NOTE *Bacteriology*

An improved loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis*

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ABSTRACT. We improved a loop-mediated isothermal amplification (LAMP) assay permitting sensitive and rapid *Mycoplasma bovis* detection. A total of 55 bacterial strains were examined in this study, including 33 *M. bovis* strains, 14 non-*M. bovis* mycoplasmas and eight non-mycoplasma bacterial strains. *M. bovis* was successfully detected by the LAMP assay within 60 min without cross-reaction to any other bacteria. Furthermore, a total of 135 nasal swab samples were tested directly using our LAMP assays, the previously reported LAMP assay, conventional PCR assay without pre-culture and comparing standard culture methods. The improved LAMP assay showed sensitivity and specificity of 97.2% and 90.9%, respectively (with a kappa coefficient of 0.8231), and the sensitivity of our revised LAMP assay was increased compared to existing methods.

KEY WORDS: cattle, loop-mediated isothermal amplification (LAMP) assay, *Mycoplasma bovis*, respiratory disease

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Mycoplasma bovis is an important pathogen that causes pneumonia in cattle [5, 8, 15, 17]. The pathogen also is associated with bovine respiratory disease complex (BRDC) when infecting in combination with viruses (such as bovine respiratory syncytial virus or parainfluenza virus) or other bacteria (such as *Pasteurella multocida* or *Mannheimia hemolytica*) [5, 15, 17]. BRDC-associated *M. bovis* tends to take the form of persistent chronic infection, resulting in large economic losses for farms [15, 17]. In *M. bovis* infection, medical treatment (e.g., prescription of antibiotics) will vary depending on the implicated pathogen [15], and therefore, it is important to diagnose at the earliest stage possible. Methods for detecting *M. bovis* include isolation by culturing, molecular detection methods (such as PCR) and immunohistochemical staining of tissue specimens [5, 15, 17]. The isolation of *Mycoplasma* from field materials requires rich medium, special technical skills and specialized equipment [10, 15]. Culturing for the detection of *M. bovis* in clinical material is often time-consuming, and the sensitivity is low [15]. Therefore, many molecular biological tests have been developed [1, 4, 5, 17]. Recently, the LAMP method was developed as a novel DNA amplification method [16]. The method is beginning to be applied as a simple and rapid diagnostic tool for infectious disease, including contagious bovine pleuropneumonia [14], porcine infection by *M. hyopneumoniae* [13] and human infection by *M. pneumoniae* [20]. *M. bovis* detection LAMP was reported by Bai *et al.* [3]. In the LAMP assay, the detection speed and sensitivity were increased by designing loop primer [16]. In the primer set of the previous *M. bovis* LAMP assay, loop primer was not designed. Therefore, we designed the primer set including loop primer and improved it for a more sensitive LAMP assay.

A total of 47 strains of mycoplasmas, included 33 *M. bovis* strains and 14 non-*M. bovis* mycoplasma strains, and 8 bacterial strains of six non-mycoplasma species obtained from cattle, were used in the present study (Table 1). These strains were confirmed with PCR used the primer specific to species of *M. bovis* [19], *M. alkalescens* [12], *M. bovirhinis* [12], *M. hyorhinis* [22], *Pasteurella multocida* [23], *Mannheimia haemolytica* [2] and/or the sequence of 16S ribosomal RNA gene [1, 7]. In addition, indirect immunoperoxidase test was carried out for *M. bovis* identification [9]. Samples of these bacteria were stored at −80°C following isolation. *M. bovis* strain PG45 was used as a reference strain in the present study. Mycoplasmas were cultured in M broth [10] at 37°C in a 5% CO_2 environment. After incubation, cells per 500 *µl* of culture medium were pelleted, resuspended in 25 *µl* of mycoplasma lysis buffer containing proteinase K [11], and incubated at 60°C for 50 min (for DNA extraction) and then at 100°C for 10 min (for proteinase K inactivation). The non-mycoplasma strains were grown overnight on Mueller-Hinton Agar (MHA, OXOID, Hampshire, U.K.) plates, and single colonies of each strain were employed for DNA extraction. DNA was extracted from these individual colonies by alkaline and heat treatment for LAMP and PCR [24]. For all strains, DNA samples were stored at −20°C until required.

LAMP reactions were performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan)

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Table 1. Bacterial strains used in the LAMP specificity tests

Species	Strain (origin)
M. bovis	PG45 (reference strain) 16Na, 52Nb, 53Na, 55Na, 57Na, 58Na, 59Na, 158Na, 184Na, 186Na, 187Na, 193Na, 204Na, 210Na, 222Nb, 223Na, 224Na, 232Na, 290Na, 291Na, 292Na, 293Na, 294Na (cattle NPS[a)]) 43La, 44La, 193La, 222La (cattle Lung) 224Ea, 229ERa, 229ELa, 230ELa, 295Ea (cattle Ear)
M. agalactiae	PG2 (reference strain)
M. alkalescens	PG51 (reference strain) 59Nf (cattle NPS)
M. bovirhinis	PG43 (reference strain) 56Nb, 70Nb (cattle NPS)
M. bovoculi	90Nd, 99Nc (cattle NPS)
A. laidlawii	PG8 (reference strain) 191Na, 235Ne (cattle NPS)
M. hyopnuemoniae	J (reference strain)
M. hyorhinis	BTS7 (reference strain) 245La (swine Lung)
Non-*Mycoplasma* bacteria	*Pasturella multocida* (n=2), *Mannhemia hemolytica* (n=2), *Moraxcella bovoculi* (n=1) , *Escherichia coli* (n=1), *Salmonella Enteritidis* (n=1), *Campylobacter jujuni* (n=1)

a) NPS; nasopharyngeal swab.

Table 2. Primers designed from the sequences (obtained from Genbank) of the oppD/F loci of *Mycoplasma bovis* PG45

Primer	Sequence (5′ to 3′)	Gene location (bp)
FIP	TGAGCTTTCCATTGTTTGTTTTTCTACTAATTGACTTTAAGTTTGACTGG	1068–1092 (F1c), 993–1017 (F2)
BIP	ATGAGATGTACTCAAACACAGATCACATTATTTCTAAACTGCGCTTG	1100–1124 (B1), 1150–1171 (B2c)
F3	CCATCTAGCACATTTTTTCCT	958–978
B3	TCTAATTCGTCAAAAGTGACAT	1172–1193
LF	AAGAAACAAAATTTTCATCAATAGA	1026–1051
LB	ATGGCTTTCTATTACGAAAAGCAA	1129–1152

as previously described [24]. Six primers recognizing eight distinct regions of the *oppD/F* target region were designed using Primer Explorer V4 software (Fujitsu System Solutions, Tokyo, Japan) (Table 2). Each 25 μl reaction mixture contained 2.0 μl of DNA sample, 8.2 μl of distilled water, 12.5 μl of reaction mixture (Eiken Chemical), 1.0 μl (8 U) Bst DNA polymerase (Eiken Chemical) and 1.3 μl of primer mix (consisting of 1.6 μM FIP and BIP primers, 0.8 μM LF and LB primers, and 0.2 μM F3 and B3 primers). Mixtures were incubated in a Loopamp real-time turbidimeter (LoopampEXIA; Termecs Co., Ltd., Kyoto, Japan) at 63°C for 60 min and then at 80°C for 2 min to terminate the reaction. The reaction was considered to be positive when the turbidity reached 0.1 within 60 min [24]. Turbidity visible with the naked eye also was considered to indicate a successful LAMP procedure.

We compared the improved LAMP, the previously reported LAMP and conventional PCR assays by characterizing nasal samples directly without pre-culturing. A total of 135 nasal samples from cattle were tested in these assays, 34 of them were collected from cattle with clinical sign (respiratory symptom and/or otitis media), 97 of them were collected from subclinical cattle housed together with clinical cattle, and 4 of them were no information about clinical symptom. DNA extraction was carried out directly from M broth without prior cultivation of the organism; the resulting DNA specimens were stored at −20°C until tested. These nasal samples also were cultured for isolation of mycoplasmas; culturing revealed the presence of *M. bovis* in 36 of these samples, while the other 99 samples were *M. bovis* culture-negative. The following four primers were used for performing the previous reported version of the LAMP assay [3]: B3: 5′-AAGCACCCTATTGATTTTTACTC-3′, F3: 5′-AGAAACAGACAAAAAATTAGTTCAC-3′, FIP: 5′-GATTTTTGCATAGCTTTTAAAGTGATTTTGAAGGC AAACTAAGAAACATAAAAGG-3′ and BIP: 5′-GACG CTTCAGTTGAAGAATTATCATTTTAATCCTTATTTTT AATGCTTTTGGC-3′; the amplification was performed at 58°C for 120 min. The reaction was determined to be positive based on above-described criteria; for the current work, we modified the procedure for the previous LAMP assay by screening for turbidity rather than for staining with SYBR Green. The PCR amplification for detection of *M. bovis* was carried out with 2.0 μl of sample DNA in a 20.0 μl reaction mixture containing 0.3 μM $MgCl_2$, 2.0 μl of 10× PCR buffer (Qiagen, Hilden, Germany), 200 μM of each deoxynucleotide triphosphate (Invitrogen), 0.5 μM of each

primer pair (5′-TAATTTAGAAGCTTTAAATGAGCGC-3′ (forward) and 5′-CATATCTAGGTCAATTAAGGCTTTG-3′ (reverse)) [3] and 0.5 U of Taq DNA polymerase (Qiagen). The PCR reaction conditions consisted of pre-heating at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 30 sec, with a final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and visualized using GelRed nucleic acid gel stain (Biotium, Inc., Hayward, CA, U.S.A.). For the evaluation of performance characteristics of these assays, results were compared to those of the culture results, which currently are considered the "gold standard" [6].

All 32 *M. bovis* field-isolate strains and the *M. bovis* type strain PG45 were positive by the new-version LAMP assay. No positive signal was observed for any of the other 14 non-*M. bovis* mycoplasma strains and 8 non-mycoplasma bacterial strains. Total time to detection, including the DNA extraction process, was only 2.5 hr. The revised LAMP assay requires only a simple incubator, such as a heating block, to provide a constant temperature of 63°C. Positive reaction by the LAMP assay (indicated by the presence of a white precipitate) could be observed with the naked eye, without requiring any additional procedure, such as SYBR Green staining. The sensitivity of our revised LAMP assay was defined 7.2×10^0 colony forming units (cfu) per reaction, and without loop primer, amplification speed and the sensitivity were decreased (detection limit was 7.2×10^2 cfu per reaction).

The two kinds of LAMP assays and the PCR assay permit direct characterization, without pre-culturing. All three assays were compared to culturing for their accuracy in characterizing a panel consisting of 135 nasal samples (Table 3). The improved LAMP assay was able to detect *M. bovis* in 35 of 36 culture-positive samples and 9 of 99 culture-negative samples. These results corresponded to sensitivity and specificity of 97.2% and 90.9%, respectively (with a kappa coefficient of 0.8231). In contrast, the previously reported LAMP assay detected *M. bovis* in only 7 of 36 culture-positive samples and 3 of 99 culture-negative samples, revealing sensitivity and specificity of 19.4% and 97.0%, respectively (with a kappa coefficient of 0.2131). The PCR assay detected *M. bovis* in 31 of 36 culture-positive samples and 7 of 99 culture-negative samples, showing sensitivity and specificity of 86.1% and 92.9%, respectively (with a kappa coefficient of 0.7767).

Our LAMP assay detected successfully *M. bovis* without cross-reaction to any other mycoplasma or non-mycoplasma bacteria. The primer was designed to target the *oppD/F* genomic region. This domain has been reported to be capable of discriminating *M. bovis* from the highly homologous (at the genome level) species *M. agalactiae* [4]. However, as the sequence of 16S ribosomal RNA genes of *M. bovis* and *M. agalactiae* is very similar (>99.8%) [18], and the study using enough numerical *M. agalactiae* field strains was desirable surely, it was thought that continuous inspection will be necessary in future.

As a result that the three kinds of DNA-based detection assays were compared to culturing, the improved LAMP assay showed high sensitivity. The following interpretation of kappa coefficient was used: <0.4-poor; 0.41–0.6-moderate; 0.61–0.8-good; and >0.8 excellent [21]; our revised LAMP assay achieved an excellent grade. The revised assay is superior to permit direct detection of signals that would be limiting in the previously reported LAMP (detection limit was 7.2×10^6 cfu per reaction) or conventional PCR assay (detection limit was 7.2×10^2 cfu per reaction). Because the loop primer used in the revised method was designed to enhance amplification speed, this revised assay was more rapid and more sensitive than the previously reported assays. We designed previously LAMP primers faithfully, but the different points were DNA extraction methods, signal detection method and LAMP reagent. The previous version of the LAMP assay required SYBR Green staining for signal detection, in turn necessitating opening the tube after thermal incubation. In contrast, the unrevised assay performed in the present work was performed with the Loopamp real-time turbidimeter using the previously reported primer. As a result, the reason was not clear, but reaction time for the unrevised assay in the present paper was longer and sensitivity was lower than for that in the previous report. In the present study, the previously reported LAMP assay was evaluated using the Loopamp real-time turbidimeter in substitution for SYBR Green staining, because the SYBR Green assay would otherwise require opening the tube cover after thermal incubation. When the Loopamp real-time turbidimeter was used as part of the revised assay, we were able to detect positive signal earlier than the endpoint analysis of the previously reported LAMP assay [24]. Use of the turbidimeter is expected to be advantageous, since the use of an assay that does not require tube opening should reduce the risk of environmental diffusion and cross-contamination during gene amplification. The slightly lower specificity (90.9%) of the LAMP assay means that some LAMP results were false-positives, or, alternatively, that the false-negative frequency of culture results was elevated. The latter condition is considered more likely: the accuracy of gene detection assays typically exceeds that of culture assays [6, 24]. The consumption of antibiotics or the presence of some special kinds of bacteria could be the source of this discrepancy in the present study. The inclusion of a loop primer is known to deeply affect amplification speed and sensitivity in LAMP assays [16]. However, because guanine-cytosine content was low in the targeting *uvrC* domain, it was thought that design

Table 3. Comparison among results of two kinds of LAMP assay, PCR assay and culturing

Assay		Culture[a)] +	Culture[a)] −	Total	Sensitivity	Specificity	Kappa coefficient
LAMP (this study)[b)]	+	35	9	44	97.2%	90.9%	0.8231
	−	1	90	91			
LAMP (Bai *et al.*)[b)]	+	7	3	10	19.4%	97.0%	0.2131
	−	29	96	125			
PCR (Bai *et al.*)[b)]	+	31	7	38	86.1%	92.9%	0.7767
	−	5	92	97			
Total		36	99	135			

a) +: *Mycoplasma bovis* isolation positive, −: no isolation. b) +: amplification positive, −: no amplification.

of loop primers was difficult in previous LAMP assay. The improved LAMP assay, which employed a loop primer, rendered the assay more sensitive than the previously reported LAMP assay. The revised assay provided enhanced sensitivity of genome detection from culture-negative samples, yielding a nominal decrease in specificity. Based on serological test being absolute method for mycoplasmal identification, it is useful that the genetic method, such as our LAMP assay, was used as one of the supplementary identification method.

The improved LAMP assay permitted single-step distinction of *M. bovis* from other bacteria, including non-*M. bovis* mycoplasmas. We also demonstrated the utility of LAMP for detection of *M. bovis* directly from nasal samples. The LAMP assay for *M. bovis* detection will serve as a practical tool in the field for controlling *M. bovis* infection and BRDC. In conclusion, we developed a LAMP assay with increased sensitivity compared to previous methods.

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