as an efficient and rapid procedure for detecting mycobacterial growth and for tesing drug susceptibility (5–8). However, this method still presents difficulty in differentiating MTBC from other mycobacteria. Disease-causing mycobacterial species must be identified rapidly and accurately to facilitate the choice of appropriate treatment regimen. New methods for the identification of *M. tuberculosis* from liquid culture are urgently needed to augment the laborious and time-consuming conventional methods based on growth characteristics and standard biochemical assays.

A number of DNA probes and nucleic acid amplification methods for detection of mycobacteria are effective, and many tests are now commercially available. These include the INNO-LiPA Mycobacteria assay (Innogenetics), Geno Type Mycobacterium (Hain Diagnostika), the Cobas Amplicor PCR system (Roche), the

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LCx *Mycobacterium tuberculosis* Ligase Chain Reaction Assay (Abbott Laboratories), BD ProbeTec strand displacement amplification (Becton Dickinson), and the Amplified *Mycobacterium tuberculosis* Direct Test (Gen-Probe Inc.) (9–12). Most are capable of identifying not only MTBC, but also a range of other mycobacteria; however, they are expensive and not easily implemented for routine use in TB endemic settings.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay capable of providing results faster than polymerase chain reaction (PCR)-based assays (13). LAMP generates a large amount of DNA with high specificity, allowing amplification to be detected by the naked eye as fluorescence or turbidity (13–17). LAMP has been used to detect a number of infectious organisms, including *M. tuberculosis* (18,19), and has been proposed as a useful tool for the rapid and accurate diagnosis of *M. tuberculosis* in resource-limited laboratories (20,21). In this study, we used an in-house LAMP assay to rapidly identify MTBC from MGIT culture in Thailand. The assay was straightforward, robust, accurate, and useful.

MATERIALS AND METHODS

Bacteria and sample processing: Bacterial strains used for evaluating the specificity of LAMP for TB (TB-LAMP) are listed in Table 1. Reference bacteria included various mycobacterial species and other bacteria. In addition, a panel of 10 clinical isolates of *M. tuberculosis* was included to serve as clinical reference strains. The clinical isolates were identified on the basis of their colony morphology, niacin accumulation, lack of nitrate reductase activity, lack of heat-stable catalase, and susceptibility to para-nitrobenzoic acid (PNB). Mycobacterial strains were cultivated in Middlebrook 7H11 medium (Becton Dickinson, Franklin Lakes, N.J., USA) while others were grown on appropriate media.

A total of 155 clinical specimens received for routine mycobacterial cultivation in the Clinical Mycobacteriology section in National Institute of Health, Thailand, were processed. Specimens consisted of 135 sputum and 20 biological samples other than sputum, such as cerebrospinal fluid (CSF), pleural fluid (PF), synovial fluid (SF), gastric fluid (GF), urine, vitreous humor (VH), and pus. Sputum specimens were homogenized and decontaminated following the standard *N*-acetyl-L-cysteine-sodium-hydroxide-sodium citrate method (2,22). In brief, 5–6 ml aliquots of the specimens were mixed with equal volumes of NALC-4% NaOH (0.5% *N*-acetyl-L-cysteine-4% sodium-hydroxide)-sodium citrate mixture in a 50 ml ridge-capped round-bottomed processing tube. This was mixed briefly by vortexing and incubated at 37°C for 15 min. After incubation, the mixture was neutralized with phosphate buffer (pH 6.8) up to a volume of 50 ml, followed by centrifugation at 3,000 × *g* for 10 min. The supernatant was decanted and the sediment was resuspended in 1 ml sterile phosphate-buffered saline (pH 6.8). Other biological specimens were inoculated directly into MGIT broth, except urine, which was subjected to decontamination prior to inoculation (2).

Mycobacterial culture and conventional identification: As part of the routine culture procedure, 0.1 ml cell suspension was inoculated on two Lowenstein-Jensen medium (L-J) slants (2.5 g of monopotassium phosphate, 0.24 g of magnesium sulfate, 0.6 g of sodium citrate, 3.6 g of L-asparagine, 30.0 g of potato flour, 12 ml of glycerol, 0.4 g of malachite green, 1,000 ml of homogenized whole egg, and 600 ml of distilled water) and 0.5 ml was inoculated into 7 ml BBL MGIT culture tubes (Becton Dickinson) supplemented with BBL MGIT PANTA antibiotic mixture and OADC enrichment (Becton Dickinson). L-J slants were incubated at 37°C and monitored for growth weekly for 8 weeks. MGIT cultures were incubated at 37°C in a BACTEC MGIT-960 instrument (Becton Dickinson) for 50 days. On the day of detection, MGIT tubes identified as positive by the instrument, with growth units of more than 75, were collected from the station. A 1-ml aliquot of each MGIT-positive culture was transferred to 1.5-ml microtube, followed by centrifugation at 10,000 × *g* for 5 min. The resulting sediments were resuspended in 50 μl distilled water. Culture smears were prepared from the suspension using the Ziehl-Neelsen (Z-N) method, and examined under a microscope for serpentine cording of acid-fast bacilli (AFB). The remainder was kept for DNA extraction and LAMP analysis. All MGIT cultures that tested positive for AFB were subcultured on two L-J slants for further identified by conventional tests of niacin accumulation, nitrate reduction, and catalase activity at 68°C, according to standard procedures (2,23). Susceptibility to PNB was also conducted to differentiate MTBC from NTM using the method described previously (23).

LAMP test: The in-house LAMP assay combined simple manual DNA extraction, isothermal amplification, and visual readout with fluorescence. A set of six primers recognizing eight distinct regions on the 16S ribosomal RNA gene of *M. tuberculosis* was used (19). The primers were composed of outer primers (F3: 5′-CTGGCTCAGGACGAACG-3′ and B3: 5′-GCTC ATCCCACACCGC-3′), inner primers (FIP: 5′-CA CCCACGTGTTACTCATGCAAGTCGAACGGAAA GGTCT-3′ and BIP: 5′-TCGGGATAAGCCTGGACC ACAAGACATGCATCCCGT-3′), and loop primers (FLP: 5′-GTTCGCCACTCGAGTATCTCCG-3′ and BLP: 5′-GAAACTGGGTCTAATACCGG-3′). TB-LAMP reactions were performed in a total volume of 20 μl consisting of 30 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol each of 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, Mass., USA), and 1 μl fluorescent detection reagent (FDR; Eiken Chemical Co., Tokyo, Japan) with 7.0 μl DNA lysates. Amplification was performed at 65°C for 60 min in a small heat block, followed by incubation at 80°C for 2 min to terminate the reaction. One negative control (including 7.0 μl of distilled water instead of lysates) and one positive control (including 7.0 μl of purified DNA of *M. tuberculosis* H37Ra [0.1 ng/μl] instead of lysates) were employed in all runs. LAMP results were examined directly by visual observation. The asssay was considered positive when

the color of the reaction mixture turned from orange to green or fluorescence was detected under a UV lamp. For further confirmation, 5 μl of some LAMP reaction mixtures were analyzed by agarose gel electrophoresis stained with ethidium bromide. Amplified DNA fragments were visualized under an ultraviolet (UV) transilluminator.

The specificity of TB-LAMP was evaluated using DNA extracted from cultivated bacteria listed in Table 1. Cell inactivation and genomic DNA preparation was performed by heat extraction. Briefly, one loop of colony was resuspended in 100 μl water. The suspension was then heated at 80°C in a dry block for 10 min. The supernatant obtained was used as the DNA template for LAMP reactions.

Identification of *M. tuberculosis* by TB-LAMP: The TB-LAMP assay was evaluated for hands-on time, reading endpoint stability, ease of use, and agreement with conventional identification of *M. tuberculosis*. DNA was extracted from the concentrated cell suspension left over from the culture smear preparation. Cell inactivation and DNA extraction was conducted by thermolysis at 80°C for 10 min. The supernatant of cell lysate was used directly in the TB-LAMP assay. Similarly, the LAMP reaction was performed as described above. The hands-on time was defined as the active working time required for examining 10 samples and 2 controls from sample preparation to the reading and recording of results. The results of *M. tuberculosis* identification by TB-LAMP were compared to those of standard biochemical tests, considered a reference identification method.

Identification by sequencing: In order to further verify the identification results of the TB-LAMP test, DNA sequencing of the partial internal transcribed spacer region (ITS) was performed on a small number of clinical isolates recovered from MGIT cultures. With primers of ITS-F: 5′-TGGATCCGACGAAGTCGTAA CAAGG-3′ and mycom-2: 5′-TGGATAGTGGTTGC GAGCAT-3′, a fragment of the highly variable region in the ITS of each isolate was amplified by PCR (24). The reaction contained 1.0 μl each of primer (stock concentration 10 μM), 200 mM deoxynucleotide triphosphate, PCR buffer, 2.5 U *Taq* polymerase (*i-Taq*; *iNtRon* Biotechnology, Inc., Kyungki-Do, Korea), and approximately 20–50 ng of DNA template in a total volume of 20 μl. PCR conditions were 94°C for 5 min, 30 cycles each of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; with a final extension at 72°C for 10 min. Cycle sequencing was performed with the BigDye terminator cycle sequencing ready reaction kit (Version 3.1; Life Technologies Co., Carlsbad, Calif., USA) and the

Table 1. Specificity of LAMP reaction for identification of *M. tuberculosis* complex

	Mycobacteria	TB-LAMP		Bacteria other than mycobacteria	TB-LAMP
1	*M. tuberculosis* H37Rv	+	30	*Achromobacter xylosoxidans* DMST 0803	−
2	*M. bovis* Ravenel	+	31	*Acinetobacter haemolyticus* DMST 1826	−
3	*M. bovis* BCG Tokyo	+	32	*Bacillus brevis* DMST 3653	−
4	*M. africanum* ATCC25420	+	33	*Bacillus cereus* DMST 1928	−
5	*M. microtti* TC77	+	34	*Corynebacterium genitalium* DMST 4366	−
6	*M. kansasii* ATCC12476	−	35	*Enterobacter aerogenes* DMST 2720	−
7	*M. kansasii* Bostrum	−	36	*Flavobacterium multivorum* DMST 0497	−
8	*M. shimoidei* ATCC27962	−	37	*Haemophilus influenzae* DMST 4560	−
9	*M. scrofulaceum* ATCC19981	−	38	*Legionella pneumophila* DMST 0455	−
10	*M. nonchromogenicum* ATCC19530	−	39	*Micrococcus roseus* DMST 4999	−
11	*M. gordonae* ATCC14470	−	40	*Neisseria gonorrhoeae* DMST 6209	−
12	*M. xenopi* ATCC19250	−	41	*Oligela ureolytica* DMST 2329	−
13	*M. chelonae* ATCC19977	−	42	*Pasteurella haemolytica* DMST 5018	−
14	*M. fortuitum* ATCC6841	−	43	*Pseudomonas aeruginosa* DMST 2960	−
15	*M. avium* ATCC17938	−	44	*Rhodococcus equi* DMST 2139	−
16	*M. avium* N238	−	45	*Saccharomyces cerevisiae* DMST 2958	−
17	*M. avium* N247	−	46	*Salmonella* Typhi DMST 1328	−
18	*M. avium* 01–16	−	47	*Shigella boydii* DMST 3395	−
19	*M. avium* P50	−	48	*Staphylococcus aureus* DMST 1339	−
20	*M. avium* Kirchberg	−	49	*Streptococcus agalactiae* DMST 4314	−
21	*M. intracellulare* ATCC15984	−	50	*Streptococcus pneumoniae* DMST 3264	−
22	*M. intracellulare* ATCC15985	−	51	*Vibrio vulnificus* DMST 1360	−
23	*M. intracellulare* ATCC15987	−	52	*Xanthomonas maltophilia* DMST 3322	−
24	*M. intracellulare* ATCC19075	−	53	*Bacillus sphaericus* DMST 4315	−
25	*M. intracellulare* ATCC19076	−	54	*Enterobacter cloacae* DMST 4546	−
26	*M. intracellulare* ATCC19078	−			
27	*M. smegmatis mc*^{2} 155	−			
28	*M. tuberculosis* clinical isolates (10)[1)]	+			
29	NTM clinical isolates (10)[1)]	−			

[1)]: Parentheses, number of clinical isolates tested.
ATCC, American Type Culture Collection.
DMST, Culture Collection from Department of Medical Sciences, Thailand.
TB-LAMP, loop-mediated isothermal amplification for *M. tuberculosis* detection.

resulting products were analyzed using an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies). Isolates were identified by comparison of ITS nucleotide sequences with those in the GenBank database.

RESULTS

Performance of TB-LAMP: The specificity of the TB-LAMP primer set was evaluated using DNA extracted from reference bacterial cultures (Table 1). We tested 27 reference mycobacterial strains, 10 clinical isolates of *M. tuberculosis*, 10 clinical isolates of NTM, and 25 non-mycobacterial species. TB-LAMP tests were positive with all MTBC strains after 60 min of incubation. No positive signal was observed for samples containing DNA of NTM or non-mycobacterial species (Table 1).

Mycobacterial growth detection by MGIT: Bacterial growth was recovered in MGIT broth from 101 sputum and 2 non-sputum biological specimens. AFB was detected by Z-N staining in all MGIT-positive cultures and no specimens were excluded from the study because of contamination with non-mycobacterial species. Rapid growth was observed in MGIT mycobacterial-positive cultures no less than 4 days after inoculation (Table 2). Time required for detection of mycobacterial growth by MGIT-960 and the rapid identification of *M. tuberculosis* in MGIT cultures by TB-LAMP is shown in Table 2. Eighty-four percent (82/98) of sputum samples were detected and confirmed to be *M. tuberculosis* positive within 20 days by combining MGIT system cultivation with the TB-LAMP assay.

Table 2. Time range of mycobaterial growth detection by BACTEC MGIT 960 and results of *M. tuberculosis* identification by TB-LAMP

Time range of growth detection (days)	No. of cultures grown from sputum	No. of samples positive by TB-LAMP
4–10	26	23
11–20	59	59
21–30	5	5
31–48	7	7
>48	4	4
Total	101	98

TB-LAMP, loop-mediated isothermal amplification for *Mycobacterium tuberculosis* detection.

Clinical efficiency of TB-LAMP in identification of *M. tuberculosis*: TB-LAMP results for 103 MGIT mycobacteria cultures positive for AFB were compared with conventional identification results derived from the analyses of niacin accumulation, nitrate reduction, heat-stable catalase activity, and susceptibility to PNB, as shown in Table 3. As controls, TB-LAMP was applied to MGIT cultures of *M. tuberculosis* H37Rv. TB-LAMP was positive in 98 samples from sputum and one each from CSF and urine samples. Of the three samples that were TB-LAMP negative, none contained *M. tuberculosis* based on biochemical and PNB susceptibility tests. Sequencing confirmed that two were *Mycobacterium avium* complex and the third was *Mycobacterium parascrofulaceum*. One sputum culture was positive based on the TB-LAMP result, while the conventional test suggested that the isolate was NTM. By colony morphological examination of the L-J medium, this sample was revealed to be a mixed culture of *M. tuberculosis* and NTM. Isolates subcultured and recovered on L-J medium had growth characteristics of *M. tuberculosis*. TB-LAMP identification gave concordant results with those of biochemical tests. Results of *M. tuberculosis* identification in 30 isolates by sequencing and LAMP were also concordant.

The average hands-on time for testing 10 samples was less than 20 min when DNA was added to LAMP premixture reaction tubes. Overall, the TB-LAMP assay could identify MTBC in a liquid-medium culture in less than 2 h; using 20 min for DNA extraction, 60 min for the LAMP reaction, and 1 min for detection. Reading endpoint stability was assessed to exclude possible nonspecific amplification during delays in recording results that might occur with routine use. The reading endpoint stability was monitored as the number of identical results read at 1 h, 2 h, and 12 h after the 60-min amplification period (data not shown). Positive results were stable; in that none of the positive samples became falsely negative within 12 h. Detection could be performed by visual observation and confirmed by gel electrophoresis (Fig. 1).

Table 3. Concordance between identification of *M. tuberculosis* by LAMP and biochemical characteristics of MGIT+ cultures

Specimen	No. of specimens	MGIT+	AFB+	TB-LAMP+	Biochemical tests			
					PNB−	Niacin+	Nitrate+	Catalase−
Sputum	135	101	101	98	97	97	97	97
Cerebrospinal fluid	9	1	1	1	1	1	1	1
Pleural fluid	2	0	ND	ND	ND	ND	ND	ND
Vitreous humor	1	0	ND	ND	ND	ND	ND	ND
Pus	1	0	ND	ND	ND	ND	ND	ND
Skin biopsy	3	0	ND	ND	ND	ND	ND	ND
Gastic wash	3	0	ND	ND	ND	ND	ND	ND
Urine	1	1	1	1	1	1	1	1
Total	155	103	103	100	99	99	99	99

MGIT, mycobacteria growth indicator tube; AFB, acid-fast bacilli (in culture smear); TB-LAMP, loop-mediated isothermal amplification for *Mycobacterium tuberculosis* detection; PNB, para-nitrobenzoic acid; ND, not done.

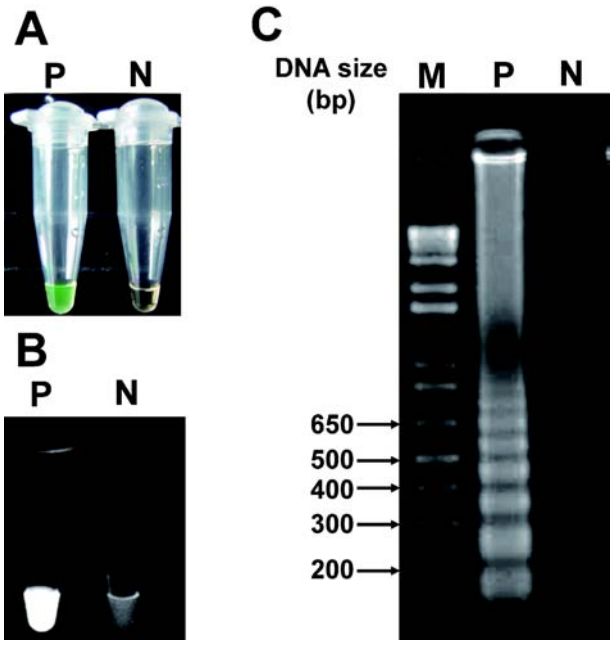

Fig. 1. Detection of TB-LAMP results in MGIT-positive cultures. (A) visual observation; (B) visualization under UV light; (C) agarose gel electrophoresis detection. P, positive; N, negative; M, markers.

DISCUSSION

The aim of this study was to assess the performance and applicability of the recently developed in-house TB-LAMP assay as a simple tool for the rapid identification of *M. tuberculosis* in culture. This study presents an alternative means for rapid definitive diagnosis of TB by combining MGIT cultivation of mycobacteria with TB-LAMP identification of MTBC. Because BACTEC MGIT 960 is increasingly being used in Thailand and elsewhere, this report was focused on MGIT mycobacterial cultures and identification using the TB-LAMP method.

The MGIT system is superior in sensitivity and turnaround time to egg-based media for detecting MTBC and potentially pathogenic mycobacteria (6–8). Although culturing gives important evidence to support clinical diagnoses, identification procedures are required to support the conclusion of *M. tuberculosis* presence within the culture. Presumptive identification of isolates recovered on solid media, such as L-J slant, can be performed based on colony morphology, but this cannot be applied with MGIT-positive cultures. In our previous study, TB-LAMP was used for direct detection of *M. tuberculosis* in sputum specimens (19); however, the direct detection of *M. tuberculosis* in sputum includes bacilli that died during anti-TB therapy. The detection of live bacilli is essential for the accurate diagnosis of active TB following proper treatment. The combination of TB-LAMP, which identifies *M. tuberculosis*, with MGIT culture, which reveals live mycobacteria, is another application of TB-LAMP that is expected to be useful.

It is important to identify *M. tuberculosis* as quickly as possible so that appropriate treatment can be prescribed. Traditionally, niacin detection is accepted as a key test for the identification of *M. tuberculosis* in routine practice. Results of biochemical tests, which include the nitrate reductase test, the heat-stable catalase test, and the PNB susceptibility test, have been considered for definitive identification. Mycobacterial growth in media containing PNB has been used to distinguish *M. tuberculosis* from NTM (5). The growth of *M. tuberculosis* is inhibited by PNB, whereas NTMs are resistant. It takes about 1–2 additional weeks of incubation to obtain a heavy growth culture suitable for reliable results, and a few more days to conduct biochemical tests; therefore, rapid detection by the MGIT system may be of limited benefit when combined with conventional identification.

The TB-LAMP assay, with its high specificity and ease of use, reduced the turnaround time for reporting results of *M. tuberculosis* identification in MGIT growth culture. This assay could be used with MGIT culture at an early growth stage (growth units as low as 75). The only equipment required for the LAMP reaction is a standard laboratory water bath or a small heat block that furnishes a constant temperature of 65°C. Results could be read with a simple UV lamp or by the naked eye, which considerably reduced costs. Visual detection eliminated the need for laborious and time-consuming post-amplification procedures, such as hybridization and electrophoresis, as well as the need for special equipment.

Compared with the standard biochemical test and PNB susceptibility, TB-LAMP was convenient, rapid, specific, and reliable for *M. tuberculosis* identification. There was no need to perform L-J subculture for TB-LAMP identification. As expected, the results of *M. tuberculosis* identification by TB-LAMP and by conventional tests were concordant. All clinical strains that were niacin positive and PNB negative were TB-LAMP positive and identified as *M. tuberculosis*. A discordant result was found in only one sample, which contained *M. tuberculosis* and other mycobacteria. TB-LAMP was positive in this sample, but niacin was negative and growth was observed on PNB culture. Although this sample was diagnosed as NTM infection based on the niacin test and PNB culture, the definitive diagnosis based on additional conventional tests and morphological examination on L-J medium was co-infection with both *M. tuberculosis* and NTM. Unlike the combination of MGIT with a conventional biochemical test, this result indicates that LAMP is able to identify *M. tuberculosis* in a mixed infection. Negative TB-LAMP results, identified in three samples to be NTM, were concordant with conventional tests. The evidence of co-infection led to the conclusion that there was no discrepancy in identification results by TB-LAMP and biochemical characteristics, including PNB susceptibility. Accordingly, application of the TB-LAMP test in the rapid identification of *M. tuberculosis* appeared to be reliable, even for mixed infections.

Since TB is one of the most important infectious diseases, a simple identification method using TB-LAMP would be a method of choice useful for TB control. From a practical point of view, TB-LAMP is suitable as a routine diagnostic tool, even in low-resource laboratories. The application of TB-LAMP, in combination with culture growth using MGIT, will enhance the mycobacterial diagnostic service by providing a rapid

means of identifying live *M. tuberculosis*. It might be used to aid in the identification of live *M. tuberculosis* in routine practice, and as a powerful tool for the early diagnosis of active TB.

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Conflict of interest None to declare.

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