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Short Communication

A Rapid Loop-Mediated Isothermal Amplification Assay Targeting *hspX* for the Detection of *Mycobacterium tuberculosis* Complex

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SUMMARY: A rapid, simple, and low-cost diagnostic tool for tuberculosis (TB) detection is urgently needed in countries with a high TB burden. Here, we report a novel loop-mediated isothermal amplification (LAMP) assay targeting the *hspX* gene for the rapid detection of *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. The specificity of this assay was evaluated using 4 reference strains of *Mycobacterium tuberculosis* complex (MTC), 22 species of non-tuberculous mycobacteria (NTM), 7 non-mycobacterial species, and 50 clinical *M. tuberculosis* isolates. All the reference MTC strains and *M. tuberculosis* clinical isolates were successfully detected by this method, and there were no false-positive results with NTM or non-mycobacterial species, which demonstrates the high specificity of this assay for MTC. The detection limit was 10 copies of MTC genome within 27 min, and the detection speed of this assay was higher than that of any other isothermal methods reported so far. Because of its speed, simplicity, sensitivity, specificity, and inexpensiveness, the TB *hspX* LAMP assay is a potential gene diagnostic method for TB detection in developing countries with a high TB burden.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the leading causes of death from infectious diseases worldwide. More than 9 million new cases of TB and nearly 1.4 million deaths resulting from TB are reported annually, with the majority occurring in developing countries (1). To control TB, a rapid, accurate, and low-cost laboratory diagnostic method is urgently needed (2).

Nucleic acid amplification techniques (NAATs) brought a breakthrough in TB diagnosis, since it took less than 1 day to obtain results by this technique, whereas the conventional diagnostic methods involving bacterial culture usually require around 1 month. Several NAATs, including the Gen-Probe Amplified *M. tuberculosis* (3), Roche Amplicor *M. tuberculosis* test (4), Cobas Amplicor test (5), and so forth have been developed for the rapid TB diagnosis. However, these tests require expensive laboratory infrastructure and sophisticated technical skill, and hence, they are not feasible for use in developing countries (6). Loop-mediated isothermal amplification (LAMP) (7) is a new isothermal NAAT that can be used in countries with resource-limited settings, because simple equipment such as a water bath or an aluminum block bath is sufficient to accomplish the reaction in this test. This method has been applied for detecting several pathogens (8–11) and can be used for the rapid detection of epidemic pathogens in peripheral healthcare settings in developing countries (12). LAMP-based assays targeting *gyrB* (13), *rrs* (14), IS*6110* (15), and *rimM* (16) sequences have been developed for detecting *Mycobacterium tuberculosis* complex (MTC); however, there is much scope for the improvement of this technique in terms of the detection limit, reaction time, and the procedure itself.

A LAMP-based assay targeting the *hspX* gene was developed for detecting the majority of MTC, because many pathogenic mycobacteria other than those belonging to MTC, such as *Mycobacterium kansasii* and *M. marinum*, do not share sequence similarity with MTC species; even *M. kansasii* and *M. marinum* show a number of differences in the selected gene segment. A primer set as shown in Table 1 and Fig. 1 was designed for the nucleotide sequence of the *hspX* gene of *M. tuberculosis* H37Rv (Rv2031c, GeneID: 887579) using the PrimerExplorer software (https://primerexplorer.jp/lamp4.0.0/index.html). This primer set along with the optimal reaction mixture consisting of the following components was used for performing LAMP: 0.2 μM of each outer primer; 1.6 μM of each inner primer; 1.2 μM of each loop primer; 2.5 μL of 10× LAMP reaction buffer; 2 μL of 100 mM $MgSO_4$; 4.5 μL of 5 M betaine; 2.5 μL dNTP (10 mM each); 1.0 μL of 8 U/μL *Bst* DNA polymerase (Lucigen); 2 μL of the target DNA; and Milli-Q water to make up the final volume to 25 μL. The

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Table 1. Primers used for TB *hspX* LAMP system and IS*6110* PCR

Target	Primer	Sequence	Length (bp)
	F3	TCATTCGCCGGACTCCG	17
	B3	GGAACCGTACGCGAATTCC	19
hspX	FIP (F1c + F2)	ACCTCGTAGCGCCCCTCTT-ACCTTCGACACCCGGTTGA	38
	BIP (B1 + B2c)	GGACGTCGACATTATGGTCCGC-GTCGAAGTCCTTCTGCTCG	41
	FL	CATCTCGTCTTCCAGCCGCA	20
	BL	GATGGTCAGCTGACCATCAAGGC	23
IS*6110*[1)]	F	CCTGCGAGCGTAGGCGTCGG	20
	R	CTCGTCCAGCGCCGCTTCGG	20

[1)]: Bhigjee et al., 2007 (17).

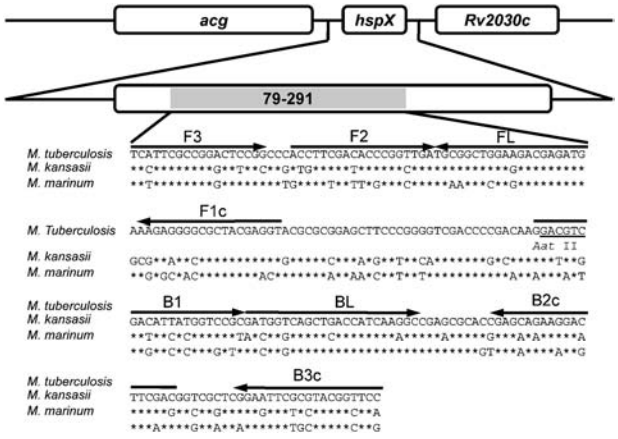

Fig. 1. Primers for the specific detection of the *M. tuberculosis* complex. Position and orientation of the primer annealing site on *hspX* gene are shown. Restriction endonuclease *Aat*II recognition sequence was underlined. Bases identical to *M. tuberculosis* are shown by asterisks.

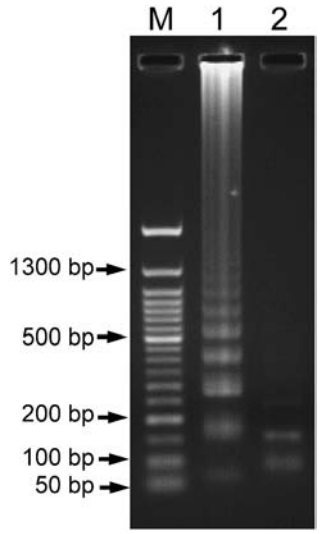

Fig. 2. Agarose gel electrophoretic analyses of TB *hspX* LAMP product. TB *hspX* LAMP product (lane 1) and its *Aat*II restriction digest (lane 2) together with DNA size marker (New England Biolabs) were analyzed on agarose gel electrophoresis followed by ethidium bromide staining and UV visualization.

optimal amplification temperature was determined to be 67°C on the basis of the threshold time, which was the time at which the optical density of the reaction mixture at 590 nm reached 0.1 using a real-time turbidimeter (Eiken Chemical Co., Tokyo, Japan).

To confirm the amplified sequence, we digested the LAMP product using the restriction enzyme *Aat*II (New England Biolabs Inc., Beverly, Mass., USA) and analyzed the resultant product by performing agarose gel electrophoresis. We mixed 1 μL of the LAMP product, 1 μL *Aat*II, 2 μL of 10× buffer, and 16 μL of Milli-Q water and incubated the mixture at 37°C for 2 h. The LAMP products, restriction-digested LAMP products, and PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and observed under UV light. In the electrophoretic analysis, the LAMP product appeared like a ladder of multiple bands characteristic of the LAMP product (Fig. 2, lane 1). The sizes of the *Aat*II-digested LAMP products were approximately 100 bp and 160 bp (Fig. 2, lane 2), which shows good agreement with the predicted sizes of 103 bp and 161 bp. These results indicate proper completion of the LAMP reaction, and we termed this assay TB *hspX* LAMP.

To evaluate the specificity of this assay, we used 4 reference MTC strains, 22 reference non-tuberculous mycobacteria (NTM), 7 reference non-mycobacterial strains (Table 2), and 50 clinical mycobacterial isolates identified to be *M. tuberculosis* by conventional biochemical tests. Genomic DNA from these bacterial strains was extracted by mechanical destruction using glass beads of 0.1-mm diameter as previously described (17). The LAMP reaction was performed under optimized conditions, and samples with a concentration of 5 ng/μL and a threshold time of less than 60 min were concluded to show positive results. The results were positive for all the MTC species, including the 50 clinical *M. tuberculosis* isolates, whereas no positive reaction was observed for the 22 NTM and 7 non-mycobacterial species.

The sensitivity of the TB *hspX* LAMP assay was investigated using a 10-fold serially diluted *M. bovis* BCG DNA (containing 5 ng to 5 fg of DNA) for each reaction. This 10-fold serially diluted DNA was equivalent to the DNA obtained from 10 to 10^6 bacterial cells, which is calculated on the basis of the size of the *M. bovis* BCG genome (4.37 mega base-pairs). The DNA amplified by LAMP could be directly detected by adding 1 μL of fluorescent detection reagent (FDR; Eiken Chemical) to the reaction mixture. The sensitivity of the LAMP assay was compared with that of IS*6110* PCR

Table 2. Strains used in the present study and the results of the specificity tests of TB *hspX* LAMP assay

	Bacterial species	Reference strain[1)]	LAMP result[2)]
MTC	*M. tuberculosis*	H37Rv (JATA)	P
	M. bovis	BCG Tokyo 172 (JBCG)	P
	M. africanum	KK 13–02 (JATA)	P
	M. microti	ATCC 19422	P
NTM	*M. intermedium*	JATA 9H–01	N
	M. mucogenicum	JATA 9P–01	N
	M. lentiflavum	JATA 9N–01	N
	M. kansasii	JATA 21–01	N
	M. marinum	JATA 22–01	N
	M. simiae	JATA 23–01	N
	M. asiaticum	JATA 24–01	N
	M. scrofulaceum	JATA 31–01	N
	M. szulgai	JATA 32–01	N
	M. gordonae	JATA 33–01	N
	M. xenopi	JATA 42–01	N
	M. nonchromogenicum	JATA 45–01	N
	M. terrae	JATA 46–01	N
	M. malmoense	JATA 47–01	N
	M. avium	JATA 51–01	N
	M. intracellulare	JATA 52–01	N
	M. shimoidei	JATA 54–01	N
	M. fortuitum	JATA 61–01	N
	M. peregrinum	JATA 61–02	N
	M. chelonae	JATA 62–01	N
	M. abscessus	JATA 63–01	N
	M. smegmatis	JATA 64–01	N
Others	*Streptococcus pneumoniae*	NBRC 102642	N
	Klebsiella pneumoniae	NBRC 3318	N
	Pseudomonas aeruginosa	NBRC 12689	N
	Staphylococcus aureus	NBRC 100910	N
	Mycoplasma pneumoniae	NBRC 14401	N
	Legionella pneumophila	Y060117–1	N
	Haemophilus influenzae	ATCC 49247	N

[1)]: JATA, Japan Anti-Tuberculosis Association; JBCG, Japan BCG Laboratory; NBRC, NITE Biological Resource Center; ATCC, American Type Culture Collection.
[2)]: N, negative; P, positive.

(18) and commercially available LAMP kit (Loopamp® Tuberculosis Complex Detection Reagent Kit; Eiken Chemical) for the same samples. Each of the results obtained using a real-time turbidimeter (Fig. 3A) and a FDR (data not shown) showed that TB *hspX* LAMP assay has the capacity to detect as low as 10 copies of *M. bovis* BCG genomic DNA, which is same as that shown by commercially available LAMP kits. Fig. 3B shows a linear relationship between the copy numbers of the template DNA expressed as log and the threshold time, with a correlation coefficient of $R^2 = 0.97$ for 10 to 10^6 copies of the genomic DNA. This result shows the possible use of TB *hspX* LAMP for semi-quantitative analysis. Agarose gel electrophoresis of the LAMP product (Fig. 3C) showed the same sensitivity as that observed for the real-time turbidimetry product. We detected 10 copies of DNA by performing the TB *hspX* LAMP assay, whereas 10^2 copies of genomic DNA were detected by performing conventional IS*6110* PCR using the same template (Fig. 3D).

Finally, the sensitivity of TB *hspX* LAMP was inves-

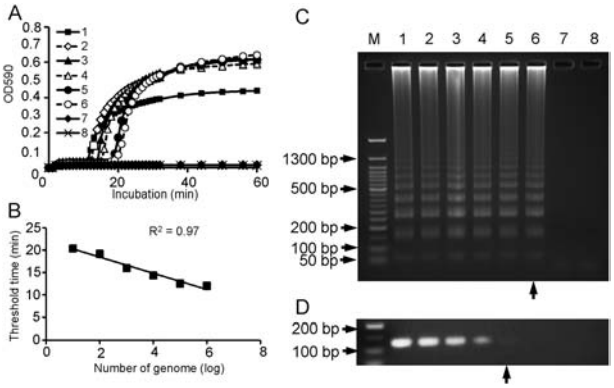

Fig. 3. Sensitivity of TB *hspX* LAMP determined by real-time turbidimeter. The amount of DNA amplification by-product (Mg-pyrophosphate) was monitored by measuring turbidity (590 nm). (A) LAMP reaction from various amounts of template DNA. 1, 10^6 copies; 2, 10^5 copies; 3, 10^4 copies; 4, 10^3 copies; 5, 10^2 copies; 6, 10^1 copies; 7, 10^0 copies; 8, negative control. (B) Copy numbers of template DNA in log (*x*-axis) were plotted against threshold times (*y*-axis). TB *hspX* LAMP (C) and IS*6110* PCR (D) products with various amount of template. Lane 1–8, reactions contained 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, and 0 copies of purified *M. bovis* BCG Tokyo 172 genomic DNA per reaction, respectively. Lane M, 50-bp DNA marker. Arrow indicated the detection limit.

tigated using spiked sputum samples. The pooled sputum sample obtained from a healthy volunteer at the Osaka Prefectural Institute of Public Health in Japan was digested with semi-alkaline proteinase (Sputazyme; Kyokuto Pharmaceuticals, Tokyo, Japan) and subjected to decontamination by *N*-acetyl-L-cysteine (NALC)-NaOH (CC-E, Japan BCG Laboratory, Tokyo, Japan) according to the manufacturer's instruction. The precipitate obtained after treatment was suspended in PBS of 1/2 the volume of original sputum. We took 200 μL each of this suspension in new tubes and spiked them with 10× serially increasing number of *M. bovis* BCG (from 2 to 20,000 bacilli). Since 200 μL of the treated sputum equivalent to 400 μL of the original sputum was spiked by the method described above, the bacterial concentration in the original sputum sample ranged from 5 to 50,000 bacilli/mL. Bacterial DNA from the spiked sputum samples was extracted using the EXTRAGEN MB Kit (TOSOH Co., Tokyo, Japan); the extraction was perfomed according to the manufacturer's instructions, with a slight modification: the final extract volume was adjusted to 20 μL. We compared the results of LAMP assay with that of IS*6110* PCR assay. We observed that 1 out of 3 TB *hspX* LAMP reactions showed positive results within 60 min when the DNA extracted from the artificial sputum containing 500 bacilli/mL was used, and a faint band was observed on electrophoresing the sample obtained by preforming IS*6110* PCR with the same DNA sample (Table 3). Thus, the detection limit for both TB *hspX* LAMP and IS*6110* PCR assays were determined to be 500 bacilli/mL.

The current conventional detection methods for TB, such as smear microscopy and microbial culture, which are routinely practiced in most laboratories, have inherent disadvantages. Smear microscopy is rapid and inexpensive but has been unsuccessful owing to its low sensitivity (detection of 5,000–10,000 acid-fast bacteria per mL). The culture method, which is considered to be the

Table 3. Comparison of the detection limit of TB *hspX* LAMP assay and IS*6110* PCR for BCG Tokyo 172 spiked sputum

Assay	Bacterial concentration (bacilli/mL) in artificial sputum sample				
	50000	5000	500	50	5
LAMP	+	+	+	−	−
PCR	+	+	+	−	−

+, positive with more than one in three LAMP reactions or visible in PCR; −, all negative in 3 LAMP reactions, not visible in PCR.

''gold standard'' for laboratory confirmation of TB, can detect only 100 TB bacilli per mL and also has the disadvantage of a delay of up to several weeks in the diagnosis of TB because of the slow growth of *M. tuberculosis*. A definite and early detection of causative mycobacterial species in clinical specimens is necessary so as to choose the appropriate therapeutic strategy for successful treatment.

The TB *hspX* LAMP assay developed in this study successfully detected the majority of MTC members without showing positive reactions for the majority of pathogenic NTM and non-mycobacterial species that cause lung infection. This result indicates the high specificity of this assay. With respect to sensitivity, this assay could detect 10 copies of *M. bovis* BCG genomic DNA within 27 min under an isothermal condition at 67°C. The sensitivity of TB *hspX* LAMP was similar to that of the internal amplification control (IAC) real-time PCR assay (19) and the commercially available LAMP kit and was 10 times higher than that of the conventional IS*6110* PCR (Figs. 3C, 3D).

In contrast to PCR, the LAMP assay seems to be practical for the developing countries because of its simplicity and low cost. Several versions of LAMP assays have been reported for the diagnosis of TB. Iwamoto et al. (13) was the first to show the applicability of LAMP for *M. tuberculosis* detection. They employed the *gyrB* gene and showed a detection limit of 100 copies/reaction in 60 min. We developed a LAMP assay based on the difference in the *rrs* gene sequence between mycobacterial species (14). The detection limit of 100 fg, which is equivalent to 20 copies of genomic DNA, was achieved within 35 min. The LAMP assay targeting the *rimM* gene was reported to have a detection limit of 1 pg genomic DNA within 45 min (15). Aryan et al. reported a LAMP assay targeting a repetitive element IS*6110* with a detection limit of 5 fg, which is equivalent to 1 copy of genomic DNA (15); however, this assay had the disadvantage of an incubation time of 75 min or longer to obtain the highest sensitivity. Longer incubation time may sometimes lead to a nonspecific reaction. The RT-LAMP-ELISA-hybridization assay targeting 16S ribosomal RNA for the detection of *M. tuberculosis* in clinical samples was described, and the detection limit of this assay was reported to be 1 copy of the ribosomal RNA per reaction (20). This novel assay is highly sensitive; however, it is important to note that the authors reported the cost to be US$ 10 per reaction, and it requires a 5-h complicated handling procedure for completion. The TB *hspX* LAMP assay showed a rapid detection time of less than 27 min of incubation and high sensitivity, with a detection limit of 10 copies/reaction. In addition, one reaction cycle of this assay costs less than US$ 1.

Sputum spiked with *M. bovis* BCG was used to explore the clinical applicability of TB *hspX* LAMP. The detection limit was 500 bacilli/mL of sputum (20 bacilli/reaction), which is similar to that obtained using purified DNA (10 copies/reaction). The sensitivity of TB *hspX* LAMP assay seems to be high enough to detect MTC not only from a positive smear (the concentration of *M. tuberculosis* is usually 5,000–10,000 bacilli/mL sputum or higher) but also from some smear-negative culture-positive sputa (100–5,000 bacilli/mL sputum). Therefore, we feel that the TB *hspX* LAMP assay is suitable for practical use in the direct gene diagnosis of TB from sputum.

Since the LAMP assays tend to yield false-positive results because of the high rate of possible contamination and nonspecific reactions by complicated primers, we recommended that at least 2 assays targeting different genes be run simultaneously to avoid misdiagnosis. The combination of TB *hspX* LAMP assay with one of the previously developed LAMP assays, for a distinct target offering high speed, high sensitivity, and high specificity will enable TB gene diagnosis to be accurate and feasible in developing countries that have a high TB burden.

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Conflict of interest None to declare.

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