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Evaluation of the Analytical Performance of the PURE-TB-LAMP Assay for Tuberculosis Detection

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ABSTRACT

Given the prevalence and lethality of tuberculosis (TB) in developing countries, there is an ongoing need for rapid, simple, and low-cost detection method that nonetheless sensitive and highly specific. The present study evaluated the basic performance of a novel TB detection method combining procedure for ultra-rapid extraction (PURE) and loop-mediated isothermal amplification for TB (TB-LAMP). The PUER-TB-LAMP assay detected four *Mycobacterium tuberculosis* complexes and did not show any cross-reactivity with 18 species of non-TB mycobacteria (NTM) or with 10 species of other bacteria that cause respiratory tract infections such as *Streptococcus pneumonia*, underscoring its high specificity for TB detection. The analytical sensitivity of the assay was 100 CFU/ml for *M. tuberculosis* strain H37Rv cell and was unaffected by the presence of excess amounts of *M. avium* cells (a typical NTM species) or human genomic DNA. Moreover, when used with a range of artificial specimens prepared by spiking known amounts of cultured *M. tuberculosis* cells, the PURE method efficiently removed various inhibitory materials from a variety of samples such as sputum, urine, simulated gastric fluid, and whole blood, demonstrating the applicability of this assay to these samples. These results suggest that the PURE-TB-LAMP assay is a highly effective and accessible TB detection method that can be useful in resource-limited communities.

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1. Introduction

Tuberculosis (TB) is among the most threatening infectious diseases in the world, with >75% of cases occurring in developing nations (WHO 2013). One of the major obstacles to controlling and eradicating TB in resource-limited countries is the lack of rapid, highly sensitive, and simple tests for the detection of the causative agent, *Mycobacterium tuberculosis*. Nucleic acid amplification tests (NAATs) have been considered as a potential means of surmounting this barrier (Huggett et al. 2009). The procedure for ultra-rapid extraction of TB by loop-mediated isothermal amplification (PURE-TB-LAMP) (Mori et al. 2013) is a new type of TB-NAAT that combines DNA extraction and amplification by LAMP (Notomi et al. 2000). The clinical performance of this assay-which is an approved in vitro diagnostic method for TB screening in Japan-has been previously evaluated (Mitarai et al. 2010) and the assay is routinely used in over 200 clinical settings. Since all steps of this assay are designed to be easy to perform and do not require any expensive equipment such as a thermal cycler—which is essential for PCR-based diagnostics—it is useful for the rapid detection of pulmonary as well as extrapulmonary TB (Kouzaki et al. 2015) at small testing facilities such as county-level microscopy centers (Ou et al. 2014). In this study, we evaluated the basic performance of the PURE-TB-LAMP assay, including its analytical sensitivity, cross-reactivity with bacteria other than those in the *M. tuberculosis* complex (MTBC), and the effect of sample components on assay performance.

2. Materials and methods

2.1. Bacterial strains and culture

Bacterial strains were purchased from RIKEN BioResource Center (Tsukuba, Japan), Microbiologics (St. Cloud, MN, USA), and The Research Institute of Tuberculosis (Tokyo, Japan) and included 26 strains of *Mycobacterium* as well as 10 of other bacteria, including respiratory pathogens (Table 1). Mycobacterial strains were inoculated in 2% OGAWA medium (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) and were cultured at 37°C, except for *M. haemophilum* JCM 15465 (30°C), *M. chelonae* JCM 6388 (28°C), and M. *marinum* JCM 17638 (25°C). Some of the non-mycobacterial strains were inoculated on 5% sheep blood agar (Eiken Chemical Co., Tokyo, Japan) and cultured in an atmosphere of 5% CO_2, including two Pseudomonas strains (30°C); *Escherichia coli, Klebsiella pneumoniae*, and *Staphylococcus aureus* (all at 35°C); and *Streptococcus pneumonia* and *Moraxella catarrhalis* (both at 35°C). *Haemophilus influenza* was inoculated on chocolate agar II (Eiken Chemical Co.) and cultured at 35°C in an atmosphere of 5% CO_2. *Legionella pneumophila* was inoculated on BCYEα (Eiken Chemical Co.) and cultured at 36°C. *M. pneumonia* was resuspended in glucose-enriched Hayflick mycoplasma medium based on PPLO broth (Difco Laboratories, Detroit, MI, USA) at 37°C in an atmosphere of 5% CO_2.

2.2. Preparation of M. tuberculosis genomic (g)DNA

Purified gDNA of *M. tuberculosis* was used to evaluate the lower detection limit of the PURE-TB-LAMP assay. gDNA was extracted from *M. tuberculosis* H37Rv KK11-291 using ISOPLANT II (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Following RNase treatment, purified DNA was resuspended in Tris-HCl (pH 8.0). The concentration was

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determined by measuring optical density at 260 nm using a spectrophotometer.

2.3. Bacterial cell suspensions

The cross-reactivity of the PURE-TB-LAMP assay was evaluated using bacterial cell suspensions. Bacteria were resuspended in phosphate buffer (PB) and washed twice by centrifugation. Cell suspensions were prepared at concentrations with a turbidity equivalent to the McFarland No. 1 standard, except for *M. pneumonia*, for which concentration was determined by real-time PCR as previously reported (Morozumi et al.2006).

2.4. Preparation of artificial samples

To prepare artificial samples with known concentrations of *M. tuberculosis* cell or gDNA, sputa, urine, 10% and 30% (v/v) blood in PB, and 10% (w/v) stool suspensions obtained from healthy volunteers were spiked with serially diluted *M. tuberculosis* H37Rv suspensions. Commercially available purified human gDNA (Promega, Madison, WI, USA) with known amounts (0.1 and 1.0 mg) as a substitute for human tissue and artificial gastric juice were also prepared. Samples were analyzed with the PURE-TB-LAMP assay after adding *M. tuberculosis* H37Rv to obtain a cell concentration of 300 CFU/ml.
To evaluate the effect of typical sample components, artificial samples were prepared as follows. Sputa with 300 CFU/ml *M. tuberculosis* H37Rv were mixed with 30% blood in PB (v/v) to produce artificial bloody sputa, and 1 mg of purified human gDNA was added to 1 ml of the same concentration of *M. tuberculosis* H37Rv cells to simulate mucous sputa with a high concentration of human gDNA. Simulated gastric fluid prepared in our laboratory according to a previous report (Ivy et al. 2012) and urine obtained from healthy volunteers were also spiked with dilute bacterial suspensions to a cell density of 300 CFU/ml.

2.5. The PURE-TB-LAMP assay

The PURE method, which was developed as a simple method for extracting/purifying DNA from samples, was carried out using the Loopamp PURE DNA Extraction kit (Eiken Chemical Co.) according to the manufacture's protocol and as previously described (Mitarai et al. 2011). Briefly, an aliquot of biological sample (including raw sputa) was added to an alkali-based extraction solution and heated for 5 min at 90°C to extract DNA from TB cells in the sample; the solution was then added to a silica-based adsorbent that binds inhibitors in the sample and neutralizes alkali without adsorbing DNA. After this treatment, the sample was added drop-wise to reaction tubes containing dried LAMP reagent inside tube caps in the Loopamp MTBC detection kit (Eiken Chemical Co.). Treated solutions were transferred to the caps to reconstitute the dried reagent, and the LAMP reaction was carried out in an LF-160 LAMP reaction incubator for 40 min at 67°C (Eiken Chemical Co.). Amplification results were obtained as "Yes/No" by detectingcalcein fluorescence (Tomita et al. 2008) with a blue light-emitting diode attached to the apparatus. The efficacy of the amplification reaction was also evaluated by measuring the time to positive (Tt) using an LA-500 under the same condition as LF-160.

3. Results and Discussion

3.1. Specificity

The specificity of the PURE-TB-LAMP assay for various strains of acid-fast bacteria was examined (Table 1). The four strains in the MTBC were detected by the PURE-TB-LAMP assay. On the other hand, there was no cross-reactivity with 18 species of non-TB mycrobacteria-including *M.avium* comp-

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lex (MAC) and *M.*

Table1. Specificity of PURE-TB-LAMP for several bacterial strains

Group	Genus/Species	Strain		Reactivity	
				turbidity	visual
Mycobacterium tuberculosis complex (MTBC)					
	Mycobacterium tuberculosis H37Rv	KK 11-291	(ATCC 27294)	+	+
	Mycobacterium tuberculosis H37Ra	KK 11-05		+	+
	Mycobacterium bovis	JATA 12-01	(ATCC 19210)	+	+
	Mycobacterium africanum	JATA 13-01	(ATCC 25420)	+	+
Nontuberculosis Mycobacteria (NTM)					
	Mycobacterium asiaticum	JCM 6409	(ATCC 25276)	-	-
	Mycobacterium kansasii	JATA 21-01	(ATCC12478)	-	-
	Mycobacterium marinum	JCM 17638	(ATCC 927)	-	-
	Mycobacterium simiae	JCM 12377	(ATCC 25275)	-	-
	Mycobacterium gordonae	JCM 6382	(ATCC 14470)	-	-
	Mycobacterium scrofulaceum	JCM 6381	(ATCC 19981)	-	-
	Mycobacterium szulgai	JCM 6383	(ATCC 35799)	-	-
	Mycobacterium xenopi	JCM 15661	(ATCC 19250)	-	-
	Mycobacterium avium	JATA 51-01	(ATCC 25291)	-	-
	Mycobacterium avium	JCM 15429		-	-
	Mycobacterium avium	JCM 15430		-	-
	Mycobacterium avium	JCM 15431		-	-
	Mycobacterium avium	JCM 15432		-	-
	Mycobacterium gastri	JCM 12407	(ATCC 15754)	-	-
	Mycobacterium haemophilum	JCM 15465	(ATCC 29548)	-	-
	Mycobacterium intracellulare	JATA 52-01	(ATCC 13950)	-	-
	Mycobacterium malmoense	JCM 13391	(ATCC 29571)	-	-
	Mycobacterium shinjukuense	JCM 14233		-	-
	Mycobacterium abscessus subsp. *bolletii*	JCM 15297		-	-
	Mycobacterium chelonae	JCM 6388	(ATCC 35752)	-	-
	Mycobacterium flavescens	JATA 67-01		-	-
	Mycobacterium fortuitum subsp. *fortuitum*	JCM 6387	(ATCC 6841)	-	-
Other bacteria					
	Streptococcus pneumoniae	ATCC 12213		-	-
	Staphylococcus aureus subsp. *aureus*	ATCC 12600		-	-
	Haemophilus influenzae	ATCC 49247		-	-
	Klebsiella pneumoniae subsp. *pneumoniae*	ATCC 13883		-	-
	Mycoplasma pneumoniae subsp. *pneumoniae* [a]	ATCC 15531		-	-
	Legionella pneumophila subsp. *pneumophila*	ATCC 33152		-	-
	Escherichia coli	ATCC 11775		-	-
	Pseudomonas aeruginosa	ATCC 27853		-	-
	Pseudomonas fluorescens	ATCC 17386		-	-
	Moraxella catarrhalis	ATCC 25238		-	-

[a] 10^8 cells/mL was used in this study.

ATCC; American Type Culture Collection, JCM; Japan. Collection of Microorganisms, JATA; Japan Anti-Tuberculosis Association

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shinjukuese, both of which have high 16S rRNA gene sequence similarity to *M. tuberculosis* (Saito et al. 2011)—or with other bacteria causing respiratory tract infections such as *L. pneumophila*, indicating that assay is highly specific for MTBC.

3.2. Lower limit of detection

To determine the lower detection limit of the PURE-TB-LAMP assay, 60 µl of artificial sputum samples with *M. tuberculosis* H37Rv cell were analyzed (Fig. 1A). The assay yielded positive results for all samples with ≥ 150 CFU/ml (n = 6) regardless of the presence or absence of control sputum. Based on the calculation of confidence intervals, the detection limit of the assay with 95% confidence was 97.4 CFU/ml (95% CI, 64.9–185.3). Moreover, almost the same result was obtained using purified *M. tuberculosis* H37Rv gDNA (Fig. 1B). The sensitivity of smear microscopy by the Ziehl-Neelsen staining method is approximately 5,000 acid-fast bacilli/ml (Watterson et al. 2000); therefore, the PURE-TB-LAMP assay is 50–100 times more sensitive than a conventional smear test. On the other hand, the detection limit of GeneXpert MTB/rif (Xpert) with 95% confidence was 131 CFU/ml (Helb et al. 2010), which was comparable to that of the assay, indicating that the two assays have similar analytical sensitivity for detecting the MTBC. The detection limit for *M. tuberculosis* was unaltered by the presence of McFarland No. *1 M. avium* cells (Fig. 2), confirming that the sensitivity of the PURE-TB-LAMP assay for MTBC detection is not influenced by MAC co-infection, which often leads the lower detection limit of TB culture tests if the major source of infection is MAC.The WHO estimated that about 1.5 million TB patients, including 360,000 who were co-infected with human immunodeficiency virus (HIV), died in 2013 (WHO 2014). HIV patients tend to exhibit lower TB infectiousness than non-infected patients in smear tests, which has led to problems with low TB detection rates using tests based on acid-fast staining (Klein et al. 1989; Elliott et al. 1993); a more sensitive method of testing for detecting TB in HIV patients is therefore needed. Higher detection rates are observed with the concentrated acid-fast as compared to the direct smear method (Peterson et al. 1999). However, decontamination by centrifugation requires staff to have a certain skill level as well as biosafety facilities and equipment, making it unsuitable for community medical institutions in developing countries (Huggett et al. 2009). Direct genetic testing of unprocessed sputum is possible with the PURE-TB-LAMP assay; moreover, as described above, its detection sensitivity is higher than that of smear tests and equivalent to that of Xpert. The PURE-TB-LAMP assay may therefore be suitable for TB testing for patients co-infected with HIV and TB in developing countries

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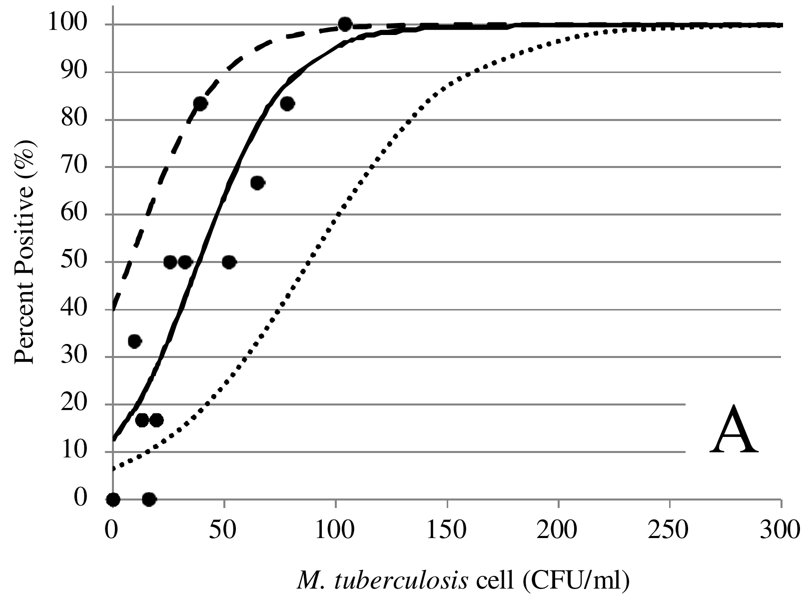

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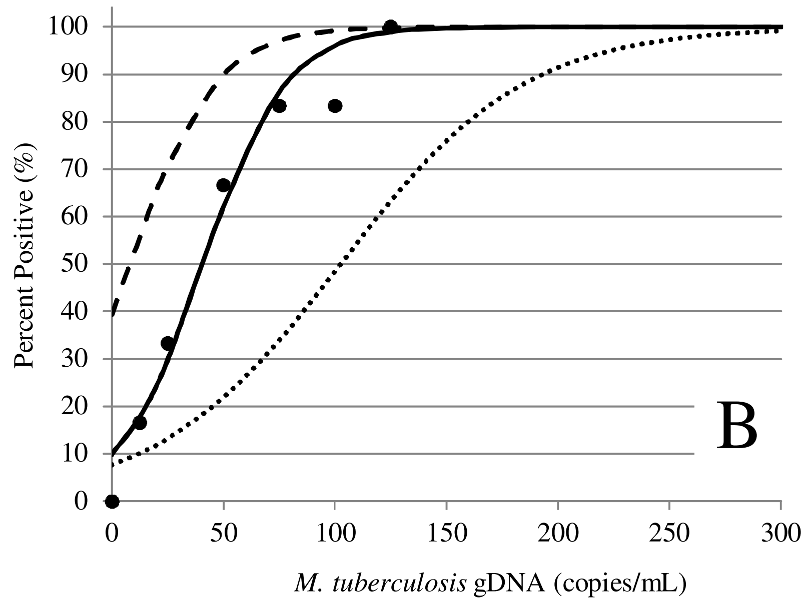

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Figure 1. Detection limit of the PURE-TB-LAMP assay based on statistical analysis. Limited dilutions of *M. tuberculosis* H37Rv cell (A) and purified gDNA (B) mixed with normal sputum were analyzed by means of these diagnostics (n = 6) and the ratio was plotted for each sample. Dashed and dotted lines indicate upper and lower 95% confidence interval (CI), respectively. (A) The detection of *M. tuberculosis* cell. 95% probability was 97.4 CFU/ml (95% CI, 64.9–185.3). (B) The detection of *M. tuberculosis* gDNA. 95% probability was 95.7 copies/ml (95% CI, 64.7–224.1).

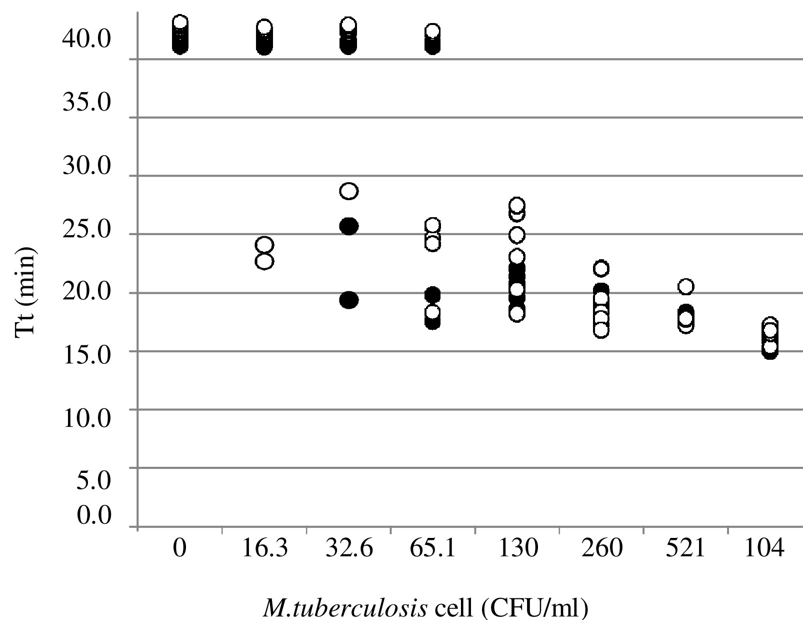

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Figure 2. Detection of *M. tuberculosis* mixed with non-TB mycobacterial cells by the PURE-TB-LAMP assay. Limited dilutions of *M. tuberculosis* H37Rv using McFarland No. 1 (approximately 107 CFU/ml) of *M. avium* JATA 51-01 were analyzed. Tt values were plotted for each concentration of *M. tuberculosis*. Open circle (○), with *M. avium*; filled circle (●), without M. avium.

The WHO estimated that about 1.5 million TB patients, including 360,000 who were co-infected with human immunodeficiency virus (HIV), died in 2013 (WHO 2014). HIV patients tend to exhibit lower TB infectiousness than non-infected patients in smear tests, which has led to problems with low TB detection rates using tests based on acid-fast staining (Klein et al. 1989; Elliott et al. 1993); a more sensitive method of testing for detecting TB in HIV patients is therefore needed. Higher detection rates are observed with the concentrated acid-fast as compared to the direct smear method (Peterson et al. 1999). However, decontamination by centrifugation requires staff to have a certain skill level as well as biosafety facilities and equipment, making it unsuitable for community medical institutions in developing countries (Huggett et al. 2009). Direct genetic testing of unprocessed sputum is possible with the PURE-TB-LAMP assay; moreover, as described above, its detection sensitivity is higher than that of smear tests and equivalent to that of Xpert. The PURE-TB-LAMP assay may therefore be suitable for TB testing for patients co-infected with HIV and TB in developing countries.

3.3. Effect of sample components

The tolerance of the PURE-TB-LAMP assay to sputa with various contaminants was evaluated by spiking experiments. Tt was unaffected by the presence of highly purulent or mucous sputa (Table 2). Furthermore, the assay showed high tolerance to sputum containing blood: although a slight delay in Tt was observed for sputa with 30% blood, there was no delay when blood content was 10%. Since patients who experience severe coughing often have bloody sputum, it is important that the assay demonstrate tolerance for blood in samples, especially in settings where it is not possible to perform N-acetyl-L-cysteine treatment. It is well-known that NAA reactions can be affected by large amounts of DNA in the sample; for instance, highly mucous and non-mucous sputa contain on average 1.3 and 0.1 mg/ml DNA, respectively (Picot et al. 1978). The assay yielded almost the same Tt values from artificial mucous sputa spiked with 1 mg human gDNA as control sputa (Table 2). These data clearly show that the PURE-TB-LAMP assay has high tolerance for the types of sample that are obtained in actual clinical practice.

The applicability of the PURE-TB-LAMP assay to specimens other than sputum was also examined. Artificial gastric fluid and urine obtained from healthy volunteers spiked with 300 CFU/ml *M. tuberculosis* H37Rv cells were processed in the same manner as the sputa samples and analyzed. The obtained Tt values were almost the same as those from sputum samples, indicating that the assay can be applied to a variety of biological samples, which is particularly useful for diagnosing TB in infants and geriatric patients who may have difficulty expectorating sputum for analysis (Coulter 2008).

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Table 2. PURE-TB-LAMP reactivity with various types of samples

Specimen	Tt (min, mean±3SD)
phospate buffer (PB)	19.8±2.7
purulent sputum	18.9±5.9
mucous sputum	18.5±3.0
artificial gastric juice	18.6±9.4
urine	21.4±4.8
10% supernatant of feces	23.2±10.9
1.0mg/mL of human genomic DNA	17.7±7.9
0.1mg/mL of human genomic DNA	17.4±1.8
10% whole blood	18.8±2.1
30% whole blood	24.5±4.1

Tt ; threshold time measured by LA-500

4. Summary

The objective of this study is to evaluate the basic performance of the PUER-TB-LAMP assay. The detection limit of the assay was higher than that of smear microscopy tests and comparable to that of Xpert. Moreover, this assay was MTBC-specific and did not demonstrate cross-reactivity with other acid-fast bacteria. In addition, the detection sensitivity was unaffected by the presence of up to 30% blood or 1 mg of human gDNA in the sample, indicating that it can be used with samples other than sputum, such as gastric fluid and urine. Therefore, the PURE-TB-LAMP assay is suitable for use in developing as well as developed countries as a rapid but effective testing method that can reinforce TB preventative measures. The clinical performance of the assay has been evaluated in some developing countries including the Republic of Haiti and the results will be published elsewhere.

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