Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients

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A number of nucleic acid amplification assays (NAAs) have been employed to detect tubercle bacilli in clinical specimens for tuberculosis (TB) diagnosis. Among these, loop-mediated isothermal amplification (LAMP) is an NAA possessing superior isothermal reaction characteristics. In the present study, a set of six specific primers targeting the *Mycobacterium tuberculosis* 16S rRNA gene with high sensitivity was selected and a LAMP system (MTB-LAMP) was developed. Using this system, a total of 200 sputum samples from Nepalese patients were investigated. The sensitivity of MTB-LAMP in culture-positive samples was 100 % (96/96), and the specificity in culture-negative samples was 94.2 % (98/104, 95 % confidence interval 90.5–97.9 %). The positive and negative predictive values of MTB-LAMP were 94.1 and 100 %, respectively. These results indicate that this MTB-LAMP method may prove to be a powerful tool for the early diagnosis of TB.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a common human disease, with 5.1 million new cases and 1.6 million deaths worldwide in 2005 (WHO, 2007). Although TB is often associated with developing nations and disadvantaged economic conditions, it is also gaining ground in many industrialized nations due to immigration, the emergence of drug-resistant strains and the AIDS epidemic.

Among the countries substantially affected by TB, Nepal has an elevated annual risk of infection, with an estimated 45 % of the total population being infected. In 2005, approximately 48 000 people suffered from active TB, of whom 22 000 had infectious smear-positive pulmonary disease (WHO, 2007). Although the introduction of the Directly Observed Treatment, Short-course (DOTS) method has reduced the number of deaths, over 6000 people in Nepal continue to die of TB every year (NTP, 2004).

Diagnosis of TB involves clinical evaluation, including the tuberculin skin test, chest radiography and bacteriological tests. Currently, bacteriological tests depend on microscopic observation of acid-fast bacilli (AFB) in clinical

Abbreviations: AFB, acid-fast bacilli; LAMP, loop-mediated isothermal amplification; NAA, nucleic acid amplification assay; TB, tuberculosis.

specimens and bacterial cultures from processed sputum samples, which has been the traditional method for many decades (Kent & Kubica, 1985). Although these techniques have been continuously refined and improved, they still have several limitations. Although sensitivity and specificity are insufficient, microscopic observation of AFB is widely used due to its rapidity. In contrast, bacteriological testing using bacterial cultures is considered the most accurate test due to its high sensitivity and specificity. However, this technique is both labour-intensive and time-consuming, requiring 6–8 weeks to achieve maximum sensitivity (Kent & Kubica, 1985). An additional technique, nucleic acid amplification assay (NAA), allows the rapid, sensitive and specific detection of *M. tuberculosis* in sputum samples by amplifying and detecting specific nucleic acid sequences (CDC, 1992). In addition to the widely used PCR, several other methods of nucleic acid amplification have been used for *M. tuberculosis* detection (Jonas *et al.*, 1993; Ruiz-Serrano *et al.*, 1998; Iwamoto *et al.*, 2003; Takakura *et al.*, 2005), including loop-mediated isothermal amplification (LAMP), a novel NAA which enables the detection of trace amounts of DNA under isothermal conditions, namely at 64 °C (Notomi *et al.*, 2000). LAMP showing high amplification efficiency has been used for the diagnosis of several diseases (Notomi *et al.*, 2000; Iwamoto *et al.*, 2003; Kuboki *et al.*, 2003; Parida *et al.*, 2004, 2005; Kimura *et al.*, 2005; Yoda *et al.*, 2007).

Here, we established an *M. tuberculosis* detection system using LAMP, and compared its sensitivity, specificity and applicability with results from culture, microscopic observation of acid-fast stained smears and chest radiography.

METHODS

Establishment of a LAMP method for the detection of *M. tuberculosis*. More than 50 of six primers recognizing eight distinct regions of the 16S rRNA gene were designed using the PrimerExplorer V3 software (Eiken Chemical; https://primerexplorer.jp/lamp3.0.0/index.html). A primer set was composed of outer primers F3 and B3, inner primers FIP and BIP, and loop primers FLP and BLP. From our preliminary experiments, a set was selected based on its high sensitivity. The specificity of the selected primer set was examined by LAMP reaction on extracted DNA from various bacterial strains, including *Mycobacterium tuberculosis*, *Mycobacterium bovis* Ravenel, *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium shimoidei*, *Mycobacterium nonchromogenicum*, *Mycobacterium xenopi*, *Mycobacterium scrofulaceum*, *Mycobacterium gordonae*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Achromobacter xylosoxidans*, *Acinetobacter haemolyticus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella boydii*, *Staphylococcus aureus* and *Streptococcus haemolyticus*. Genomic DNA from bacterial strains was prepared from colonies by mechanical disruption as previously described (Suzuki *et al.*, 1995) and dissolved in 300 μl TE buffer consisting of 10 mM Tris/HCl (pH 8.0) and 1 mM EDTA. LAMP reactions were performed in a total volume of 25 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 $MgSO_4$ and 8 U *Bst* DNA polymerase (New England Biolabs) with specified amounts of bacterial DNA. The mixture was incubated at 64 °C for 60 min in a Loopamp real-time turbidimeter (LA-200; Teramecs). The reaction was considered positive when turbidity was greater than or equal to 0.1 within 60 min. The sensitivity of the primer set was examined by LAMP reaction on serially diluted DNA preparations from cultured *M. tuberculosis* H37Ra.

In addition, the sensitivity of the primer set was examined using sputum samples from patients. The sediment layer of *N*-acetyl-L-cysteine-NaOH-treated sputum was suspended in distilled water using standard procedures (Kent & Kubica, 1985) and serially diluted with sterile distilled water to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} concentrations. Following this, 0.1 ml of each dilution was cultured on Löwenstein–Jensen (L–J) medium at 37 °C for 4 weeks to count bacterial colonies. DNA was extracted from the sputum samples using a DNA extraction kit (DNeasy Blood & Tissue kit; Qiagen) and used for LAMP reaction. Results were visualized with fluorescence detection reagent (Eiken) according to the manufacturer's instructions. The relation between the number of colonies observed on the L–J medium and the MTB-LAMP results was then evaluated to assess sensitivity on sputum samples.

Evaluation of MTB-LAMP using sputum samples. This study was carried out from September 2004 to October 2005 in Nepal. Samples were collected and examined by acid-fast smear and bacterial culture at the National Tuberculosis Center (NTC), DNA extraction was performed at the Mycobacterial Research Laboratory, Anandaban Hospital, and MTB-LAMP reactions were carried out at the Everest International Clinic and Research Center. One hundred AFB-positive sputum samples from pulmonary TB patients and 100 AFB-negative sputum samples from patients with chest pain, cough and fever were collected at the NTC. During sample and data collection, study objectives and expected outcomes were explained to each study participant before receiving their consent. About 4 ml of mucoid or muco-purulent early morning sample was collected, followed on the same day by microscopy and sample culture preparation. A 50 μl sputum sample was smeared directly on a slide, covering an area of approximately 2.0×1.0 cm. For the detection of AFB, a standard fluorochrome method (Truant *et al.*, 1962) was employed. Results were interpreted based on WHO guidelines. The remaining sample was treated by conventional methods as above and used for culture growth and MTB-LAMP. For culture growth, 0.1 ml sputum suspension was inoculated into two culture tubes containing 2 % Ogawa medium and incubated at 37 °C. When colonies were observed during incubation, acid-fastness of bacilli was determined by a smear test. When no colonies appeared after weekly observation for 8 weeks, the result was considered negative.

Mycobacterial DNA was extracted from concentrated sputum samples by freezing and boiling (Woods & Cole, 1989). LAMP reactions were performed as described above using 5 μl extracted DNA. Results were visualized using fluorescence detection reagent. Patients with lung cavity observed by chest radiography were considered X-ray-positive.

RESULTS AND DISCUSSION

The diagnosis of TB relies primarily on microscopic analysis, which is a rapid method of detecting AFB. However, this method is limited in sensitivity and ability to identify infecting species. In contrast, although the bacterial culture method can efficiently identify species, it is a time-consuming procedure. A test that combines the rapidity of microscopy and sensitivity of bacterial culture methods

would facilitate the initiation of clinical TB treatment. Here, detection of *M. tuberculosis* using the MTB-LAMP system was developed and evaluated.

Development of LAMP for the detection of *M. tuberculosis*

Among more than 50 sets of primers examined during our preliminary study, a set was selected based on its high sensitivity for purified DNA of *M. tuberculosis* H37Ra. As shown in Fig. 1(a), these primers targeted the first variable region of the *rrs* gene. Positions and orientations of F3, F2, FL, F1c, B1, BL, B2c and B3c primers (Notomi *et al.*, 2000) are shown by arrows in Fig. 1(b). The sequences of MTB-FIP, MTB-BIP, MTB-F3, MTB-B3, MTB-FLP and MTB-BLP primers are shown in Fig. 1(c).

We first examined the specificity of MTB-LAMP for genomic DNA from various bacterial species. Efficient DNA amplification by MTB-LAMP was observed in the *M. tuberculosis* complex including *M. tuberculosis*, *M. bovis*,

M. africanum and *M. microti* (Fig. 2a). In contrast, no DNA amplification was observed with the remaining bacterial species evaluated. This absence of amplification was also observed using larger amounts of bacterial genomic DNA (10 ng) (data not shown). These results suggest that the primer set used for LAMP is highly specific for the *M. tuberculosis* complex. The specificity of MTB-LAMP was further confirmed using DNA from *M. tuberculosis*, the *M. avium* complex and *M. kansasii* clinical isolates. MTB-LAMP reactions were positive for all 121 *M. tuberculosis* samples and negative for all 17 *M. avium* complex and 18 *M. kansasii* clinical isolates (Table 1). These results clearly demonstrate the high specificity of the LAMP system in detecting *M. tuberculosis*. Further confirmation of the selected primer set sensitivity was obtained by serially diluting DNA from *M. tuberculosis* H37Ra. Specific DNA amplification by MTB-LAMP was observed with starting DNA amounts as low as 100 fg (Fig. 2b). Because the weight of 4.5 billion base pairs from one bacterial genome is estimated at 5 fg, this result suggests that as few as 20
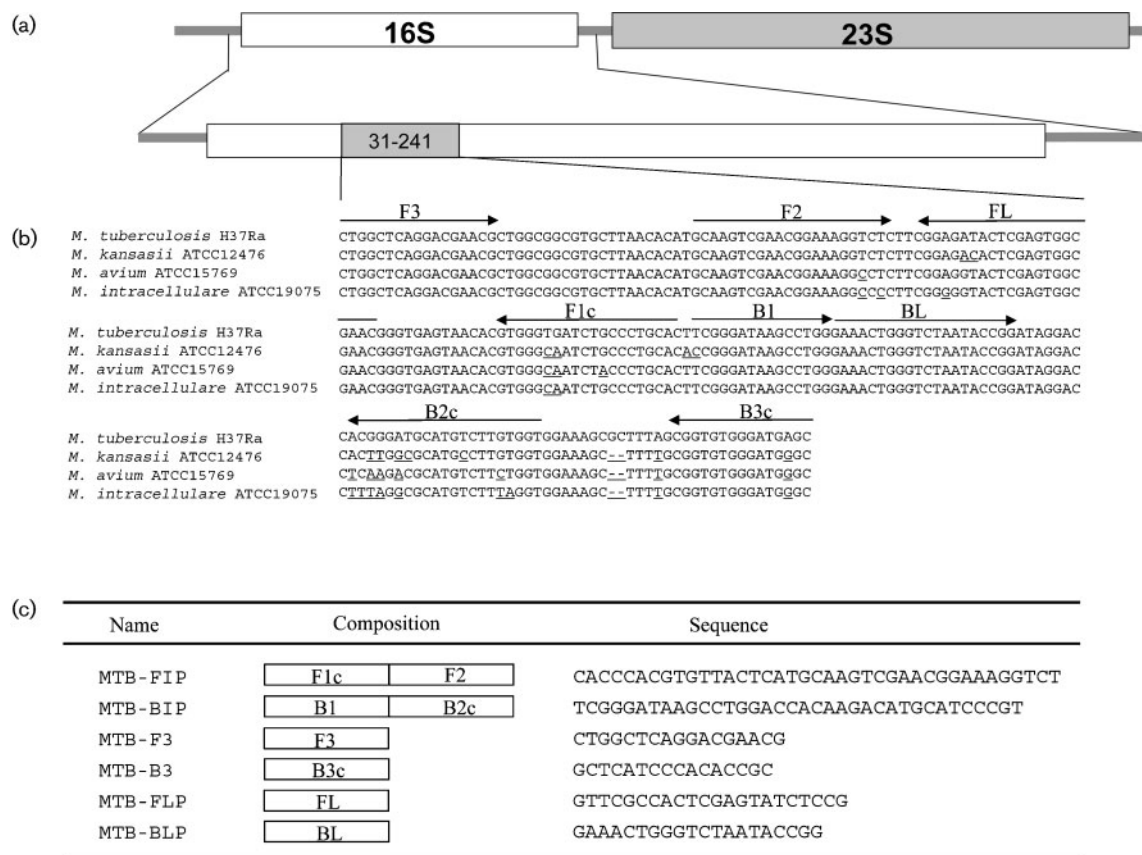

Fig. 1. Primers for the specific detection of the *M. tuberculosis* complex. (a) Position of the target on the 16S rRNA (*rrs*) gene for MTB-LAMP (near first variable region: 31–241). (b) Position and orientation of the primer annealing site on the first variable region of the *rrs* gene. Bases different from those in *M. tuberculosis* H37Ra are underlined. (c) Composition and sequence of six primers used for MTB-LAMP. F1c and F2 in Fig. 1(b) were combined to form the MTB-FIP primer; B1 and B2c in Fig. 1(b) were combined to form the MTB-BIP primer. F3, B3c, FL and BL in Fig. 1(b) were used as MTB-F3, B3, FLP and BLP primers, respectively.
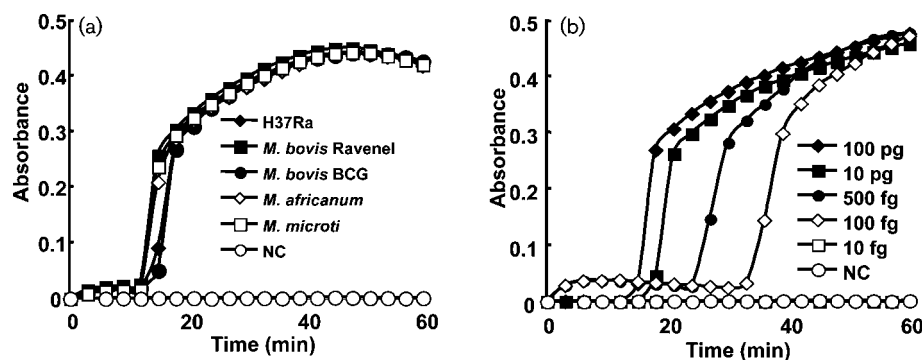
Fig. 2. Specificity and sensitivity of MTB-LAMP determined by real-time turbidimetry. The amount of DNA amplification by-product (Mg-pyrophosphate) was monitored by measuring turbidity (590 nm). NC, Negative control. (a) Specificity of MTB-LAMP analysed using extracted DNA from various bacterial species. Only results for the *M. tuberculosis* complex are shown. No positive reaction was observed with DNA from the other bacterial species. (b) Sensitivity of MTB-LAMP analysed using various concentrations of *M. tuberculosis* H37Ra DNA.

copies of *M. tuberculosis* DNA can be detected using our primer set. Using a bacterial culture of the same NaOH-treated sputum samples, the minimum detectable number of live *M. tuberculosis* species in a sputum sample by MTB-LAMP was determined to be 10 (data not shown). These observations show that MTB-LAMP is more sensitive than a LAMP method used in a previous study, in which the detection of *M. tuberculosis* targeting the *gyrB* gene was estimated to require a minimum of 50 copies of DNA (Iwamoto *et al.*, 2003).

Evaluation of MTB-LAMP for the detection of *M. tuberculosis* in clinical specimens

Application of MTB-LAMP to clinical specimens was evaluated by comparing LAMP results with acid-fast smear tests, bacterial cultures and chest radiographs of 200 patients in Nepal. Results are summarized in Table 2. In culture-positive (C$^+$) samples, *M. tuberculosis* was detected by MTB-LAMP in all 90 smear-positive (S$^+$) and 6 smear-negative (S$^-$) samples. The sensitivity of MTB-LAMP in C$^+$ specimens was 100 % (96/96). This result suggests that MTB-LAMP may be superior to the acid-fast smear test and comparable with bacterial culture testing, though the number of C$^+$ and S$^-$ samples was not large enough to

strongly conclude this. In culture-negative (C$^-$) samples, MTB-LAMP reactions were positive in 1 of 5 S$^+$ X-ray-negative (X$^-$) cases; 2 of 13 S$^-$ X$^+$ cases; and in 3 of 81 S$^-$ X$^-$ cases. No positive MTB-LAMP reaction was observed in the five S$^+$ X$^+$ patients. MTB-LAMP specificity in C$^-$ samples was 94.2 % (98/104; 95 % confidence interval 90.5–97.9 %). The positive and negative predictive values of MTB-LAMP were 94.1 and 100 %, respectively. These results from C$^-$ patients may suggest that MTB-LAMP is possibly highly sensitive in the detection of a small number of tubercle bacilli released from hidden foci in sputum.

M. tuberculosis was detected by MTB-LAMP in all C$^+$ and a number of C$^-$ samples. Compared with results from standard culture testing, acid-fast staining and chest radiography, our results demonstrate the high clinical performance of MTB-LAMP in the rapid detection of *M. tuberculosis* from sputum samples. A study by Iwamoto *et al.* (2003) on LAMP sensitivity in sputum samples indicated that the detection limit of their LAMP assay is equivalent to that of the Amplicor assay (Roche Diagnostics). In some C$^+$ sputum samples, however, their system was unable to detect *M. tuberculosis*. In contrast, the sensitivity of our MTB-LAMP appears to be superior

Table 1. Reactivity of MTB-LAMP with 10 ng DNA from *M. tuberculosis*, *M. avium* complex and *M. kansasii* clinical isolates

Clinical isolates	LAMP reaction	
	+	−
M. tuberculosis	121/121	0/121
M. avium complex	0/17	17/17
M. kansasii	0/18	18/18

Table 2. Comparison of MTB-LAMP results with smear, culture and chest radiograph results

The table shows the percentage of *M. tuberculosis* LAMP-positive cases (no. of positive/total cases).

	Culture (+)		Culture (−)	
	X-ray (+)	X-ray (−)	X-ray (+)	X-ray (−)
Smear (+)	100 % (90/90)	0 % (0/0)	0 % (0/5)	20 % (1/5)
Smear (−)	100 % (5/5)	100 % (1/1)	15 % (2/13)	3.7 % (3/81)
	100 % (96/96)		5.8 % (6/104)	

because of the detection of *M. tuberculosis* in all 96 C$^+$ samples (100 % sensitivity). A multicentre study on *M. tuberculosis* detection showed the feasibility of using the LAMP method in developing countries (Boehme *et al.*, 2007). The study evaluated a prototype LAMP assay targeting the *gyrB* gene and using a simplified manual DNA extraction method. The sensitivity of MTB-LAMP was 97.7 % (173/177) in S$^+$ and C$^+$ sputum samples and 48.8 % (21/43) in S$^-$ and C$^+$ sputum samples. This is lower than in the present results, with a sensitivity of 100 % (90/90) and 100 % (6/6) for C$^+$ S$^+$ and S$^-$ sputum samples, respectively. Although our MTB-LAMP method is more time-consuming regarding sputum sample manipulation, it has the advantage of being highly sensitive.

In conclusion, this MTB-LAMP assay is an NAA method that allows direct identification of *M. tuberculosis* in processed sputum samples. Due to its ease of use in developing countries and its high sensitivity and specificity, this assay may facilitate the identification of *M. tuberculosis* in S$^+$ cases, as well as the confirmation of diagnosis in suspected but sputum S$^-$ clinical TB cases.

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