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Development of visual dye-based loop-mediated isothermal amplification assays for *Mycobacterium avium* subsp. *paratuberculosis* detection in ruminants

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ABSTRACT

Importance: *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD) in ruminants, is a potential zoogenic pathogen. At the asymptomatic subclinical stage, infected animals periodically shed the pathogen through feces and milk, serving as silent carriers for disease transmission. In Republic of Korea, where treatment for JD is not pursued and culling is the primary management strategy for infected animals, early detection is crucial for effective disease control.
Objective: In this study, we aimed to develop a new primer set for the loop-mediated isothermal amplification (LAMP) method for facilitating visual-based detection of MAP.
Methods: We designed LAMP primers for the MAP-specific gene IS*1311* and optimized the reaction conditions to enhance diagnostic sensitivity and specificity. Practical application was evaluated using 170 field samples of feces and intestinal tissues from cattle and goats.
Results: The LAMP method demonstrated sensitivity superior to that of the conventional polymerase chain reaction (PCR) method and equal to that of real-time-PCR targeting the F*57* gene, detecting as low as 0.1 pg MAP DNA per reaction. Notably, we integrated a mixed dye composed of calcein and hydroxynaphthol blue at an optimal ratio, enabling clear visual distinction of results under natural light. In practical application, the LAMP assay demonstrated a true positive rate of 100% and true-negative rate of 92.22%.
Conclusions and Relevance: The developed mixed dye-LAMP method facilitates immediate, sensitive diagnosis by providing a user-friendly readout. By reducing diagnostic time and enhancing visual clarity without specialized equipment, it significantly improves JD control in the livestock industry.

Keywords: IS*1311*; Johne's disease; mixed dye-loop-mediated isothermal amplification; diagnosis

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Conflict of Interest

The authors declare no conflicts of interest.

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johne's disease (JD) in ruminants, a condition marked by chronic gastrointestinal inflammation leading to persistent diarrhea and progressive weight loss. JD is not only found in livestock but also in wildlife such as moose, tule elk, and rabbits [1]. Various MAP-infected wild animal species can transmit the pathogen to livestock sharing the same pastures, serving as potential reservoir hosts. Furthermore, the possible association with human inflammatory bowel disease, Crohn's disease, raises concerns about MAP as a zoonotic pathogen [2]. MAP is transmitted through diverse routes: fecal–oral (the primary route), intrauterine [3], and bio-aerosol [4], with bacteria-contaminated manure constituting the main infection reservoir. Calves younger than 6 months of age are particularly susceptible to MAP infection [5], which can occur upon contacting feces-contaminated teats/udders or consuming MAP-containing colostrum [6]. Chronic progression of JD results in an asymptomatic subclinical stage during which the pathogen is intermittently shed through feces and milk; thus, silent carriers are important mediators of disease transmission [7], and this characteristic increases the economic burden on farms [8]. In the Republic of Korea, JD is primarily identified through antibody testing, and infected cattle are managed by culling rather than treatment, making early detection of infected animals even more important.

Multiple methods exist to detect MAP in clinical samples including microscopy (Ziehl-Neelsen staining), culture-based, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA). Culture is considered the gold standard for JD diagnosis but requires a prolonged incubation period of 8 to 16 weeks due to MAP's slow growth rate and the necessity for exogenous mycobactin [9]. ELISA has been widely adopted for monitoring JD by detecting MAP-specific antibodies in milk or serum, owing to its simplicity, speed, and cost-effectiveness. Nevertheless, according to the United States Department of Agriculture, its sensitivity for detecting MAP-specific antibodies (30%–65%) is lower than that of bacterial culture from fecal samples (60%–65%) [10]. Recent developments in molecular diagnostic methods enable precise microbial species or subspecies identification with rapid detection and high sensitivity. Several such methods have been proposed for MAP detection in feces including conventional, nested, and real-time PCR [11-14]. Conventional PCR requires a thermocycler and 1–2 h for the amplification process, followed by a separate gel electrophoresis step for result confirmation. Real-time PCR, which requires similar processing time, supports result confirmation through real-time monitoring but necessitates a relatively expensive thermocycler equipped with real-time detection capabilities. The loop-mediated isothermal amplification (LAMP) assay is more sensitive than conventional PCR owing to its use of 6–8 primers, with a detection limit as low as 10 copies per microliter [15]. LAMP requires only a simple heat block to maintain a constant temperature of 60 to 65°C to sustain enzyme activity. Moreover, it utilizes various detection techniques such as turbidity monitoring [16,17], fluorescent DNA-binding dyes such as SYBR Green I [18], and visual colorimetric indicators including calcein and hydroxynaphthol blue (HNB) [19,20]. However, using each dye typically results in insufficient contrast between positive and negative samples, making it challenging to obtain objective results. Recently, a technique for combining these two dyes at optimal ratios was proposed to improve the clarity of LAMP visual interpretation under natural light [21].

LAMP diagnostic methods targeting IS*900* or ISMap*02* for MAP detection have already been developed [22-24]. However, these methods have limitations due to the extensive genomic

similarity with other Mycobacterium species [25-28], which can lead to false-positive results. To address these limitations, our study introduces a LAMP method targeting the IS*1311* gene to enhance the specificity and accuracy of MAP detection. We developed primer sets targeting the IS*1311* gene and optimized the ratio of colorimetric dyes (HNB and calcein) to ensure clear color changes for easy result interpretation. The sensitivity was compared with those of conventional and real-time PCR using a reference strain (K-10). For field sample validation, we confirmed the true-positive (TPR) and true-negative (TNR) rates of the optimized mixed LAMP method using 80 positive and 90 negative samples based on PCR results from fecal and tissue samples of cattle and goats.

METHODS

Ethics statements

Animal management and sample collection were performed in accordance with the standards of the Animal Ethics Committee of the National Institute of Animal Science (NIAS), Republic of Korea, which approved the study protocol (approval No. NIAS 2022-0559).

Animal collection, MAP cultivation, and DNA extraction

Cattle and goats were transferred to the experimental animal facility upon the confirmation of antibody positivity (S/P > 55) during JD monitoring using ELISA (Paratuberculosis screening Ab Test; IDEXX Laboratories, Inc., USA). These animals were sourced from three farms across the Republic of Korea. Field strains of MAP were obtained from the ileum of three goats that were euthanized after exhibiting end-stage JD symptoms such as diarrhea, emaciation, and lethargy. The reference (K-10) and three field MAP strains were verified by performing 16S sequencing to confirm independent colony growth. The sequences of the 16S rRNA gene from the three field strains (accession numbers OP881810, OP881812, and OP881815). The four MAP field strain, kindly provide by Prof. Yoo from Seoul National University, were included in this study. Their whole genome sequence from four field strain (accession numbers CP033428, CP033427, CP033911, and CP033909) were registered in the National Center for Biotechnology (NCBI) database. MAP strains were cultured in Middlebrook 7H9 broth (Merck KGaA, Germany) supplemented with oleic acid, albumin, dextrose, catalase (OADC; Sigma, USA) and mycobactin J (Allied Monitor, Inc., USA) at 37°C in a 5% CO_2 incubator. Following an 8–12 week growth period, when the liquid cultures exhibited sufficient turbidity, aliquots were transferred to Löwenstein–Jensen medium plates supplemented with glycerol and egg homogenate (Kisan Bio, Korea). MAP genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Germany) in combination with lysozyme. Briefly, two to three MAP colonies were picked and directly suspended in 180 μL lysis buffer (20 mg/mL lysozyme, 2 mM ethylenediaminetetraacetic acid, 20 mM Tris-Cl [pH 8.0], and 1.2% Triton X-100). After 30 min incubation (37°C), DNA was extracted following the manufacturer's instructions.

LAMP primer design for IS*1311* detection

Initial primer screening and optimization processes were performed using the MAP reference strain ATCC BAA-968 (K-10, isolated from cattle feces, kindly provided by Prof. Han Sang Yoo, Seoul National University). The LAMP primer set for IS*1311* was designed using PrimerExplorer V5 (Eiken Chemical Co., Japan). Bioneer (Korea) synthesized six primers including two external (F3 and B3), two internal (FIP and BIP), and two loop (LF and LB) primers. The selected primer binding locations and sequences are shown in **Fig. 1** and **Table 1**.

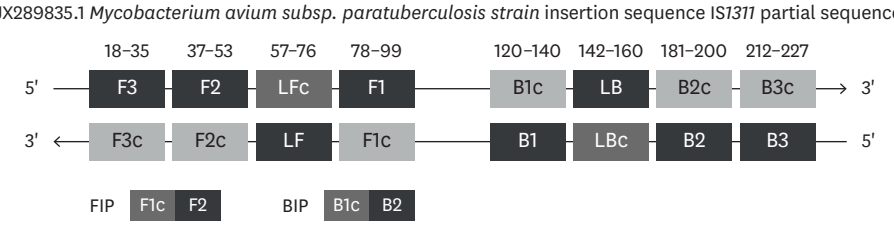

Fig. 1. Schematic representation of LAMP assays.
Spatial arrangement and locations of the primers within the IS*1311* DNA sequence.
LAMP, loop-mediated isothermal amplification.

Table 1. Sequence of designed loop-mediated isothermal amplification primers for detecting IS*1311*

Target	Primer	Sequence (5′–3′)	Length (bp)
IS*1311*	F3	AGACCACCCGATGGTTGA	18
	B3	TGCACGGCACCTCCAC	16
	FIP (Flc+F2)	CGGCCTCTATCCGAACAGCACTCCCGAGCCCTTGCAGTA	39
	BIP (B1c+B2)	CGGTGTCCAGATCGGCGCAAAACGATTTGGTCAAAGCCCTC	41
	LF	CCTATGTGTTCCTCGACGCC	20
	LB	CCGGGAGACCTCGCTTTTG	19

The IS*1311* nucleotide sequence was acquired from NCBI GenBank under accession number JX289835.1.

LAMP reaction optimization

To determine the optimal LAMP reaction conditions, various parameters were systematically evaluated using HNB dye (Merck KGaA). The reaction time varied at intervals from 10 to 60 min in 10 min increments using reference strain DNA (K-10) ranging from 1 ng to 1 fg. Additionally, the $MgSO_4$ (Thermo Fisher Scientific, USA) concentration was tested at 4, 6, 8, and 10 mM, whereas the dNTP (Thermo Fisher Scientific) concentration was varied at 0.6, 1.0, 1.4, and 1.8 mM. The other reaction mixture components were fixed as 10× Isothermal Amplification Buffer II (New England Biolabs [NEB], USA); 8 units of Bst 2.0 WarmStart DNA Polymerase (NEB); 1.6 μM each of FIP (Flc+F2) and BIP (Blc+B2), 0.2 μM of F3 and B3, and 0.4 μM of LF and LB primers; 0.8 mM betaine (Thermo Fisher Scientific); and 150 μM HNB along with 1 μL template DNA (25 μL total volume). All procedures were performed on ice; the reaction was terminated for 5 min (85°C). The synthesized products were stored at 4°C for 10 min to enhance the visibility of any color changes and subsequently analyzed through agarose gel electrophoresis (1.5% w/v) using the Gel-Doc XRS+ system (Bio-Rad, USA).

LAMP assays with single or mixed dye

To determine the optimal color differentiation dye between positive and negative samples, LAMP assay sensitivity using mixed-dye, calcein, and HNB was compared. Initially, the ideal mixing ratio of calcein and HNB was tested. Calcein dye dissolved with 500 μM $MnCl_2$ was fixed as 25 μM [20,22] and mixed with HNB at different final concentrations (150, 200, 250, and 300 μM). Dilution ratios ranging from 1:6 to 1:12 were evaluated using both negative (nuclease-free water) and positive (0.1 ng MAP DNA) samples. Except for the dye conditions, the mixed dye-loaded LAMP assays were conducted under the established optimal parameters of 30 min reaction time, 8 mM $MgSO_4$, and 1.4 mM dNTPs. For calcein reactions, all conditions were identical except for calcein concentration (25 μM). The tube reaction images were acquired using a smartphone (iPhone 11; Apple Inc., USA) under natural light, and single calcein dye photographs were taken under 365 nm blue light. The color differences

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were quantified as absolute differences in red, green, and blue (RGB) values using image J (Version 1.53i; National Institutes of Health, USA).

LAMP assay analytical specificity and sensitivity

LAMP assay specificity was examined using various bacterial DNA samples including MAP (n = 9), non-MAP *Mycobacterium* subsp. (n = 3), and non-*Mycobacterium* species (n = 3) (**Supplementary Table 1**). The 9 MAP strains, either isolated or obtained, were confirmed to express IS*1311* through conventional PCR and ISMap*02* through nested PCR [13]. The analytical sensitivity of LAMP assays loaded with each dye (mixed dye, HNB, and calcein) were compared with those of PCR and real-time PCR by serially diluting the standard strain (K-10) from 1 ng to 1 fg. PCR was performed targeting the same genes, whereas real-time PCR targeted F*57*. For these comparisons, reference strain (K-10) DNA was serially diluted from 1 ng to 1 fg in ten-fold steps. PCR and real-time PCR were adapted from previous studies [29,30]. Briefly, for IS*1311* PCR, the protocol involved initial denaturation at 94°C (3 min), then 37 cycles of denaturation at 94°C (30 sec), annealing at 62°C (15 sec), and extension at 72°C (5 min). A TaqMan probe-based F*57* real-time PCR assay utilized the AccuPower Plus DualStar qPCR PreMix & Master Mix (with UDG) (Bioneer). The procedure was initiated with a UDG activation step at 37°C (2 min), followed by pre-denaturation at 95°C 5 min). The amplification protocol included 40 cycles of denaturation at 95°C (15 sec) and an annealing and extension step at 60°C (35 sec). For the ISMap02 nested PCR, the protocol involved an initial denaturation at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 7 min. For the second amplification, the protocol included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 7 min.

LAMP assay validation using cattle and goat field samples

Blood and feces were sampled monthly from January 2022 to June 2023 to screen for JD in cattle (n = 19) and goats (n = 19) at the experimental animal facility. To prevent cross-contamination between individuals, sanitary gloves were changed when feces were collected directly from the rectum. Sera were separated using a serum-separating tube (BD, USA) and used in ELISA. During the study period, intestinal tissues (duodenum, ileum, jejunum, colon, and cecum) and mesenteric lymph nodes from cattle (n = 3) and goats (n = 10) necropsied at the end-stage of JD were collected and stored at −80°C. In total, 80 IS*1311* and ISMap*02* PCR-positive (14 fecal and 66 tissue samples) and 90 PCR-negative (62 fecal and 28 tissue samples) samples that were PCR negative were used for LAMP validation (**Supplementary Table 2**). Portions of each fecal (2 g) and tissue (1 g) sample were prepared for DNA extraction using the Fast DNA Spin kit for feces (MPBio, USA) and the QIAamp DNA Mini Kit, respectively, according to manufacturer protocol. Subsequently, the DNA concentrations was measured using an Epoch and Take3 spectrometer (BioTek, USA), and the DNA samples were stored at −20°C until further use.

Statistical analysis

All PCR, real-time PCR, and LAMP assays were performed in triplicate to ensure the reliability and reproducibility of the results. LAMP assay diagnostic sensitivity and specificity, in comparison to those of PCR, were calculated using MedCalc statistical Software version 20.305 (MedCalc Software Ltd, Belgium) [31]. TPR and TNR were determined as follows:

$$\text{TPR} = \frac{\text{True } p\text{ositive (TP)}}{\text{True } p\text{ositive (TP)} + \text{False } n\text{egative (FN)}} \times 100$$

$$\text{TNR} = \frac{\text{True } n\text{egative (TN)}}{\text{True } n\text{egative (TN)} + \text{False } p\text{ositive (FP)}} \times 100$$

TPs were defined as samples that were positive by both PCR and LAMP. FNs were samples that were positive by PCR but negative by LAMP. TNs were those that were negative by both assays. FPs were negative by PCR but positive by LAMP. A one-way analysis of variance (ANOVA) was conducted to determine whether statistically significant differences in color occurred in LAMP assays. Post-hoc comparisons were performed using the Tukey HSD test to identify specific group differences. All statistical analyses were performed using SPSS (version 27; IBM, USA) with significance set at $p < 0.05$.

RESULTS

Optimization of LAMP assay conditions

In the LAMP assay using HNB, the reaction time and DNA quantity were varied stepwise to determine the minimum amplification time (**Supplementary Fig. 1A**). The reaction tubes showed no color changes during the 10 min reactions. At 20 min, partial amplification occurred at high DNA concentrations (1–0.1 ng), resulting in a color change from purple to blue. From 30 min onward, DNA amplification was evident across all concentrations from 1 ng to 1 fg. Consequently, the minimum amplification time for the LAMP assay was established as 30 min. Subsequently, our findings confirmed that $MgSO_4$ and dNTP concentrations affect the color change in the LAMP assays (**Supplementary Fig. 1B**). Specifically, 8 and 10 μM $MgSO_4$ displayed distinct color difference between the negative (purple) and positive (blue) samples. The clearest color distinction for dNTPs was observed at 1.0 and 1.4 μM. Therefore, we finalized the concentrations of $MgSO_4$ and dNTP at 8 and 1.4 mM, respectively, for use in subsequent LAMP experiments.

Enhanced visualization in LAMP assays

When the calcein and HNB ratio was adjusted from 1:6 to 1:12, the color contrast between positive and negative samples was visually distinguishable at all ratios (**Fig. 2A**). Although no significant differences existed between groups, a dilution ratio of 1:10 (25 μM of calcein and 250 μM of HNB) yielded the highest visual contrast (**Fig. 2B**). Subsequently, visual and quantitative comparisons of color differentiation between negative and positive samples were conducted using the established mixed dye, HNB, and calcein in the LAMP assays (**Fig. 2C**). Under natural light, the color differentiation between positive and negative samples was visually distinguishable with mixed dye and HNB, whereas calcein required blue light for clear differentiation. When the color differences were quantified under natural light, the mixed dye showed significantly ($p < 0.05$) higher color contrast than those of the two single dyes. No significant difference was observed between HNB and calcein under natural light (**Fig. 2D**).

LAMP assay analytical specificity and sensitivity

Based on the optimized experimental conditions, we validated the specificity of mixed dye-loaded LAMP assay for IS*1311*. Specific DNA amplification occurred only for MAP strains (numbers 1–9); no cross-reactions occurred with other *Mycobacterium* (numbers 10–12) or

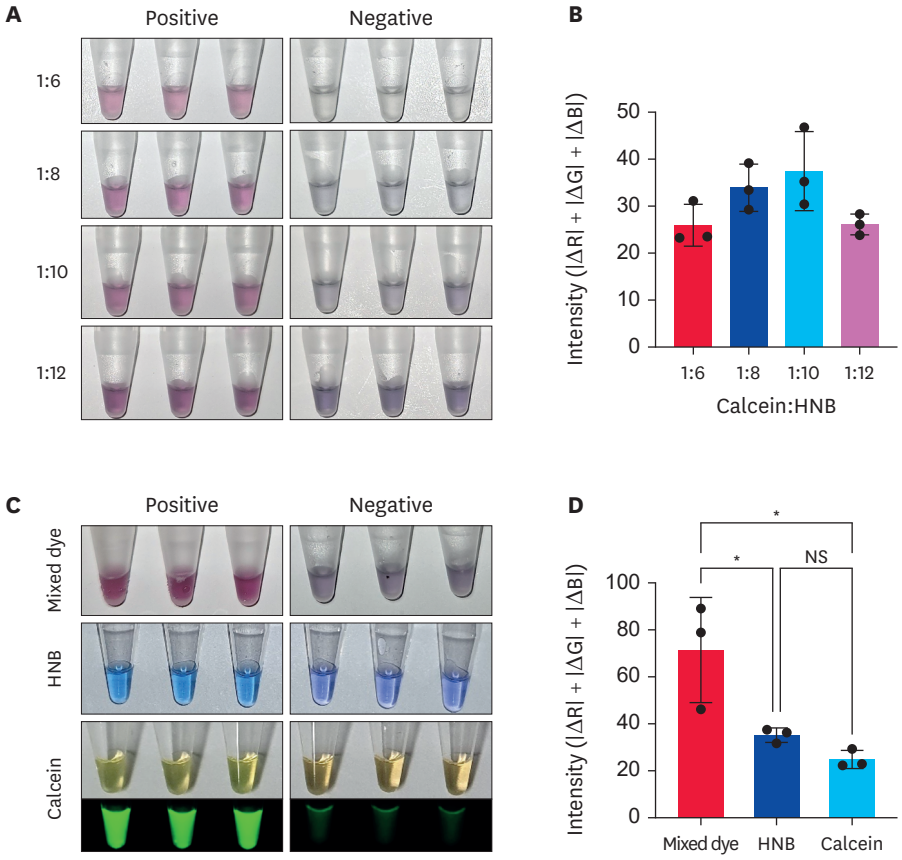

Fig. 2. Visual and quantitative analysis of color contrast between positive and negative samples at different mixing ratios of mixed dye, HNB, or calcein in LAMP assays.
(A) Visual analysis of LAMP assays with varying mixing ratios (calcein:HNB; 1:6, 1:8, 1:10, and 1:12). Positive samples contain 0.1 ng of MAP DNA; negative samples contain nuclease-free water. The color change from gray to pink--purple indicates DNA amplification. (B) Quantitative analysis of RGB color differences between positive and negative samples as determined using ImageJ. (D) Visual analysis of mixed dye, HNB, or calcein-loaded LAMP assays. For calcein, an additional row shows the results under blue light (365 nm) for comparison. (E) Quantitative analysis of color intensity differences between positive and negative samples as determined using ImageJ. The mixed dye showed a significantly higher color contrast than that of either single dye. No significant difference (NS) was observed between HNB and calcein under natural light. All LAMP reactions were performed in triplicate; error bars represent standard deviation (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.
HNB, hydroxynaphthol blue; LAMP, loop-mediated isothermal amplification; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; RGB, red, green, and blue; ANOVA, analysis of variance; NS, not significant.
*$p < 0.05$.

non-*Mycobacterium* (numbers 13–15) species isolates (**Fig. 3**). Especially, *IS1311* was successfully amplified in both Bison type (n = 4) and Cattle type MAP strains (n = 5). LAMP assay sensitivities were determined by comparing the detection limits of serial dilutions of the MAP reference strain (K-10) with those from previously established PCR and real-time PCR methods (**Fig. 4**). The detection limits for IS*1311* conventional PCR were 1 pg, whereas those of LAMP assays were 0.1 pg. Similarly, real-time PCR targeting the F*57* gene could detect DNA as low as 0.1 pg but failed to detect DNA in one out of three replicates.

Validation of the TPR and TNR of the LAMP assay

We evaluated the TPR and TNR of the developed mixed dye LAMP assays using MAP-positive (n = 80) and MAP-negative (n = 90) samples via IS*1311* PCR. The IS*1311* LAMP assay, which

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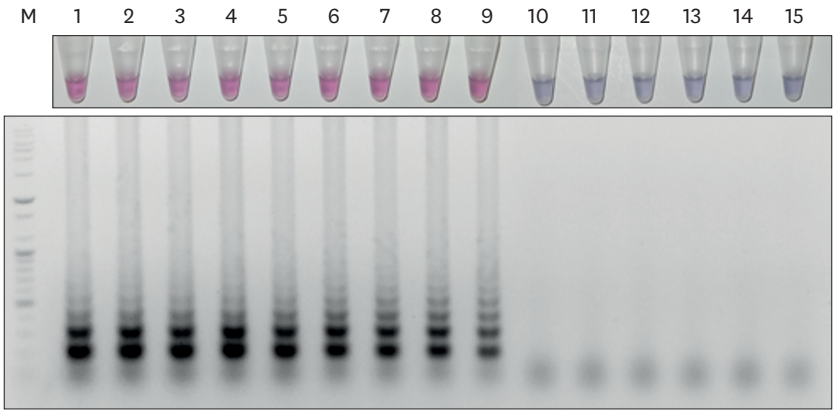
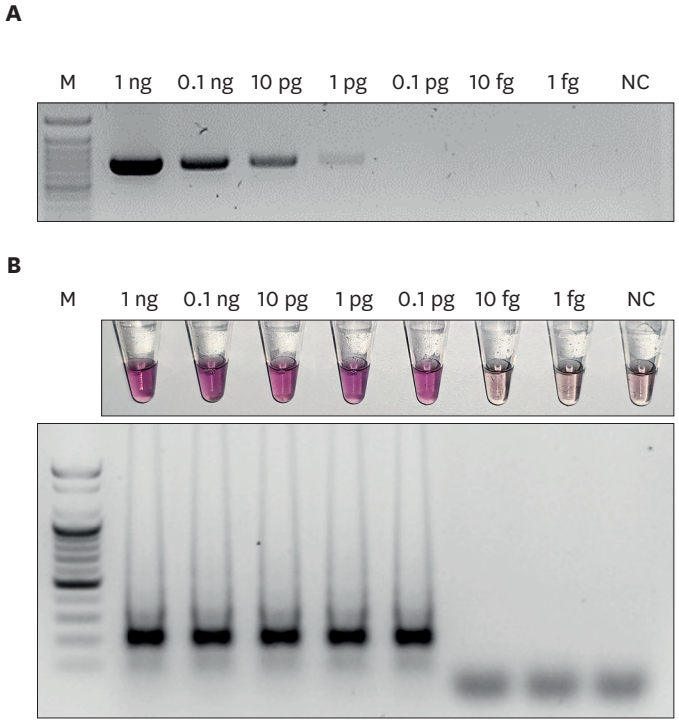
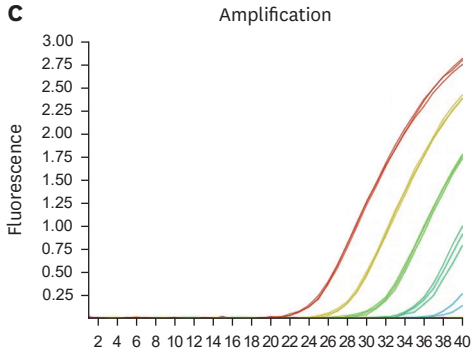


Fig. 3. Analytical specificity of the IS*1311* LAMP assay using mixed dye.
Lane 1, MAP strain K-10; lane 2, MAP strain SU-29; lane 3, MAP strain 15-25; lane 4, MAP strain 20-13; lane 5, MAP strain 19-13; lane 6, MAP strain CN7/15; lane 7, MAP strain CN9/15; lane 8, MAP strain JB16/15; lane 9, MAP strain JJ1/13; lane 10, *Mycobacterium avium* (W-986); lane 11, *M. avium* (C-29); lane 12, *Mycobacterium intracellulare* (D-100); lane 13, *Corynebacterium pseudotuberculosis* (JBNU CP-001); lane 14, *Escherichia coli* O157:H7; lane 15, *Staphylococcus aureus*.
LAMP, loop-mediated isothermal amplification; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; JBNU, Jeonbuk National University.





Genomic DNA	CT values		
	Repeat 1	Repeat 2	Repeat 3
1 ng	22.75	22.55	22.47
0.1 ng	26.44	26.27	25.84
10 pg	29.70	29.26	30.02
1 pg	33.63	34.37	33.89
0.1 pg	Undetected	38.28	37.41
10 fg	Undetected	Undetected	Undetected
1 fg	Undetected	Undetected	Undetected
NC	Undetected	Undetected	Undetected

Fig. 4. Comparison of the limit of detection between conventional PCR, LAMP assay, and reverse transcription PCR.
Ten-fold serial dilutions of template DNA (MAP; K-10) from 1 ng to 1 fg were used for all assays. (A) Gel electrophoresis was performed following conventional IS*1311* PCR. (B) Visual analysis and gel electrophoresis were conducted following IS*1311* LAMP assay (C) Real-time PCR sensitivity assessment for the F*57* gene; the upper panel shows the amplification plot, and the lower panel displays the corresponding CT values for three replicates at each concentration.
PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; CT, cycle threshold; NC, negative control.

determined the diagnosis based on visual color change (**Supplementary Fig. 2**), reported seven false-positive PCR samples, resulting in a TNR of 92.22% (95% confidence interval, 84.63%–96.82%). Ninety PCR-positive samples were identified as positive, resulting in a TPR of 100% (**Table 2**).

Table 2. True-positive and true-negative rates of LAMP in comparison with those of PCR

LAMP	PCR	
	Positive	Negative
Positive	80	7
Negative	0	83

True-positive and true-negative rates of mixed dye-loaded LAMP assay for the IS*1311* gene compared to those for PCR. Cross-table values show true positives (pos/pos), true negatives (neg/neg), false positives (pos/neg), and false negatives (neg/pos) of the LAMP assay compared to those of PCR.
LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

DISCUSSION

JD poses significant challenges owing to its chronic progression and subclinical shedding, which facilitates transmission among livestock. Particularly, goats rarely show signs of diarrhea until later-stage infection [24]. Therefore, developing diagnostic methods enabling sensitive and precise identification of infected animals, suitable for use with both cattle and goat samples, is essential for reducing JD spread and mitigating its economic impact on farms. For early diagnosis, fecal samples are commonly used [12]. However, accurate post-mortem diagnosis using tissues from animals that died showing suspected JD symptoms such as diarrhea, emaciation, and poor coat condition is crucial for preventing further disease spread.

In this study, we successfully developed and validated a LAMP assay targeting the IS*1311* gene for MAP detection. Compared to conventional PCR, the newly developed LAMP assay demonstrated superior diagnostic sensitivity and short amplification time. Use of mixed dye-based colorimetric detection streamlined the diagnostic process, highlighting its potential as a practical alternative for MAP detection in field diagnostic applications.

The LAMP diagnostic method is influenced by various reaction conditions including temperature, time, and reagent concentrations [16]. We found that a 20 min reaction time of 20 min was insufficient to amplify DNA samples with concentrations of ≤ 10 pg, whereas ≥ 30 min reaction time allowed DNA sample detection as low as 1 fg. This result aligns with the generally accepted range for LAMP reactions (30–60 min) [32,33]. Our findings highlight the crucial role played by Mg^{2+} in achieving maximum LAMP assay amplification efficiency. Low $MgSO_4$ concentrations led to false-positive results in negative samples, whereas insufficient dNTPs prevented DNA amplification even in positive samples. Mg^{2+} stabilizes dNTP binding and enhances Bst DNA polymerase activity [34]. Insufficient Mg^{2+} decreases enzyme activity, slowing DNA synthesis and increasing non-specific binding, resulting in observed false positives. This is consistent with previous findings [35,36], emphasizing the significant role of Mg^{2+} and dNTPs in LAMP reactions.

Here, the mixed dye system (calcein + HNB) enhanced the visual distinction between positive and negative samples compared to those of conventional HNB and calcein single-use systems, as quantitatively confirmed by comparing RGB color difference values. Although the mixed-dye system has been previously reported for LAMP assay-mediated foodborne pathogen detection [22], our findings demonstrate the potential applicability of the mixed-dye system for detecting various pathogens. Additionally, the optimal mixing ratio in the developed LAMP method was 1:10, with 25 μM calcein and 250 μM HNB. This differs from previous studies (25 μM calcein and 300 μM HNB), indicating that the optimization process likely varies between diagnostic methods.

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The LAMP assay targeting IS*1311* showed no cross-reactions with tested non-MAP mycobacteria. The detection limit (0.1 pg of DNA) was 10-fold more sensitive than that of conventional PCR (1 pg). The detection limit for IS*1311* PCR [13], which targets a different sequence region [31], was also 1 pg. Notably, the forward primer sequence in the IS*1311* PCR method matches the B1c sequence in our developed IS*1311* LAMP primer sets, highlighting the improved sensitivity of the LAMP assay. Our results demonstrate that even when targeting the same region. Additionally, although utilizing the first PCR product for nested PCR enhances the detection limit to 1 fg [13], this protocol requires approximately 3 h for IS*1311* amplification and additional gel electrophoresis for result analysis. In contrast, our LAMP method offers rapid amplification within 30 min and immediate visual diagnosis, indicating its better suitability for field conditions. Similarly, although F*57*-targeted real-time PCR also yields a 0.1 pg detection limit, only two out of three technical replicates successfully detected MAP. This inconsistency highlights the newly developed IS*1311* LAMP primer set as a superior alternative for MAP detection to reverse transcription PCR.

This study has several limitations. First, some samples that were negative by IS*1311* PCR and ISMap*02* nested PCR were determined to be positive by the LAMP assay. These results could indicate true positives that were only detectable by the LAMP method due to the relatively lower sensitivity of PCR, or they could represent false-positive results from the LAMP method. To confirm true positivity, bacterial culture should be performed; however, due to the limited availability of samples, this was not possible. Second, this study utilized a relatively limited sample set, including repeated sampling from housed herds and a restricted number of non-MAP strains. This limitation highlights the need for further validation of the developed diagnostic method with a broader range of samples including MAP-free and MAP-contaminated farms in future studies. Finally, this study focused on achieving improved colorimetric changes compared to conventional dye-based LAMP methods. However, relying on dye-based indicators indirectly reflects DNA amplification and may not ensure the detection of specific targets. Probe-based LAMP assays have been developed [16,21,37] to address this limitation by accurately detecting specific targets. Therefore, future studies incorporating probe-based LAMP targeting IS*1311* are expected to enhance diagnostic accuracy further.

In conclusion, the novel LAMP assay targeting the IS*1311* gene demonstrated high diagnostic sensitivity and rapid amplification, making it a practical tool for MAP detection. Integrating this LAMP assay with advanced probe-based technologies could enhance its accuracy and applicability in broader diagnostic settings.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Bacterial cultures used for specificity analysis including MAP species, non-MAP *Mycobacterium* subsp., and non-*Mycobacterium* species

Supplementary Table 2

IS*1311* and ISMap*02* PCR-positive and PCR-negative samples used for LAMP validation

Supplementary Fig. 1

Optimal amplification time and buffer conditions in LAMP assay.

Supplementary Fig. 2

Visual color changes in IS*1311* mixed dye-LAMP assays.

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