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Development of a loop-mediated isothermal amplification assay for the detection of *Mycobacterium bovis*

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ABSTRACT

Bovine tuberculosis caused by *Mycobacterium bovis* is an important zoonosis. In this study, a simple, rapid method for detecting this organism was developed based on loop-mediated isothermal amplification of the *mpt83* gene. The technique will be of value in the clinical and field-based diagnosis of *M. bovis* and can differentiate it from other bacteria such as *Corynebacterium diphtheriae, Streptococcus pneumoniae,* β-haemolytic streptococci, *Pseudomonas aeruginosa, Yersinia pseudotuberculosis, Staphylococcus aureus,* and *Klebsiella pneumoniae.* The limit of detection was 10 copies/μL and the results were corroborated by PCR. The method was highly specific and more sensitive than PCR.

Introduction

Bovine tuberculosis (TB) caused by *Mycobacterium bovis* causes significant disease in cattle and wildlife populations worldwide and is also a zoonosis, spreading through inhalation of infectious droplets and following the ingestion of raw milk (Thoen et al., 2006). The World Health Organization estimated that there were 9.2 million new cases of human infection with *M. bovis* in 2006,[1] so there is a clear need for rapid and reliable diagnosis and control of this disease.

The standard laboratory diagnosis of TB relies on the detection of acid-fast bacilli by microscopic examination of Ziehl–Neelsen-stained smears and on the culture of the organism on appropriate media. Smear microscopy can have low sensitivity and periods of 6–8 weeks are frequently required to culture *M. bovis* (Christie and Callihan, 1995). The high cost of molecular detection methods such as PCR, where specialised equipment and reagents are required (Chakravorty and Tyagi, 2005; Chakravorty et al., 2005a,b), has resulted in an increased effort to find alternative diagnostic methods applicable in the field.

Loop-mediated isothermal amplification (LAMP) is a novel method of amplifying nucleic acid sequences (Notomi et al., 2000) that has been applied for the detection of bacteria (Pandey et al., 2008; Saleh et al., 2008; Yamazaki et al., 2008). The technique relies on auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi et al., 2000; Nagamine et al., 2002a) and has the advantages of amplifying DNA under isothermal conditions (63–65 °C), producing a result within 60 min (Notomi et al., 2000), and only requiring the use of a conventional water-bath, which keeps the cost of the method low relative to other molecular detection techniques. In this study, we assessed the capacity of a modified LAMP method to detect *M. bovis* in sputum.

Materials and methods

Bacteria, samples and DNA extraction

The bacteria used were identified by standard biochemical tests (Table 1). Experiments involving bacteria were performed in a biosafety level-3 laboratory. Bacterial DNA was extracted by precipitation of sputum or from suspensions of clinical samples. Two methods were used to prepare the DNA for LAMP and PCR. Firstly, to extract DNA for sensitivity studies we used the TaKaRa MiniBEST Bacterial Genomic DNA extraction kit (Takara Bio. Co.) according to the manufacturer's instructions and DNA was eluted in a final volume of 100 μL of buffer. In the second method, DNA was extracted from one or two colonies of each bacterium following 20 min boiling to obtain the crude lysate (Mohran et al., 1998). Following extraction, DNA was immediately stored at −70 °C prior to its use in the one-step PCR or LAMP reaction.

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[1] See: www.who.int/tb/publications/global_report/2008/chapter_1/en/index3.html.

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Table 1
Bacteria used in the assessment of the specificity of loop-mediated isothermal amplification (LAMP) reaction.

Bacteria	Source[a]	Number
Mycobacterium bovis	GLAMI	1
Mycobacterium bovis (isolate 1)	SCAUVCSL, lung	10
Mycobacterium bovis (isolate 2)	SCAUVCSL, lung	10
Corynebacterium diphtheriae	GLAMI	1
Streptococcus pneumoniae	GLAMI	1
β-*Haemolytic streptococci*	GLAMI	1
Pseudomonas aeruginosa	GLAMI	1
Yersinia pseudotuberculosis	GLAMI	1
Staphylococcus aureus	GLAMI	1
Klebsiella pneumoniae	GLAMI	1

[a] GLAMI: Guangdong Laboratory Animals Monitoring Institute, SCAUVCSL: South China Agriculture University Veterinary Clinical Surgery Laboratory.

Table 2
Comparison of loop-mediated isothermal amplification (LAMP) assay with the tuberculin skin test (TST) and PCR in detecting *Mycobacterium bovis* in bovine sputum.

Farm	Specimen number[a]	Number of positives by TST	PCR	LAMP
1	50S	10	8	10
	50B	10	9	10
2	50S	15	10	15
3	50S	15	9	15
4	50S	0	0	0

[a] S: sputum, B: conjunctival swab.

A total of 250 samples were collected from four dairy farms (Table 2). Of these, 200 were from cows that had negative tuberculin skin test (TST) reactions and included 100 oral, 40 conjunctival and 60 sputum swabs from each cow. Forty oral and 10 conjunctival swabs were taken from cows that had positive TST reactions. DNA was obtained from the samples following boiling for 20 min, de-contamination with N-acetylcysteine–sodium hydroxide and concentration by centrifugation. All samples were examined using both the conventional PCR and LAMP assays.

Protocols for LAMP and PCR assays

A set of six primers consisting of two outer (F3 and B3), a forward inner (FIP), a backward inner (BIP) and two loop (loop F and loop B) primers were designed based on the sequence of the *M. bovis* cell surface lipoprotein *mpt83* (Hewinson et al., 1996), using PrimerExplorer V4.[2] These primers recognise eight distinct regions on the target DNA (Fig. 1). The FIP consists of a complementary sequence of F1c and a sense sequence of F2 and the BIP consists of a sense sequence of B1 and a complementary sequence of B2c.

LAMP reactions were carried out in a conventional water-bath by mixing 40 pM each of FIP and BIP primer, 5 pM each of F3 and B3 primer, 20 pM each of Loop F and Loop B primer, 8.75 mM of deoxynucleoside triphosphate, 8 U of *Bst* DNA polymerase (8000 U/mL) (New England Biolabs), 10× Thermopol reaction buffer and 1 μL of extracted DNA. Amplification was performed at 63 °C for 60 min, followed by heating at 80 °C for 2 min to terminate the reaction. LAMP products were analysed by 1.5% agarose gel electrophoresis.

LAMP amplicons in the reaction tube were visualised by adding 1 μL of 1/10-diluted original SYBR Green I (Xiamen Biov Gentech Co.). The solution turned green in the presence of a LAMP amplicon, but otherwise remained orange. Confirmation of amplified product was by agarose gel electrophoresis. Aliquots (5 μL) of LAMP products were subject to electrophoresis on 1.5% agarose gels, followed by staining with GoldView (Beijing SBS Gentech Co.). The relative sensitivity of direct visualisation and electrophoresis were compared using serially diluted LAMP products.

The PCR reaction was carried out in a mixture containing 10 mM of deoxynucleoside triphosphates (dNTPs), 8 U of Taq polymerase, 1 μM each of primers F and R, and a template consisting of 1 μL of extracted DNA. The amplification protocol was 5 min at 94 °C, followed by 34 cycles of 94 °C for 40 s, 50.3 °C for 40 s, 72 °C for 1 min and a final elongation step for 10 min at 72 °C. The PCR products were analysed by 2% agarose gel electrophoresis.

Sensitivity and specificity of LAMP identification

The detection limit of the LAMP assay was compared with that of PCR using the same templates at identical concentrations in triplicate. DNA transcripts corresponding to the *M. bovis mpt83* gene were generated to use as standards. DNA was extracted from *M. bovis* using the TaKaRa MiniBEST extraction kit. The amplified product was cloned into the pMD18-T vector (TaKaRa Co.) according to the manufacturer's instructions and was sequenced to verify its accuracy. The recombinant plasmid was linearised, gel-purified and used as template in the Fastfilter Plasmid Midi kit (Omega, Feyou Biotechnology Co.) according to the manufacturer's instructions. The length of each DNA transcript was verified by agarose gel analysis, and 10-fold dilutions of *M. bovis* DNA plasmid (spanning 10–10^5 copies/μL) were used to provide a set of templates to test the sensitivity of the LAMP identification of *M. bovis*.

To determine the specificity of the method, the LAMP assay was carried out under the conditions described above with DNA templates from eight different bacteria (Table 1). Each organism was examined at least twice.

Validation of LAMP assay

The ability of the LAMP assay to identify *M. bovis* was validated using a total of 21 strains (Table 1) grown on Middlebrook 7H9 agar plates supplemented with glycerol and polysorbate 80 and tested for *M. bovis* using the LAMP assay. The results were compared with those obtained using conventional PCR. Two parallel experiments were carried out in which DNA templates were extracted by one of the two methods described above.

Results

Sensitivity of LAMP compared to PCR assay

The LAMP reaction with the specific primers at 63 °C for 60 min produced multiple bands of different size on electrophoresis because the products consisted of several inverted-repeat structures. Thus, whereas LAMP produced a ladder-like pattern, the PCR product was a specific DNA band corresponding to an 850 bp amplicon. The detection sensitivity of the LAMP assay was 10-fold greater than that of conventional PCR (100 relative to 10 copies/reaction) (Fig. 2).

Specificity of LAMP assay

The LAMP reaction was highly specific for *M. bovis* (Fig. 3). Amplification products exhibited a typical ladder-like pattern on electrophoresis indicating the formation of stem-loop DNA with inverted repeats (Notomi et al., 2000). Conversely, the assay was negative when distilled water (the negative control) and the bacteria listed in Table 1 were assessed.

Validation of LAMP assay

Twenty-one *M. bovis* strains tested positive by the LAMP assay and the results determined by direct visualisation were in agreement with those obtained by electrophoresis (Fig. 4). The results obtained by LAMP and conventional PCR were also identical and identification of *M. bovis* was not influenced by the extraction method.

Evaluation of LAMP assay using clinical samples

The diagnostic sensitivity of the LAMP method was assessed by testing the 250 samples as well as by PCR and TST (Table 2). Overall, the sensitivity of LAMP was higher than that of PCR and its specificity was similar to that obtained by PCR and TST. Of the 50 samples from TST-positive animals, all were positive by the LAMP assay, but 14 of these were negative on PCR. The detection rate by PCR and LAMP was therefore 72% and 100%, respectively.

Discussion

Although the TST is useful in the diagnosis of bovine TB, confirmation relies on the identification of the aetiological agent (Shinnick and Good, 1995). The diagnostic process

[2] See: https://primerexplorer.jp/lamp4.0.0/index.html.