Detection of *Mycobacterium tuberculosis* complex in sputum specimens using a loop-mediated isothermal amplification assay in Korea

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Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* complex (MTC), remains one of the leading causes of death in the world. In Korea, the current prevalence of multidrug-resistant TB (MDR-TB) poses a major problem. The most common method for diagnosing TB in developing countries is sputum smear microscopy; however, the sensitivity of this test is relatively low and it usually requires well-trained laboratory staff. Cultures of MTC require up to several weeks in sophisticated facilities, such as Biosafety Level 3. Effective diagnostic techniques are necessary to control TB. In Korea, we evaluated a loop-mediated isothermal amplification (LAMP) assay targeting the *hspX* gene (TB-*hspX*-LAMP) of MTC. For clinical evaluation, culture confirmation, smear microscopy and TB-*hspX*-LAMP were performed on 303 sputum specimens obtained from suspected TB patients in Korea. The sensitivity, specificity, positive predictive value and negative predictive value of TB-*hspX*-LAMP were 71.1, 98.8, 91.4 and 95.1 %, respectively, compared with TB culture, which is the gold standard for diagnosis of TB. In contrast, the comparable values of smear microscopy were 24.4, 98.1, 68.8 and 88.2 %, respectively. Therefore, we concluded that TB-*hspX*-LAMP was superior to the use of smear microscopy for the detection of MTC in sputum specimens in clinical settings in Korea.

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INTRODUCTION

Tuberculosis (TB), an airborne infectious disease caused by *Mycobacterium tuberculosis* complex (MTC, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium canetti*, *Mycobacterium pinnipedii*) (Behr *et al.*, 1999; CDC, 2009), still remains one of the leading causes of death by infectious diseases worldwide. In 2013, 9 million people developed TB throughout the world; 1.5 million of this group died from it, of which 360 000 were human immunodeficiency virus-positive (WHO, 2014).

Early, accurate diagnosis of MTC is important for the control of TB (Brodie & Schluger, 2005). The most common method for diagnosing TB in developing countries is sputum smear microscopy (George *et al.*, 2011). Smear microscopy offers the advantages of rapid detection time and simplicity. However, the sensitivity of smear microscopy is relatively low and it requires well-trained laboratory staff.

Culture methods can detect as few as 100 *Mycobacterium* cells per 1 ml specimen (Hofmann-Thiel *et al.*, 2010) and TB culture is still considered the gold standard for the

Abbreviations: CI, confidence interval; KUMC, Korea University Medical Center; LAMP, loop-mediated isothermal amplification; MDR, multidrug-resistant; MTC, *Mycobacterium tuberculosis* complex; NAAT, nucleic acid amplification test; NPV, negative predictive value; NTM, non-tuberculosis mycobacteria; PPV, positive predictive value; TB, tuberculosis.

detection of MTC. However, the culture methods require up to several weeks in sophisticated facilities, such as Biosafety Level 3, and delays in the diagnosis of TB may harm many patients due to the current prevalence of multidrug-resistant TB (MDR-TB) (WHO, 2013a).

Nucleic acid amplification tests (NAATs) have been considered the most valuable diagnostic tool for the identification of TB (Amin *et al.*, 2011). Conventional NAATs, which are comparable with the culture confirmation method, have a high sensitivity and specificity for the detection of MTC. However, they require exclusive equipment, and the use of complicated techniques and procedures (Aryan *et al.*, 2010). Therefore, they are not suitable for resource-limited laboratory settings in developed and developing countries. A rapid, simple, sensitive and effective diagnostic technique needs to be developed to control global TB-related problems.

In 2000, a novel nucleic acid amplification method known as loop-mediated isothermal amplification (LAMP) was developed (Notomi *et al.*, 2000), and was considered a breakthrough technique for the diagnosis of TB and other infectious diseases (Mori & Notomi, 2009; Notomi *et al.*, 2015; Tomita *et al.*, 2008). As the LAMP technique uses strand-displacing DNA polymerases that have high activity under isothermal temperatures ranging from 60 to 68 °C, no expensive equipment is required (Iwamoto *et al.*, 2003). The LAMP reaction produces stem–loop DNA structures with several inverted repeats and cauliflower-like structures with multiple loops. The products of amplified LAMP reactions can be measured by a change in the turbidity of the reaction mixture because magnesium pyrophosphate accumulates in the reaction mixture as a byproduct of the amplification reaction.

The LAMP assay has several exceptional features compared with other TB diagnostic tools. (1) The LAMP assay uses four different primers to identify six distinct regions on the target gene, resulting in a greater specificity than conventional NAATs. (2) A small amount of DNA can be detected due to the high amplification capacity of LAMP. (3) The LAMP assay does not require the use of complicated procedures, equipment or machines. Therefore, the LAMP assay is considered more rapid, simpler and more economical than other TB diagnostic methods.

We clinically evaluated a LAMP method that targeted the *hspX* gene of MTC (TB-*hspX*-LAMP) (Bi *et al.*, 2012). Although several clinical evaluations of LAMP assays have been published (Boehme *et al.*, 2007; George *et al.*, 2011; Mitarai *et al.*, 2011; Pandey *et al.*, 2008), to the best of our knowledge, this is the first study to clinically evaluate the use of the LAMP method to detect the *hspX* gene of MTC and is also the first report of the LAMP method for diagnosing TB in Korea. Sputum specimens obtained from patients at the Korea University Medical Center (KUMC) who were suspected of having TB were subjected to culture confirmation, smear microscopy and the LAMP method. The clinical sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated and compared with the culture results.

METHODS

Clinical sputum specimens. This study was approved by the Ethics Committee of Clinical Trials at the KUMC (Institutional Review Board no. AS12084). From 3 September to 21 December 2012, a total of 303 sputum specimens were obtained from patients with suspected TB at the KUMC. Using the obtained specimens, an experienced laboratory technician performed smear microscopy according to the acid-fast bacillus stains, including auramine-O and Ziehl–Neelsen stainings, as described previously (Hänscheid *et al.*, 2007).

The clinical specimens were also processed as described previously (Mitarai *et al.*, 2011). Briefly, an equal volume of decontamination-digestion agent, NALC (*N*-acetyl-L-cysteine)/2 % NaOH, was added to the sputum in a 50 ml falcon tube (BD Diagnostics). For homogenization, the mixture was vortexed and subsequently neutralized with PBS (pH 6.8). For concentration, the mixture was centrifuged at 3000 ***g*** for 15 min at 4 °C. Aliquots of 100 µl of sediment were inoculated onto 2 % Ogawa solid medium and bacterial growth was checked once per week for 6–8 weeks. The isolated bacteria were discriminated between MTC and non-TB mycobacteria (NTM) using a SD BIOLINE TB Ag MPT64 Rapid kit (Standard Diagnostics). Aliquots of 500 µl of sediment were used for DNA extraction. The sediment was recentrifuged at 3000 ***g*** for 1 min, the supernatant subsequently discarded and the pellet processed for DNA extraction using an AdvanSure TB kit (LG Life Science) (Aldous *et al.*, 2005).

Bacterial strains. Genomic DNA used in this study consisted of 12 reference strains, including a *M. tuberculosis*-type strain (*M. tuberculosis* ATCC 27294), four NTM reference strains and seven other reference strains. The four NTM reference strains were *Mycobacterium scrofulaceum* ATCC 19981, *Mycobacterium kansassi* ATCC 12478, *Mycobacterium intracellulare* ATCC 13950 and *Mycobacterium fortuitum* ATCC 6841. The other seven reference strains consisted of *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, *Escherichia faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 700603, *Klebsiella oxytoca* ATCC 700324, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Five clinical isolates of *Salmonella typhi*, *Salmonella flexneri*, *Vibrio cholerae, Haemophilus influenzae* and *Neisseria meningitidis* were also used. Bacterial genomic DNA was prepared according to the standard protocol (Wilson, 2001). For the sensitivity test, a 10-fold serial dilution series (2.5 fg to 25 ng μl^{-1}) of *M. tuberculosis* ATCC 27294 genomic DNA was prepared. The amount of DNA was measured using the ND-1000 spectrophotometer (NanoDrop Technologies) and the number of genomic copies was calculated based on a molecular size of *M. tuberculosis* of 4.4 Mbp (Cole *et al.*, 1998). As genomic DNA-spiked clinical specimens, DNA-extracted sputum samples negative by culture, smear microscopy and LAMP were spiked with the *M. tuberculosis* DNA.

LAMP assay. The LAMP reaction was carried out using *hspX* gene-specific LAMP primers (Bi *et al.*, 2012). The LAMP reaction mixture (25 µl) consisted of 1.6 µM FIP and BIP primers, 0.2 µM F3 and B3 primers, 0.8 µM LF and LB primers, 0.8 M betaine (Sigma-Aldrich), 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1 % Tween 20, 1.4 mM dNTPs (Roche Diagnostics), 8 U *Bst* DNA polymerase (New England Biolabs), and 2 µl template DNA (Lee *et al.*, 2015). Using a Loopamp real-time turbidimeter (LA-500; Eiken), the mixture was incubated at 67 °C for 60 min, followed by inactivation at 80 °C for 5 min. The laboratory space was separated into a mixture zone, a DNA zone, an amplification zone and an electrophoresis zone to prevent cross-contamination.

Analysis of LAMP products. The turbidity of amplified product was proportional to the amount of amplified DNA (Mori *et al.*, 2004). Amplified products were detected through visualization of the white precipitate, using a Loopamp real-time turbidimeter (LA-500; Eiken) and agarose gel electrophoresis. We used the application software for the turbidimeter to obtain the amplification time required to exceed a turbidity level of 0.1 (T_t), according to the manufacturer's protocol (Mori *et al.*, 2004). For the detection limit study, a colorimetric visual inspection dye (Miyamoto *et al.*, 2015) dried down in the caps of the reaction tubes (D-Quick; Kaneka) was used. According to the manufacturer's recommendation, we used 0.1 % Triton X-100 for the LAMP reagent instead of 0.1 % Tween 20. After the reactions were performed, the LAMP amplicons were mixed with the dye by inverting the tubes and the colour change was observed.

To confirm the structure, the amplified products were digested with the restriction enzyme *Sma*I (Fig. 1, New England Biolabs). The reaction products were further analysed by sequencing using the F2 and B2 primers (F2, ACCTTCGACACCCGGTTGA; B2, GTCGAAGTCCTTCTGCTCG).

Statistical analysis. The clinical sensitivity, specificity, PPV and NPV of the TB-*hspX*-LAMP and the smear microscopy techniques were compared with those of the conventional culture method (the gold standard was the culture results). The 95 % confidential interval (CI) was calculated using the standard interval estimation and the modified Jeffreys prior interval for small n was used as described previously (Brown *et al.*, 2001). A linear regression line was obtained by plotting T_t against the log of the initial template DNA.

RESULTS AND DISCUSSION

According to the 2014 WHO global tuberculosis report (WHO, 2014), tuberculosis is widespread in many developing countries and there are still a considerable number of infected patients in developed countries. In the past half-decade, the number of MDR-TB patients rapidly increased in Korea (WHO, 2013a). Therefore, a rapid, simple, reliable and accurate diagnostic technique to identify MTC is required to control MDR-TB. The most sensitive procedure, the culture method (gold standard), and the easiest technique, the smear microscopy method, have been utilized to diagnose cases of TB throughout the world. The current study evaluated a LAMP assay targeting the *hspX* gene of MTC. Compared with the smear microscopy test, this method showed a higher sensitivity, specificity, PPV and NPV.

LAMP-based assays targeting the *gyrB* (Iwamoto *et al.*, 2003), 16S rRNA (Pandey *et al.*, 2008), *IS6110* (Aryan *et al.*, 2010) and *rimM* (Zhu *et al.*, 2009) sequences have been developed for the detection of MTC. Several studies of the clinical evaluation of LAMP assays targeting *gyrB* (Boehme *et al.*, 2007; Mitarai *et al.*, 2011), 16S rRNA (Pandey *et al.*, 2008) and *rimM* (George *et al.*, 2011) have been reported. The *hspX* gene is also a consensus gene for MTC and a LAMP-based assay targeting the *hspX* gene was developed previously (Bi *et al.*, 2012). Bi *et al.* (2012) reported a high specificity and a detection limit of 10 genome copies within 27 min. The detection speed of this assay was higher than that of any other isothermal methods. However, this method has not yet been clinically evaluated.

Detection limit of TB-*hspX*-LAMP

We confirmed that the detection limit of TB-*hspX*-LAMP was 10 copies of genomic DNA per reaction (triplicate results) and 100 copies of genomic DNA-spiked clinical specimens (duplicate results; Fig. 2). These results were identical to those in a previous report (Bi *et al.*, 2012).

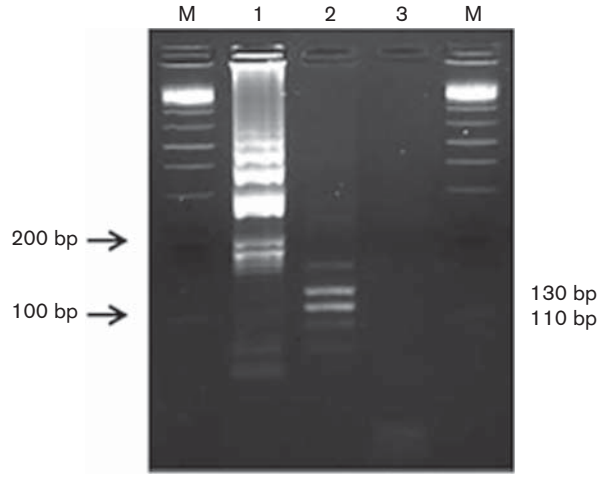

Fig. 1. Confirmation of amplified LAMP product. This figure demonstrates that amplified TB-*hspX*-LAMP products were digested into ~100 and 130 bp fragments by *Sma*I. Lane M, 1 kb ladder (Invitrogen); lane 1, amplified TB-*hspX*-LAMP product; lane 2, *Sma*I-digested LAMP product; lane 3, negative control (without template).

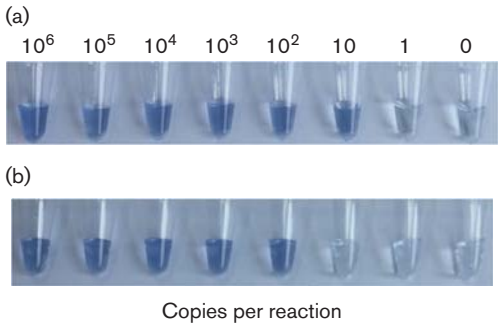

Fig. 2. Colorimetric visual inspection dye monitoring of the detection limits of TB-*hspX*-LAMP using (a) genomic DNA (*M. tuberculosis* ATCC 27294) and (b) genomic DNA-spiked clinical specimens as templates. The original colourless appearance of the visual inspection dye (Kaneka) changed to blue if the reaction was positive; if it was negative, the dye remained colourless.

Analytic specificity of TB-*hspX*-LAMP

The LAMP reaction demonstrated a positive result only with *M. tuberculosis* and negative results were observed with other bacteria. No false-positive results were seen with NTM or non-mycobacterial species, indicating that the TB-*hspX*-LAMP primer set was specific to only MTC, as reported previously (Bi *et al.*, 2012).

Analysis of the LAMP-amplified products

Amplified product was observed by the naked eye without the use of equipment due to the appearance of a white precipitate of magnesium pyrophosphate as a byproduct of the amplification. We confirmed that the relationship between T_t and the log of the amount of template DNA was linear and the correlation coefficient (r^2) was 0.9937. The quantity of DNA in a sample could be estimated by measuring the reaction time. This phenomenon was almost the same as in the previous report (Bi *et al.*, 2012). For the detection limit study, we used a new colorimetric visual inspection dye, Leuko crystal violet (LCV) (Miyamoto *et al.*, 2015). The LCV was obtained after sodium sulfite treatment of crystal violet. Throughout the study, evaluation of the LAMP reactions demonstrated complete agreement amongst a white precipitate recorded by visual inspection, the real-time turbidimeter, electrophoretic analysis and LCV. Using LCV, the colourless dye changed to blue, which could be visualized under natural light without the need for UV light. If there was no amplification, the dye remained colourless (Fig. 2). Therefore, a positive LAMP reaction is indicated by the colour change from colourless to blue. The dye described here will be especially appropriate for use in resource-limited settings, including developed and developing countries. Moreover, to confirm the structure, LAMP products were digested with *Sma*I, and the size of the digested product fragments was approximately 110 and 130 bp (Fig. 1), as expected. The amplified products were also subjected to DNA sequencing to verify the LAMP products. The DNA sequences of the amplified products were identical to expected target sequences.

Clinical evaluation of the LAMP reaction with 303 sputum specimens

A total of 303 sputum specimens were tested and the number of culture-positive samples was 45 (14.9 %). Amongst the 45 culture-positive samples, there were 32 5 (71.1 %) TB-*hspX*-LAMP-positive samples and 11 (24.4 %) smear-positive samples. All of the 11 smear-positive, culture-positive samples were TB-*hspX*-LAMP positive (100 %). TB-*hspX*-LAMP-positive samples accounted for 21 (61.8 %) of the 34 smear-negative and culture-positive samples. TB-*hspX*-LAMP-negative specimens accounted for 255 (98.8 %) and smear-negative samples accounted for 253 (98.1 %) of the 258 culture-negative samples. The sensitivity of LAMP in smear-positive, culture-positive specimens was 100 % (11/11 specimens; 95 % CI 71.5–100). In the smear-negative, culture-positive specimens, the sensitivity of LAMP was 61.8 % (21/34 specimens; 95 % CI 45.4–78.1). Of the smear-negative, culture-positive specimens, 13 were reported by LAMP to be false negative, suggesting that a loss of DNA occurred during the DNA preparation process. A clinical follow-up of the LAMP-positive, culture-negative patients with suspected TB (three patients) was not performed, but one of the LAMP-positive and culture-negative specimens was also positive for the smear test (Table 1). These specimens may include non-viable MTC.

The overall performances of the LAMP, smear and culture tests used for the detection of MTC are shown in Table 1. The sensitivity, specificity, PPV and NPV of TB-*hspX*-LAMP were 71.1 % (32/45 specimens; 95 % CI 57.9–84.4), 98.8 % (255/258; 95 % CI 97.5–100), 91.4 % (32/35; 95 % CI 82.2–100) and 95.1 % (255/268; 95 % CI 92.6–97.7), respectively, compared with culture as the gold standard for the diagnosis of MTC. In contrast, the results of the smear microscopy were 24.4 % (11/45; 95 % CI 11.9–37.0), 98.1 % (253/258; 95 % CI 96.4–99.7), 68.8 % (11/16; 95 % CI 44.4–86.9) and 88.2 % (253/287; 95 % CI 84.4–91.9), respectively. The high PPV and NPV of the LAMP test indicated that the LAMP test was superior to smear microscopy for the detection MTC in sputum specimens.

The 98.8 % (255/258) clinical specificity of the TB-*hspX*-LAMP reaction was comparable or somewhat increased compared with previous reports (WHO, 2013b). It seems likely that 1.2 % (3/258) of LAMP positive in culture-negative samples were non-viable MTC in the samples.

Within the 45 culture-positive samples, the sensitivity of the TB-*hspX*-LAMP reaction for the 11 smear-positive samples (100 %, 11/11) and the sensitivity of the 34 smear-negative samples (61.8 %, 21/34) were also comparable to or relatively increased compared with those in previous reports (WHO, 2013b). The two clinical sensitivity results of the TB-*hspX*-LAMP reaction could be enhanced by improving the template DNA extraction and purification methods. For example, a commercially available ultrarapid DNA extraction method (PURE; Eiken) could be used to improve the sensitivity. In this case, the homogenization steps followed by centrifugation can be omitted and this would prevent the loss of bacteria during the process, as reported by Mitarai *et al.* (2011).

From a public health and infection control viewpoint, the LAMP method has an advantage in the clinical laboratory as it permits immediate distinction of MTC infection (with the need for patient quarantine) from NTM infection (without such need for quarantine). Conventionally, the diagnosis of TB at the hospital is based on smear microscopy, X-ray and culture confirmation. The culture methods require up to several weeks and continue with MTC identification by biochemical or molecular methods. Smear microscopy is still a fundamental technique for detection of TB patients in many settings in the world as a simple and rapid means of detection. However, the

Table 1. Comparison of LAMP, smear microscopy (S) and culture (C) results from 303 sputum specimens

Method	Culture*			Sensitivity in S+C+ [% (95 % CI)]	Sensitivity in S–C+ [% (95 % CI)]	Sensitivity in C+ [% (95 % CI)]	Specificity in C– [% (95 % CI)]	PPV [% (95 % CI)]	NPV [% (95 % CI)]
	Positive	Negative	Total						
LAMP†									
Positive	32	3	35	100 (71.5–100)	61.8 (45.4–78.1)	71.1 (57.9–84.4)	98.8 (97.5–100)	91.4 (82.2–100)	95.1 (92.6–97.7)
Negative	13	255	268	11/11	21/34	32/45	255/258	32/35	255/268
Smear									
Positive	11	5	16			24.4 (11.9–37.0)	98.1 (96.4–99.7)	68.8 (44.4–86.9)	88.2 (84.4–91.9)
Negative	34 (21)‡	253 (2)	287 (23)			11/45	253/258	11/16	253/287
Total	*45*	*258*	*303*						

*Culture was carried out on 2 % Ogawa solid medium: positive, *M. tuberculosis* was isolated; negative, no *M. tuberculosis* was isolated.
†LAMP reaction was performed using a Loopamp real-time turbidimeter (LA-500; Eiken): positive, amplification occurred; negative, amplification did not occur.
‡Number of LAMP-positive in parentheses.

sensitivity is low and it does not efficiently distinguish MTC from NTM in sputum specimens. We were able to perform LAMP diagnostics at the same time as smear microscopy. The LAMP detected only MTC, and showed excellent PPV and NPV.

In resource-limited settings, the conventional diagnostics of TB are difficult to implement. Our LAMP technique does not need experienced skill or effort. It will contribute to simplifying TB diagnosis.

Moreover, it takes a long time to perform a TB drug-susceptibility test because the culture of TB should be carried out in advance. The use of TB-*hspX*-LAMP would be advantageous in Korea because it has the potential to reduce the primary detection time before the drug-susceptibility test and to therefore improve the treatment of TB patients.

In Korea, the use of solid media is still popular instead of liquid culture, which is currently recommended as a standard method. We used solid media in this study. Despite this limitation, we confirmed the superiority of the LAMP method compared with smear microscopy.

We conclude that the use of TB-*hspX*-LAMP was superior to the use of smear microscopy for the detection of MTC in sputum specimens in clinical settings. Therefore, this method will contribute to an improvement in the treatment of TB patients in Korea and may provide a suitable alternative to smear microscopy for the detection of MTC.

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