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Use of visual loop-mediated isotheral amplification of *rimM* sequence for rapid detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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ABSTRACT

Mycobacterium tuberculosis and *Mycobacterium bovis* are pathogenic bacterial species in the genus *Mycobacterium* and the causative agents of most cases of tuberculosis (TB). Detection of *M. tuberculosis* and *M. bovis* using conventional culture- and biochemical-based assays is time-consuming and laborious. Therefore, a simple and sensitive method for rapid detection has been anxiously awaited. In the present study, a visual loop-mediated isothermal amplification (LAMP) assay was designed from the *rimM* (encoding 16S rRNA-processing protein) gene sequence and used to rapidly detect *M. tuberculosis* and *M. bovis* from clinical samples in South China. The visual LAMP reaction was performed by adding calcein and manganous ion, allowing the results to be read by simple visual observation of color change in a closed-tube system, and which takes less than 1 h at 65 °C. The assay correctly identified 84 *M. tuberculosis* isolates, 3 *M. bovis* strains and 1 *M. bovis* BCG samples, but did not detect 51 non-tuberculous mycobacteria (NTM) isolates and 8 other bacterial species. Sensitivity of this assay for detection of genomic DNA was 1 pg. Specific amplification was confirmed by the ladder-like pattern of gel electrophoresis and restriction enzyme *Hha*I digestion. The assay successfully detected *M. tuberculosis* and *M. bovis* not only in pure bacterial culture but also in clinical samples of sputum, pleural fluid and blood. The speed, specificity, sensitivity of the *rimM* LAMP, the lack of a need for expensive equipment, and the visual readout show great potential for clinical detection of *M. tuberculosis* and *M. bovis*.

1. Introduction

Tuberculosis (TB) is an ancient scourge of humankind, caused by *Mycobacterium tuberculosis* and infrequently by other species of the *Mycobacterium tuberculosis* complex (MTC), such as *Mycobacterium bovis* (Behr et al., 1999). TB is a major cause of illness and death worldwide, especially in Asia and Africa, with 9.2 million new cases and 1.7 million deaths occurring in 2006 globally (WHO, 2008). Control of TB relies on diagnosis and treatment of infected patients. However, detection of *M. tuberculosis* and *M. bovis* is a notorious problem for public health and clinical laboratories because of their slow growth rate, which may take 4 to 12 weeks for blood or sputum culture (Iwamoto et al., 2003). Therefore, there is a great need of a simplified method for rapid, sensitive and specific detection of *M. tuberculosis* and *M. bovis*.

One of the most potential candidates is a novel nucleic acid amplification method termed Loop-mediated isothermal amplification (LAMP), which relies on auto cycling strand displacement DNA synthesis performed by the enzyme *Bst* polymerase (Notomi et al., 2000). The technique uses four primers that recognize six regions of the target DNA and the amplification products are stem-loop DNA structures with several inverted repeats of the target DNA. LAMP is a simple, rapid and cost-effective nucleic acid method, and the resulting amplicons can be detected by confirming the presence of generated white precipitate (magnesium pyrophosphate) or visualized by adding SYBR Green I after reaction (Mori et al., 2001; Enosawa et al., 2003; Iwamoto et al., 2003). Since its invention, LAMP has been used to detect a number of infectious agents, including *Vibrio parahaemolyticus* (Yamazaki et al., 2008), dengue virus (Parida et al., 2005), *Trypanosoma brucei rhodesiense* (Njiru et al., 2008), *Plasmodium falciparum* (Poon et al., 2006), *M. tuberculosis* (Pandey et al., 2008) and *M. avium* (Iwamoto et al., 2003).

However, the detection system of LAMP need to be improved because the white precipitate is rather faint for visual detection and opening tube to add SYBR Green I involves a high risk of workspace contamination with amplicons. Since the large amounts of products are a repeat of the same sequence, the highly sensitive LAMP reaction may lead to incorrect result upon contamination of even a small

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quantity of amplicons. Therefore, there is a need to develop a closed reaction system for easy readout of LAMP result. Recently, Tomita et al. (2008) reported an improved LAMP by adding calcein (a fluorescent metal indicator) and manganous ion (Mn^{2+}) to the reaction, in which the result allowed visualization by color change without opening the tube or any detection device. The reaction solution initially appears orange because the calcein is quenched by combining with manganous ion. When the amplification proceeds, the calcein is deprived of manganous ions by newly generated pyrophosphate ions ($P_2O_7^{4-}$), which results in the emission of fluorescence and thus the solution appears green. The improved LAMP not only simplified the detection system but also minimized the risk of workspace contamination.

In the present study, we developed a visual *rimM*-LAMP for specific detection of *M. tuberculosis* and *M. bovis*, and succeeded their detection in clinical sputum, pleural fluid and blood samples. The sensitivity, specificity and applicability of the visual *rimM* LAMP were evaluated. Our results indicate that the visual *rimM* LAMP is sensitive and specific and has the potential to be developed into an early diagnosis tool for TB.

2. Materials and methods

2.1. Bacterial strains

A total of 147 bacterial strains were used, including 84 *M. tuberculosis*, 3 *M. bovis*, 51 NTM, 1 *M. bovis* BCG and 8 other bacterial species. All of the *M. tuberculosis*, *M. bovis* and NTM strains were well characterized clinical isolates collected from patients with suspected TB between Jan 2007 and Mar 2008 in South China. The 51 NTM isolates included 24 *M. avium-intracellular* complex, 7 *M. gordonae*, 7 *M. chelonei,* 5 *M. abscessus*, 1 *M. scrofulaceum*, 1 *M. smegmatis* and 6 *M. nonchromogenicum*. The 8 other bacterial species used to determine the specificity of LAMP detection were commonly encountered bacteria deposited in our laboratory, including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Pasteurella multocida*, *Streptococcus pneumoniae*, *Sarcina lutea, Bacillus subtilis* and *Legionella pneumophil*a.

2.2. Template preparation from positive culture

Genomic DNA was extracted as described previously (Xue et al., 2004). Briefly, isolated colonies from the Löwenstein–Jensen medium were treated with a 300 µl lysis solution (4.5 g/L Tween-20, 4.5 g/L NP-40, 0.2 g/L proteinase K and 1 × PCR buffer), incubated at 55 °C for 3 h and then heat-killed at 95 °C for 5 min. Cell lysates were extracted by the phenol/chloroform method. The purified genomic DNA was used as template for LAMP and PCR.

2.3. DNA oligonucleotides

In order to obtain a specific gene marker for *M. tuberculosis* and *M. bovis*, genome comparison of bacteria and BLAST analysis on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) were performed, and the results showed 16S rRNA-processing protein gene (*rimM*) (GeneID: 887188) sequence was specific and highly conserved in *M. tuberculosis* and *M. bovis*, indicating that *rimM* could be a new candidate gene for detection of *M. tuberculosis* and *M. bovis*. Four primers for LAMP were designed based on the *rimM* gene sequence according to the criteria described previously (Notomi et al., 2000; Tomita et al., 2008), including two outer primers (F3 and B3), one forward inner primer (FIP) and one backward inner primer (BIP) (Table 1). They recognize six distinct regions on the target DNA (Fig. 1). FIP consists of F1c sequence complementary to F1, a TTT linker and F2 sequence; BIP consists of B1c sequence complementary to B1, a TTTT linker and B2 sequence.

2.4. Optimization of the visual LAMP conditions

The visual LAMP was carried out in a 25 µl volume by adding two more reagents (calcein and manganese chloride) to the conventional LAMP reaction mixture. The reaction system was described previously (Notomi et al., 2000; Tomita et al., 2008). The final reaction mixture comprised of 0.8 µM (each) of FIP and BIP primers, 0.2 µM (each) of each F3 and B3 outer primers, 400 µM of each dNTP, 1 M betaine (Sigma), 25 µM calcein (Sigma), 0.5 mM $MnCl_2$ (Sigma), 1 × thermopol buffer (New England Biolabs), 8 U of *Bst* DNA polymerase large fragment (New England Biolabs) and 1 µl of genomic DNA (~100 ng). To find the optimum temperature and time for the visual LAMP amplification, the reactions were carried out in a thermocycler at 63, 64, 65, 66 and 67 °C for 25, 35, 45 and 60 min. The reaction was terminated by increasing the temperature to 80 °C for 4 min. A positive control (purified DNA of *M. tuberculosis*) and a negative control (distilled water) were included in each run.

2.5. PCR reaction

PCR reaction with F3 and B3 primers was performed in a 25 µl reaction mixture containing 2.5 µl 10 × *Ex Taq* buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.625 U *TaKaRa Ex Taq* DNA polymerase (TaKaRa, DaLian, China) and 1 µl genomic DNA as a template. The conditions used for amplification were as follows: initial denaturing at 94 °C for 5 min; 30 cycles of amplification with 0.5 min at 94 °C, 0.5 min at 55 °C and 1 min at 72 °C; and ending with a final extension for 7 min at 72 °C.

2.6. Detection and analysis of visual LAMP and PCR products

Amplification products generated from LAMP can be directly detected by visual inspection under daylight or UV light. To confirm the structure of the LAMP products, the amplicons were analyzed by gel electrophoresis in 2% agarose gel. To confirm the specificity of the LAMP products, the amplicons were purified using a high pure PCR purification kit (Invitrogen, Carlsbad, CA, USA) and then digested with restriction enzyme *Hha*I (New England Biolabs) at 37 °C for 3 h, followed by electrophoresis in 2% agarose gel.

The PCR products were electrophoresed in 2% agarose gel-stained with ethidium bromide and were visualized under UV light. The target PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced by using an ABI Prism 3730 DNA sequencer (Perkin-Elmer, Wellesley, MA, USA).

Table 1
Nucleotide sequences for the visual *rimM* LAMP primers.

Primer type	Sequence(5′-3′)	Length	Target
F3	CTAAGGGGCCTTTTGACGG	19	*rimM* gene
B3	CACCACTTCGGTGACGACAC	20	*rimM* gene
FIP (F1c + F2)	TCCAGCGAGTCGCACCAACAGTTTGGCAGTGCGGTGAGTTACGTC	45	*rimM* gene
BIP (B1c + B2)	GACGCCGATGACTTGCCCCCTTTTTCGCCGTCTGGACCATAAGC	44	*rimM* gene