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Full Length Research Paper

Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Mycoplasma bovis*

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A loop-mediated isothermal amplification (LAMP) assay targeting *uvrC* of *Mycoplasma bovis* was developed and evaluated. The assay specifically amplified only *M. bovis*; no cross-reactivity was observed for other *Mycoplasma* species or respiratory bacterial species. The sensitivity of the assay in pure cultures was 10-fold higher than that of polymerase chain reaction (PCR), with a detection limit of 34 CFU per reaction. The accuracy of the assay was further validated by both restriction analysis and nucleotide sequencing of the amplified product. The assay was applied to 98 specimens collected from cattle with respiratory disease or from healthy individuals and compared with a PCR-based assay. The sensitivity and specificity of the LAMP assay in terms of PCR was 100 and 74%, respectively. In conclusion, we successfully developed a rapid, specific, and sensitive LAMP test for *M. bovis* detection in a clinical setting.

Key words: Loop-mediated isothermal amplification, polymerase chain reaction, *uvrC* gene, *Mycoplasma bovis*, cattle.

INTRODUCTION

Mycoplasma bovis is an important but sometimes overlooked cattle pathogen. It causes major economic losses mainly by causing pneumonia, arthritis, and reduced weight gain in calves; mastitis in cows; and reproductive problems in both cows and bulls (Tenk et al., 2006; Caswell and Archambault, 2007). It is found worldwide and has spread into Africa, including Sudan (Abbas, 1996), Morocco (Taoudi et al., 1988) and the Republic of South Africa (Gilbert and Oettlé, 1990). Because *M. bovis* is largely unresponsive to antibiotic treatment, and because co-infection with other pathogens during subsequent stages make treatment more difficult (Nicholas and Ayling, 2003), a rapid, reliable, and practical diagnosis is essential for effective *M. bovis* prevention and control.

The diagnosis of *M. bovis* infection is generally determined by culture, serologic, and molecular methods. Culture and metabolism-based assays are both time-consuming and complex. In addition, the isolation of *M. bovis* from chronically ill animals is sometimes difficult because antibiotic treatment or secondary infections can inhibit isolation (Cai et al., 2005; Tenk et al., 2006). Serological methods are insufficiently sensitive, and cross-reactions with other *Mycoplasma* species can result in doubtful serological test results (Ghadersohi et al., 2005; Rosengarten et al., 1994). Molecular methods such as conventional polymerase chain reaction (PCR), multiplex PCR, and real-time PCR have been used to detect *M. bovis* (Cai et al., 2005; Foddai et al., 2005; Sung et al., 2006). Although, PCR-based assays for *M. bovis* are rapid and precise, they require agarose gel electrophoresis, which is time-consuming and tedious (Yamazaki et al., 2008). Real-time PCR assays are faster and involve less handling of the products compared with conventional PCR assays; however, because of an expensive thermal cycle with a fluorescence detector, this application is still not very common (Saito et al., 2005).

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Table 1. Bacteria and results of the LAMP assay.

Organism	Strain	LAMP result
Mycoplasma bovis	HB0801, clinical isolates (n = 19)	+ (20/20)
Mycoplasma agalactiae	ATCC 35890, CVCC 344	- (0/2)
Mycoplasma arginini	CVCC 346	- (0/1)
Mycoplasma ovipneumoniae	ATCC 29419	- (0/1)
Mycoplasma mycoides subsp. *Capri*	CVCC 368	- (0/1)
Mycoplasma gallisepticum	Clinical isolate	- (0/1)
Bacillus anthracis	Clinical isolate	- (0/1)
Escherichia coli	Clinical isolate (n = 2)	- (0/2)
Mycobacterium bovis	ATCC BAA-935	- (0/1)
Streptococcus pneumoniae	Clinical isolates (n = 2)	- (0/2)
Staphylococcus aureus	ATCC 25923, clinical isolate	- (0/2)
Mannheimia haemolytica	Clinical isolate	- (0/1)
Pasteurella multocida	Clinical isolate (n = 5)	- (0/5)
Salmonella typhimurium	ATCC 14028	- (0/1)
Arcanobacterium pyogenes	Clinical isolate	- (0/1)
Bacillus proteus	Clinical isolate	- (0/1)

+, positive reaction; -, negative reaction (positive number/number of strains tested); ATCC, American Type Culture Collection; CVCC, China Veterinary Culture Collection.

Loop-mediated isothermal amplification (LAMP) can amplify DNA with high specificity, efficiency, and speed under simple laboratory conditions (Notomi et al., 2000). It is conducted under isothermal conditions of approximately 60℃. Moreover, LAMP products can be detected not only by electrophoresis and staining, but also by visual observation of turbidity or fluorescence (Saito et al., 2005). The LAMP assay was initially evaluated for the detection of hepatitis B viral DNA (Notomi et al., 2000). Although the LAMP assay was recently applied to the direct detection of *Campylobacter fetus* (Yamazaki et al., 2009), *M. bovis* (Zhang et al., 2011), and *Riemerella anatipestifer* (Zheng et al., 2011), no assay for the detection of *M. bovis* has been described. Here, we describe a rapid and simple LAMP assay for the detection of *M. bovis*. We evaluated the efficacy of this LAMP assay in the direct detection of specimens obtained from cattle.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 43 strains were prepared for the evaluation of primer specificity (Table 1). *M. bovis* strain HB0801, which was used for standardization of the LAMP assay, was isolated from local cattle with respiratory disease, identified by chemical testing and genomic sequencing, and confirmed by the China Center for Type Culture Collection. The *Mycoplasma* species were cultured in Hayflick liquid medium supplemented with 10% (v/v) donor equine serum (HyClone, Logan, UT, USA) and penicillin (final concentration 800 IU/ml) in an atmosphere of 5% CO_2 at 37℃. *M. bovis* was grown in 7H9 (BD, Franklin Lakes, NJ, USA) liquid culture in a biosafety level 3 facility. All other bacteria were grown on Trypticase Soy Agar (BD).

Clinical specimens

A total of 98 specimens were examined. 59 samples (51 nasal swabs, 2 tracheal swabs, and 6 lung samples) were collected from cattle suspected of having *M. bovis* infection. In addition, 15 nasal samples were collected from healthy cattle. Both specimens were stored at -80℃ until required.

Primer design

Oligonucleotide primers targeting *uvrC* (GenBank accession no. AF003959) for LAMP were designed using LAMP primer design software (http://primerexplorer.jp/e/index.html). The 2 outer primers were designated as forward outer primer (F3) and backward outer primer (B3). The inner primers were designated as forward inner primer (FIP) and backward inner primer (BIP). FIP consisted of the complementary sequence of F1 (F1C) and sense sequence of F2. F1C contained an AluI restriction enzyme site. BIP consisted of the complementary sequence of B1 (B1C) and sense sequence of B2. The final locations of the primers were from 1,775 to 1,983 nt within *uvrC*; the sequences of the primers used for the amplification of *M. bovis* are shown in Table 2 and Figure 1. The sequences of the primers targeting *uvrC* are shown in Table 2.

DNA preparation

DNA for LAMP and PCR were prepared according to Mohran's method with a slight modification (Mohran et al., 1998). DNA from pure cultures was extracted with the use of a boiling water bath for 10 min, immediately placed in an ice bath for 2 min, and centrifuged at 13,000 × g for 1 min. In addition, 1 ml of sterilized phosphate-buffered saline (PBS) was added to each nasal or tracheal swab and vortexed for 5 to 10 min. Lung tissue (0.5 g) was excised from the lesion edges, and 1 ml of sterile PBS was added to the tissue and homogenized with glass homogenizers. The supernatant from the swabs or lung tissue was filtered through a 0.45 µm filter membrane. The filtrate was sequentially centrifuged for 5 min at

Table 2. Sequences of the primers used in this study.

Method	Primer	Sequence (5'→3')
LAMP	B3	AAGCACCCTATTGATTTTTACTC
	F3	AGAAACAGACAAAAAATTAGTTCAC
	FIP	GATTTTTGCATAGCTTTTAAAGTGATTTTGAAGGCAAACTAAGAAACATAAAAGG
	BIP	GACGCTTCAGTTGAAGAATTATCATTTTAATCCTTATTTTTAATGCTTTTGGC
PCR	UvrC1	TAATTTAGAAGCTTTAAATGAGCGC
	UvrC2	CATATCTAGGTCAATTAAGGCTTTG

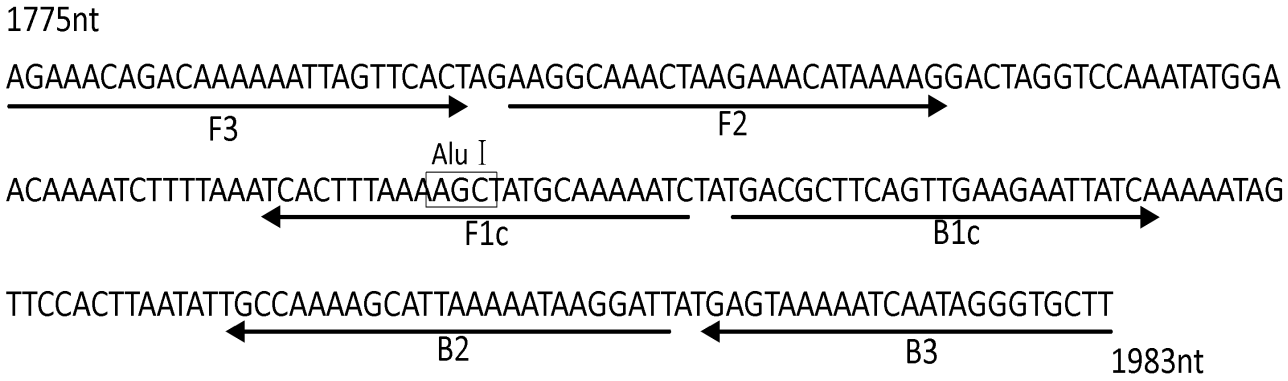

Figure 1. Oligonucleotide primers used for the LAMP assay of *M. bovis* targeting *uvrC*. The arrowed letters indicate the sequences and locations of the primers. The box indicates the *Alu*I restriction site.

13,000 × g for 30 min, and the pellet was dissolved in 50 µl of sterile PBS. It was then subjected to a boiling water bath, ice bath, and centrifugation as described above. The final supernatant containing the DNA was stored at -80℃ until use.

LAMP assay

The LAMP reaction was carried out in a 25 µl reaction volume. The system contained 1.4 M betaine (Sigma, St. Louis, MO, USA), 200 µM dNTPs (TransGen Biotech), 2.5 µl of 10× *Bst* buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $[NH_4]_2SO_4$, 2 mM $MgSO_4$, and 0.1% TritonX-100 [pH 8.8]), 8 U of *Bst* DNA polymerase large fragment (New England Biolabs), 0.64 µM each of FIP and BIP, 0.08 µM each of F3 and B3, and 1 µl of template DNA. The reaction mixture was incubated in a heating block. Amplification was performed at 58℃ for 60 min followed by heating at 80℃ for 5 min to terminate the reaction.

Analysis of the LAMP products

The products of the LAMP reaction were detected either as ladder-like patterns using 2% agarose gel electrophoresis or by direct visualization of a color change after SYBR Green I staining. The solution turned green in the presence of a LAMP amplicon and remained orange without any amplification. The accuracy of the reaction was confirmed by restriction endonuclease digestion with *Alu*I (a restriction site was present in F1); the predicted fragment sizes were 87 and 130 bp. In addition, the structures of the amplified products were confirmed by direct sequencing.

Determination of the sensitivity of the LAMP assay

To compare the sensitivity of the LAMP and PCR assays, *M. bovis* HB0801 was diluted serially 10-fold to a final concentration of 3.4×10^6 to 3.4×10^0 CFU/µl. A total of 1 ml of the bacteria for each dilution was boiled for 10 min, immediately placed in an ice bath for 2 min, and centrifuged at 13,000 × g. Next, 1 µl of the supernatant for each dilution was used as the template for amplification. Sensitivity tests of the LAMP and PCR assays were conducted in triplicate; the detection limits were defined as the last positive dilutions.

Determination of the specificity of the LAMP assay

To determine the specificity of the method, the LAMP assay was carried out under the conditions described above using DNA from different bacteria (Table 1). The assay was carried out in triplicate. For further confirmation, the LAMP products were digested with 5 U of *Alu*I. The original amplicon and digested products were subjected to 2% agarose gel electrophoresis.

PCR assay

A PCR assay targeting *uvrC* was performed in a 20 µl reaction volume containing 0.4 µM each of UVRC1 and UVRC2, 1 U of TaqMix DNA polymerase, and 1 µl of template DNA. Amplification was achieved under the following conditions: 95℃ for 5 min followed by 30 cycles of 95℃ for 1 min, 55℃ for 1 min, and 7℃ for 30 s. A final extension was done at 72℃ for 10 min. The 238 bp

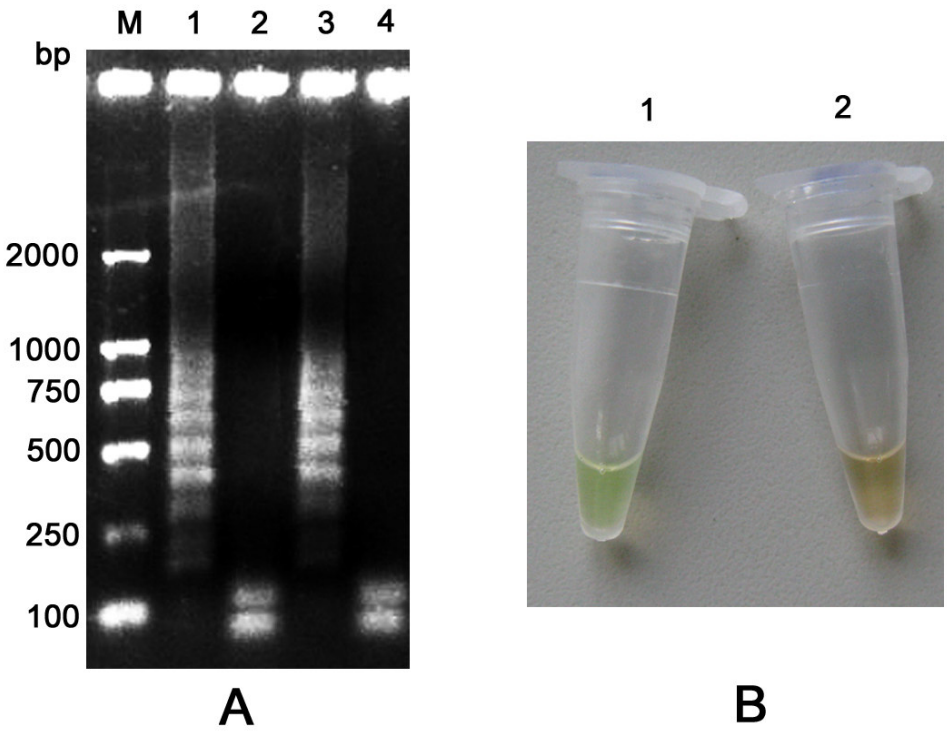

Figure 2. (A) Electrophoretic analysis of the LAMP products. Lane M, DL 2000 DNA marker; Lanes 1 and 3, repeated positive amplification of *M. bovis* HB0801 by LAMP assay; Lanes 2 and 4, repeated *Alu*I digestion of the LAMP products for the positive samples into 2 bands of 87 and 130 bp. (B) Visual appearance of the LAMP reactions after the addition of SYBR Green I. The left tube (green) was positive, while the right tube (light orange) was negative.

products were subjected to 2% agarose gel electrophoresis.

RESULTS

Specificity of the LAMP assay

A total of 43 known bacterial strains were analyzed to evaluate the specificity of the LAMP assay. By observation of SYBR Green I, *uvr*C was detected in all 20 *M. bovis* strains, and no cross-reactivity was observed for other *Mycoplasma* species or respiratory bacterial species (Table 1). Furthermore, the LAMP products were subjected to 2% agarose gel electrophoresis, and a characteristic ladder of multiple bands was observed (Figure 2A). The amplified product was digested using *Alu*I, and the fragment sizes were in good agreement with the sizes predicted from the expected DNA structures (Figure 2A). In addition, the sequence obtained matched the expected nucleotide sequence perfectly (data not shown).

Sensitivity of the LAMP assay

To ascertain the detection limit of our LAMP assay for *M. bovis*, serial 10-fold dilutions of *M. bovis* HB0801 were tested by LAMP and PCR. Our results show a detection limit of 3.4×10^1 CFU per reaction for LAMP and 3.4×10^2 CFU per reaction for PCR. Thus, LAMP was 10-fold more sensitive than PCR. Amplification by LAMP showed a ladder-like pattern, whereas PCR produced a 238 bp amplicon, which is equal to the expected length of the gene fragment (Figure 3).

Evaluation of the LAMP assay using clinical specimens

The LAMP assay was evaluated for the ability to detect *M. bovis* in clinical specimens using 98 samples from cattle, and the results were compared with those obtained using PCR (Table 3). The specificity of the LAMP assay was further confirmed by restriction analysis as well as nucleotide sequencing of the amplified product (data not shown). The LAMP assay detected 54 positive samples and 44 negative samples; the PCR assay detected 29 positive samples and 69 negative samples. Of the 98 samples, 29 were positive and 44 were negative by both tests. However, the LAMP assay was able to detect *M. bovis* DNA in 25 additional specimens that were negative by PCR. The sensitivity and specificity of the LAMP assay in terms of PCR was 100 and 74%, respectively. Of 51

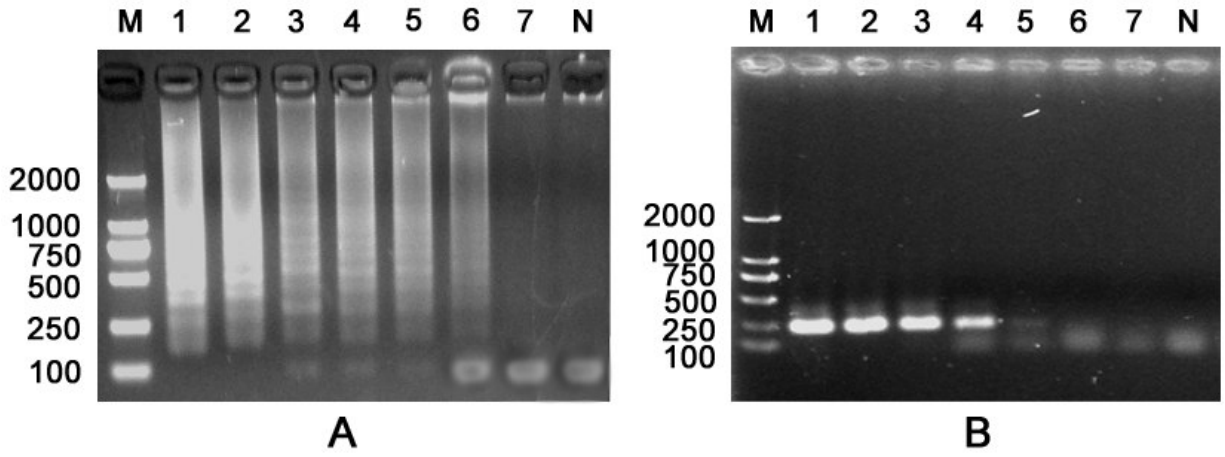

Figure 3. Sensitivity of the electrophoretic analysis of (A) LAMP- and (B) PCR-amplified products. Lane M, DL 2000 DNA marker; Lane 1, genomic DNA from 3.4×10^6 CFU of *M. bovis*; Lane 2, 3.4×10^5; Lane 3, 3.4×10^4; Lane 4, 3.4×10^3; Lane 5, 3.4×10^2; Lane 6, 3.4×10^1; Lane 7, 3.4×10^0; and Lane N, negative control.

Table 3. Comparative evaluation of LAMP and PCR for the detection of *M. bovis* in clinical specimens.

Specimen type	Number of sample tested	% (number) of positive sample for assay	
		LAMP	PCR
Nasal swabs	51	90 (46)	45 (23)
Lung tissue	6	100 (6)	67 (4)
Tracheal swabs	2	100 (2)	100 (2)
Healthy nasal swabs	39	0	0
Total	98	55 (54)	30 (29)

nasal swabs, 90% (46/51) were positive by LAMP compared to 45% (23/51) positive by PCR. Of 6 lung tissue samples, 100% (6/6) were positive by LAMP compared to 67% (4/6) positive by PCR. Two tracheal swabs were positive by LAMP and PCR. None of the healthy nasal swabs tested were positive, indicating the specificity of the 2 systems.

DISCUSSION

In this study, we developed a novel LAMP assay for the rapid detection of *M. bovis* in culture isolates or clinical specimens. LAMP is a simple diagnostic tool in which the reaction takes place in a single tube. The primers, buffer, and DNA polymerase are mixed together, and the mixture is incubated in a regular laboratory water bath or heat block that provides a constant temperature (Saito et al., 2005). The culture and identification of *M. bovis* takes 3-10 days to produce results (Cai et al., 2005). Conventional PCR assays require 4 to 5 h for amplification, electrophoresis, and staining. The overall time required for DNA extraction from samples and amplification using the LAMP assay is approximately 1 h. Further, amplification by the LAMP assay can be judged by direct visual assessment without the need for electrophoresis (Yamazaki et al., 2008; Inácio et al., 2008). The LAMP assay was found to be quicker and simpler than conventional culture or PCR.

In the present study, the assay specifically amplified only *M. bovis*; no cross-reactivity was observed for other *Mycoplasma* species or other respiratory bacterial species. These results demonstrate the highly efficient detection and strong specificity for the amplification of *M. bovis*. The efficiency of amplification was 34 CFU per reaction, which is more than 10 times that of conventional PCR.

A total of 74 specimens were tested simultaneously by LAMP and PCR, and the positive detection rates for LAMP (55%) were much higher than those for PCR (30%). Moreover, *M. bovis* was detected among the various types of clinical specimens. These results demonstrate that LAMP is the most sensitive method and can meet the requirement for speed in the clinical field.

LAMP primers were designed for this assay in a highly conserved region of the *M. bovis uvrC* gene. In a previous study, Subramaniam et al. (1998) established a PCR assay using *uvrC* and demonstrated that it clearly differentiated *M. bovis* and *M. agalactiae*. Thomas et al. (2004) used PCR to amplify the *uvrC* gene of 92 strains, sequenced 20 of them, and confirmed that *uvrC* is a suitable target for the diagnosis of *M. bovis* using PCR.

The strong sensitivity and specificity obtained in the present study further confirmed that *uvrC* is a suitable conserved target for the diagnosis of *M. bovis* using LAMP.

The exquisite sensitivity of the LAMP method makes it vulnerable to contamination. Controls are necessary in LAMP procedure. In addition, a pre-incubation of the multiplex LAMP reaction mix with UNG could prevent LAMP products from carry-over contamination effectively (He and Xu, 2011). More studies on preventing the carry-over contamination in the process of LAMP should be explored.

In conclusion, a LAMP assay was developed and evaluated for the detection of *M. bovis*, and it was found to be markedly more sensitive, specific, and rapid than conventional PCR. The assay can be carried out in a local laboratory without the need for specialized equipment. Additional studies of larger numbers of clinical samples of different types are needed before it can be used in the field.

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