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

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(Received 4 August 2015/Accepted 12 April 2016/Published online in J-STAGE 24 April 2016)

**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.

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

doi: 10.1292/jvms.15-0459; J. Vet. Med. Sci. 78(8): 1343–1346, 2016

**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]. It is beginning to be applied as a simple and rapid diagnostic test for infectious disease, including contagious bovine pleuropneumonia [14], porcine infection by 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% CO₂ 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)
Six primers recognizing eight distinct regions of the \textit{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 was also 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 signs (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 \textit{M. bovis} in 36 of these samples, while the other 99 samples were \textit{M. bovis} culture-negative.

The following four primers were used for performing the previous reported version of the LAMP assay [3]: B3: 5'-AAGCACCTATGATTTTACT-3', F3: 5'-AGAAACAGACAAAAATTAGTTCAC-3', FIP: 5'-GATTTTGCAATGTTTAAAGTGATTTTGAAGGCAGATTTTGACTGG-3', BIP: 5'-GACTCTTCAGTTGAAGAATTATCATTTTAATCCTTATTTTAATGCTTTTGGC-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 \textit{M. bovis} was carried out with 2.0 µl of sample DNA in a 20.0 µl reaction mixture containing 0.3 µM MgCl₂, 2.0 µl of 10× PCR buffer (Qiagen, Hilden, Germany), 200 µM of each deoxynucleotide triphosphate (Invitrogen), 0.5 µM of each

### Table 1. Bacterial strains used in the LAMP specificity tests

| Species              | Strain (origin)                                  |
|----------------------|-------------------------------------------------|
| \textit{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)                              |
|                      | 43La, 44La, 193La, 222La (cattle Lung)           |
|                      | 224Ea, 229Ea, 229ELa, 230ELa, 295Ea (cattle Ear)|
| \textit{M. agalactiae}| PG2 (reference strain)                          |
| \textit{M. alkalescens}| PG51 (reference strain)                      |
|                      | 59Nf (cattle NPS)                               |
| \textit{M. bovirhinis}| PG43 (reference strain)                         |
|                      | 56Nb, 70Nb (cattle NPS)                         |
| \textit{M. bovoculi} | 90Nd, 99Nc (cattle NPS)                         |
| \textit{A. laidlawii} | PG8 (reference strain)                         |
|                      | 191Na, 235Ne (cattle NPS)                       |
| \textit{M. hyopneumoniae} | J (reference strain)                            |
| \textit{M. hyorhinis} | BTS7 (reference strain)                         |
|                      | 245La (swine Lung)                              |
| Non-Mycoplasma bacteria | Pasturella multocida (n=2), Mannheimia hemolytica (n=2), Moraxcella bovoculi (n=1), Escherichia coli (n=1), Salmonella Enteritidis (n=1), Campylobacter jejuni (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](image)

| Primer | Sequence (5' to 3') | Gene location (bp) |
|--------|---------------------|--------------------|
| FIP    | TGAGTTTCTTCCATTTGTTTGGGAAAGTGTGACTGG            | 1068–1092 (F1c), 993–1017 (F2) |
| BIP    | ATGAGATGTACAAAAACAACAGATCATATTCTAATTCTGACTGG    | 1100–1124 (B1), 1150–1171 (B2c) |
| F3     | CCATCTACGCAATTTTCTCCT | 958–978            |
| B3     | TCTAATTCGCAAAGTAGGGAACAT | 1172–1193         |
| LF     | AAGAAACAAAAATTTATCAATAGA | 1026–1051         |
| LB     | ATGGCTTTCTATTCAGAAAGCAA | 1129–1152         |
IMPROVED LAMP ASSAY FOR DETECTION OF M. BOVIS

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 as 7.2 × 10⁶ colony forming units (cfu) per reaction, and without loop primer, amplification speed and the sensitivity were decreased (detection limit was 7.2 × 10⁵ 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 all 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 × 10⁶ cfu per reaction) or conventional PCR assay (detection limit was 7.2 × 10⁶ 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 mrvC domain, it was thought that design

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

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

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Note: LAMP = Loop-mediated amplification; PCR = Polymerase chain reaction; 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 testing 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 \textit{M. bovis} from other bacteria, including non-\textit{M. bovis} mycoplasmas. We also demonstrated the utility of LAMP for detection of \textit{M. bovis} directly from nasal samples. The LAMP assay for \textit{M. bovis} detection will serve as a practical tool in the field for controlling \textit{M. bovis} infection and BRDC.

In conclusion, we developed a LAMP assay with increased sensitivity compared to previous methods.

ACKNOWLEDGMENT. We appreciated to Dr. Kobayashi of the National Institute of Animal Health who provided mycoplasma reference strains (PG45, PG2, PG51, PG43, PG8, J and BTS7).

REFERENCES

1. Alberti, A., Addis, M. F., Chessa, B., Cubeddu, T., Profiti, M., Rosati, S., Ruiu, A. and Pittau, M. 2006. Molecular and antigenic characterization of a \textit{Mycoplasma bovis} strain causing an outbreak of infectious keratoconjunctivitis. J. Vet. Diagn. Invest. 18: 41–51. [Medline] [CrossRef]

2. Alexander, T. W., Cook, S. R., Yanke, L. J., Barker, C. W., Morley, P. S., Read, R. R., Gow, S. P. and McAllister, T. A. 2008. A multiplex polymerase chain reaction assay for the identification of \textit{Mannheimia haemolytica}, \textit{Mannheimia glucosida} and \textit{Mannheimia ruminalis}. Vet. Microbiol. 130: 165–175. [Medline] [CrossRef]

3. Bai, Z., Shi, L., Hu, C., Chen, X., Qi, J., Ba, X., Peng, Q., Chen, Y., Chen, H. and Guo, A. 2011. Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of \textit{Mycoplasma bovis}. Afr. J. Biotechnol. 10: 12333–12338.

4. Bashiruddin, J. B., Frey, J., Königsson, M. H., Johansson, K. E., Hotzel, H., Diller, R., de Santis, P., Botelho, A., Aylng, R. D., Nicholas, R. A., Thihaouc, F. and Sachse, K. 2005. Evaluation of PCR systems for the identification and differentiation of \textit{Mycoplasma agalactiae} and \textit{Mycoplasma bovis}: a collaborative trial. Vet. J. 169: 268–275. [Medline] [CrossRef]

5. Caswell, J. L. and Archambault, M. 2007. \textit{Mycoplasma bovis} pneumonia in cattle. Anim. Health Res. Rev. 8: 161–186. [Medline] [CrossRef]

6. Clothier, K. A., Jordan, D. M., Thompson, C. J., Kinyon, J. M., Frana, T. S. and Strait, E. L. 2010. \textit{Mycoplasma bovis} real-time polymerase chain reaction assay validation and diagnostic performance. J. Vet. Diagn. Invest. 22: 956–960. [Medline] [CrossRef]

7. Edwards, U., Rogall, T., Blöcker, H., Emde, M. and Böttger, E. C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17: 7843–7853. [Medline] [CrossRef]

8. Gourlay, R. N., Thomas, L. H. and Howard, C. J. 1976. Pneumonia and arthritis in gnotobiotic calves following inoculation with \textit{Mycoplasma agalactiae} subsp \textit{bovis}. Vet. Rec. 98: 506–507. [Medline] [CrossRef]

9. Imada, Y., Uchida, I. and Hashimoto, K. 1987. Rapid identification of mycoplasmas by indirect immunoperoxidase test using small square filter paper. J. Clin. Microbiol. 25: 17–21. [Medline] [CrossRef]

10. Kobayashi, H., Morozumi, T., Munthali, G., Mitani, K., Ito, N. and Yamamoto, K. 1996. Macrolide susceptibility of \textit{Mycoplasma hyorhinis} isolated from piglets. Antimicrob. Agents Chemother. 40: 1030–1032. [Medline] [CrossRef]

11. Kobayashi, H., Munthali, G., Miyamoto, C., Morozumi, T., Mitani, K., Ito, N., Shiono, H. and Yamamoto, K. 1996. A simple preparation of mycoplasmal DNA template for PCR from biological samples using effective surfactants. J. Vet. Med. Sci. 58: 477–479. [Medline] [CrossRef]

12. Kobayashi, H., Hirose, K., Worarak, A., Paugts, P., Ito, N., Morozumi, T. and Yamamoto, K. 1998. \textit{In vitro} amplification of the 16S rRNA genes from \textit{Mycoplasma bovirhinis}, \textit{Mycoplasma alkalescens} and \textit{Mycoplasma bovigenitalium} by PCR. J. Vet. Med. Sci. 60: 1299–1303. [Medline] [CrossRef]

13. Li, J., Minion, F. C., Petersen, A. C., Jiang, F., Yang, S., Guo, P., Li, J. and Wu, W. 2013. Loop-mediated isothermal amplification for rapid and convenient detection of \textit{Mycoplasma hyorhinis}. World J. Microbiol. Biotechnol. 29: 607–616. [Medline] [CrossRef]

14. Mair, G., Vilei, E. M., Wade, A., Frey, J. and Unger, H. 2013. Isothermal loop-mediated amplification (LAMP) for diagnosis of contagious bovine pleuro-pneumonia. BMC Vet. Res. 9: 108. [Medline] [CrossRef]

15. Maunsell, F. P., Woolums, A. R., Francoz, D., Rosenbusch, R. F., Step, D. L., Wilson, D. J. and Janzen, E. D. 2011. \textit{Mycoplasma bovis} infections in cattle. J. Vet. Intern. Med. 25: 772–783. [Medline] [CrossRef]

16. Nagamine, K., Hase, T. and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16: 223–229. [Medline] [CrossRef]

17. Nicholas, R. A. J. and Ayling, R. D. 2003. \textit{Mycoplasma bovis}: disease, diagnosis, and control. Res. Vet. Sci. 74: 105–112. [Medline] [CrossRef]

18. Pettersson, B., Uhlén, M. and Johanson, K. E. 1996. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA sequences and definition of a new cluster within the hominis group. Int. J. Syst. Bacteriol. 46: 1093–1098. [Medline] [CrossRef]

19. Rifatbegović, M., Assunção, P., Poveda, J. B. and Pasić, S. 2007. Isolation of \textit{Mycoplasma bovis} from the respiratory tract of cattle in Bosnia and Herzegovina. Vet. Rec. 160: 484–485. [Medline] [CrossRef]

20. Saito, R., Misawa, Y., Moriya, K., Koike, K., Ubuyaka, K. and Okamura, N. 2005. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of \textit{Mycoplasma pneumoniae}. J. Med. Microbiol. 54: 1037–1041. [Medline] [CrossRef]

21. Spekhorst, L. M., Visschedijk, M. C., Alberts, R., Festen, E. A., van der Wouden, E. J., Dijkstra, G., Weersma R. K., Dutch Initiative on Crohn and Colitis 2014. Performance of the Montreal initiative on Crohn and Colitis 2014. Performance of the Montreal classification for inflammatory bowel diseases. World J. Gastroenterol. 20: 15374–15381. [Medline] [CrossRef]

22. Stakenborg, T., Vicca, J., Butaye, P., Imberechts, H., Peeters, J., de Kruif, A., Haesebrouck, F. and Maes, D. 2006. A multiplex PCR to identify porcine mycoplasmal present in broth cultures. Vet. Res. Commun. 30: 239–247. [Medline] [CrossRef]

23. Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitriou, J. M. and Dawkins, H. J. 1998. Development of PCR assays for species- and type-specific identification of \textit{Pasteurella multocida} isolates. J. Clin. Microbiol. 36: 1096–1100. [Medline] [CrossRef]

24. Yamazaki, W., Kumeda, Y., Uemura, R. and Misawa, N. 2011. Evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of \textit{Vibrio parahaemolyticus} in naturally contaminated seafood samples. Food Microbiol. 28: 1238–1241. [Medline] [CrossRef]
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Title:
An improved loop-mediated isothermal amplification assay for the detection of Mycoplasma bovis

Date:
2016-08-01

Citation:
Higa, Y., Uemura, R., Yamazaki, W., Goto, S., Goto, Y. & Sueyoshi, M. (2016). An improved loop-mediated isothermal amplification assay for the detection of Mycoplasma bovis. JOURNAL OF VETERINARY MEDICAL SCIENCE, 78 (8), pp.1343-1346. https://doi.org/10.1292/jvms.15-0459.

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