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Application and Evaluation of LAMP-PCR for the Diagnosis of Silkworm Pebrine Disease

Jong Woo Park*, Pu Reun Kook, Jeong Sun Park, Yeong Hee Cho, Seul Ki Park, Hyeok Gyu Kwon, Ji Hae Lee, Sang Kuk Kang, Seong-Wan Kim, Kee Young Kim, and Seong-Ryul Kim

Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Wanju Gun, Jeollabukdo, 55365, Republic of Korea

Abstract

For stable silkworm breeding and high-quality sericulture product production, the detection of Pebrine disease in silkworm eggs is critical. Current diagnostic methods can be time-consuming and complex. This study aimed to develop a simplified and rapid diagnostic method using loop-mediated isothermal amplification (LAMP) technology to detect pebrine infection in silkworm mother moths. Eight primer candidates targeting the ribosomal gene region of microsporidia were designed and evaluated for specificity and detection sensitivity. A simplified nucleic acid extraction method was established, and isothermal amplification was performed using the selected primers. Of these, primers ID30 and ID45 showed no polymerization, while ID5, ID18, and ID76 exhibited nonspecific reactions, making them unsuitable. Primers ID1, ID6, ID45, and ID82 successfully amplified DNA only in the presence of pebrine, with ID82 demonstrating the best reproducibility and sensitivity, detecting as low as 2.5 pg/ul of DNA through electrophoresis and 5 pg/ul via a colorimetric change with phenol red. The entire process, from nucleic acid extraction to detection, was completed within 60 min. The use of the ID82 primer set in LAMP technology offers a promising and efficient approach for the rapid diagnosis of pebrine disease, potentially enhancing quality control in sericulture.

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Introduction

Microsporidia are unicellular eukaryotes responsible for pebrine disease in insects such as silkworms and honeybees, leading to significant economic losses in sericulture and apiculture industries (Lee *et al*., 2008; Didier 2005). Since their discovery in 1845, their taxonomic classification has evolved from Protista-Protozoa to Fugi-Microspordia, reflecting their complex nature (Gill and Fast, *2006*). Microsporidia are obligate intracellular parasites with a wide host range, including insects and humans, with approximately 1,100 known species. Many of these species are highly pathogenic, causing fatal infections in their hosts, including silkworms, where they are the causative agent of pebrine disease *Nosema bombycis*) (Didier, 2005; Huang *et al*., 2024). Transmission of pebrine occurs primarily through oral and transovarial routes, making it critical to detect

***Corresponding author.**
Jong Woo Park
Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Wanju Gun, Jeollabukdo, 55365, Republic of Korea
Tel: +82-63-238-2945 / FAX: +82-63-238-3833
E-mail: jwpark0824@korea.kr

and prevent infection in silkworm eggs (Bao *et al*., 2019; Wang *et al*., 2009).

Current diagnostic methods for microsporidia infections in insects, including visual and microscopic examination, enzyme-linked immunosorbent assays (ELISA) using antiserum, and culture identification, have significant limitations. Visual and microscopic methods can only detect infections in the late stages, rendering early intervention impossible (Garcia, 2022; and Nguyen *et al*., 1997). ELISA is time-consuming, costly, and prone to cross-reactivity, reducing its specificity (Ghosh *et al*., 2014). Culture methods are slow, requiring extended time for species identification and numerous biochemical tests. Polymerase chain reaction (PCR), while more sensitive, necessitates specialized equipment (thermocycler) and prolonged processing time, limiting its field applicability (Ghosh and Weiss, 2009).

To address these limitations, loop-mediated isothermal amplification (LAMP) offers a promising alternative. Unlike PCR, LAMP does not require temperature cycling, allowing for gene amplification at a constant temperature of 60~65 ℃ using simple equipment, such as a water bath (Dai *et al*., 2019, Notomi *et al*., 2000). This method can provide rapid detection within 1 h, making it highly suitable for field use.

Given these advantages, this study aims to develop LAMP primers targeting the ribosomal gene region of microsporidia. The goal is to replace the conventional microscope-based virulence tests for a more efficient and reliable method for detecting microsporidia and diagnosing pebrine disease in silkworms, thereby ensuring the stable production and distribution of high-quality silkworm eggs.

Materials and Methods

Silkworm sample preparation

Silkworm samples were collected from the National Institute of Agricultural Sciences between 2022 and 2023. Samples targeted silkworms exhibiting symptoms of pebrine disease, including loss of scales on the abdomen and back. Following confirmation of pebrine disease through nucleic acid extraction and PCR testing, these samples were selected for further analysis.

Genomic DNA extraction

For save time and accommodate multiple sample processing, crude nucleic acid extraction, the tail of a microsporidia-infected silkworm was excised, and 200 µl of 2% KOH (Sigma-Aldrich, USA) was added. The sample was homogenized using a pipette tip or a plastic bag. The homogenate was centrifuged at 1,500 xg for 5 min using an TOMY MX-307 centrifuge, and the supernatant was discarded. To the pellet, 50 µl of extraction solvent (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% NP-40, 2 mg/ml Proteinase K) was added, followed by incubation at 65 ℃ for 30 min. Proteinase K was then inactivated by heating at 95 ℃ for 10 min. The resulting solution was used as a template for PCR. For detection limit analysis, 10 ml of 2% KOH solution was added to five microsporidia-infected moths, which were then ground using a mortar and pestle. The homogenate was filtered using a WHIRL-PAK filter bag (Nasco, USA), centrifuged at 1,500 ×g for 5 min, and the sediment was washed thrice with 2% KOH and twice with distilled water. The sediment was resuspended in 3 ml of distilled water, and 10^5 spores were counted using a hemocytometer. Nucleic acids were extracted using the PURE Fungal gDNA extraction kit (Infusion tech, Anyang-si, Gyeonggi-do, Korea) follow the manufacturer's instructions. DNA concentration and purity were using a Nanophotometer P300 (IMPLEN, Schatzbogen, München, Germany), with the DNA stored at -20 ℃ until use.

Primer design for PCR

PCR and LAMP primers were designed using PrimerExplorer V5 (https://primerexplorer.jp/lampv5e/index.html), based on the *N. bombycis* large subunit ribosomal RNA gene (GenBank: AY259631.1). Eight primers were selected, including SA45 primers, based on sequences published by Science Asia (Die *et al*., 2019).

LAMP-PCR reaction and confirmation

PCR was performed using the primer set listed in Table 1, with conditions set to 94 ℃ for denaturation, 57 ℃ for annealing, and 72 ℃ for extension, for a total of 30 cycles. Gene amplification was performed using 2 µl of crude nucleic acid extract and Ex Taq DNA polymerase (Takara, Japan) in a Takara Thermal Cycler. Amplified products were analyzed on a 2% agarose gel. The LAMP reaction was conducted in a total volume of 20 µl, containing 1.6 µM FIB and BIP, 0.2 µM F3 and B3, 0.4 µM F2 and B2, 1 µl template DNA, and 10 µl of WarmStart Colorimetric LAMP 2X Master Mix (NEB, USA), with distilled water added to reach the final volume. The mixture was incubated

Table 1. Primers for PCR and LAMP

Name	Position	Sequence (5'-3')
Pebrine	F	GGA TGA ACA GAA GCG AAA GC
	R	AAT TAA GCC GCA CAA TCC AC
ID1	FIP	CACATTTCATCTCTCTCCTAACCAT-TGATTGATGCAGTTAAAAAGTCTG
	BIP	ATAACCCTAACTGGATGAACAGAAG-TCCTCTAGCTTACGTCCTT
	F3	ACTTGTTCCGATAGTGTGTA
	B3	CAATGGTATCTAATCATCTTCGA
ID5	FIP	TCTTCCAATAAACGGTCGTTTATCT-ATCTGGTAAGACCCGAAAC
	BIP	TAAGTCATTCTGACGTGCAAATCG-GTGAACCAGCTACCACAT
	F3	GACAGTATTAAAGAATCATAGGTGA
	B3	TCAACTGTCCTAAGAGACATT
	F2	ATCTGGTAAGACCCGAAAC
	B2	GTGAACCAGCTACCACAT
ID6	FIP	ACCCCGCGTTGAGTCAAATTAA-GAAATTGACGGAAGAATACCAC
	BIP	GGTGCATGGCCGTTTTCAAT-AGGGTCTCACATCTTGTTG
	F3	GGGGATAGTATGATCGCAAG
	B3	TCATATGTATCACTACATCTGTCT
ID18	FIP	GCCATGCACCACTATCATGATAAAA-ATTGTGCGGCTTAATTTGAC
	BIP	CGTTTTCAATGGATGCTGTGAAGT-TCATATGTATCACTACATCTGTCT
	F3	GGAAGAATACCACAAGGAGTG
	B3	GGACCTGTTTTAATCCTCTCC
ID30	FIP	GACCTGTTTTAATCCTCTCCTTCAT-TAATTTCAACAAGATGTGAGACC
	BIP	GTTGCACGCGCAATACAATAATA-TTTACTAGCAATTCCATGTTCAA
	F3	TTTTCAATGGATGCTGTGAA
	B3	AAGAACAGGGACTCATTCA
ID45	FIP	TGGTCCGTGTTTCAAGACGG-TTGTGAAAATGGTAGTATTAGCT
	BIP	AGGTGATTTGTCATCTGGTAAGAC-TCTTCCAATAAACGGTCGT
	F3	GAATAAGTACTGCGAAGGAAC
	B3	GCACGTCAGAATGACTTAGG
	F2	TTGTGAAAATGGTAGTATTAGCT
	B2	TCTTCCAATAAACGGTCGT
ID76	FIP	TAGGAATCTCGTTGCTCCATTCATG-TATGACTCTCTTAAGGTAGCC
	BIP	GTCCCTATTTAGAGCTATGTGAAGC-TCTTCTTTCCCCGCTGAT
	F3	CATATATAAACGGCGGGAGTA
	B3	ACTAGAGTAAAGCTCAACAGG
	F2	TATGACTCTCTTAAGGTAGCC
	B2	TCTTCTTTCCCCGCTGAT
ID82	FIP	TAGGAATCTCGTTGCTCCATTCATG-CTCTTAAGGTAGCCAAATGC
	BIP	GTCCCTATTTAGAGCTATGTGAAGC-TCTTCTTTCCCCGCTGAT
	F3	GGCGGGAGTAACTATGACT
	B3	ACTAGAGTAAAGCTCAACAGG
	F2	CTCTTAAGGTAGCCAAATGC
	B2	TCTTCTTTCCCCGCTGAT
SA45	FIP	GACCTGTTTTAATCCTCTCCTTCA-TAATTTCAACAAGATGTGAGACC
	BIP	GTTGCACGCGCAATACAATAATA-TTTACTAGCAATTCCATGTTCAA
	F3	TCGAATGGATGCTGTGAA
	B3	AAGAACAGGGACTCATTCA

at 65 ℃ for 60 min, and the reaction was confirmed through color change was it visually confirmed and 2% agarose gel electrophoresis. Reproducibility was verified by performing 30 independent repeats of the LAMP-PCR assay on samples previously determined to be positive for microsporidia by PCR. The detection limit for *Nosema bombycis* DNA was assessed by serially diluting DNA samples from 5 ug/ul and analyzing the reaction in both PCR and LAMP.

Results and Discussion

Pebrine disease sample

Moths infected with microsporidia exhibited brown-tinged hair loss and small spots on their wings, as shown in Fig 1B. The severity of these symptoms varied. Previous studies by Yun and Lim (1985) identified *N. bombycis* along with two related microsporidia, K79 and S80, in Korea. However, research on the genetic differences between K79 and S80 has been limited to pathological and morphological analyses. In this study, three distinct microsporidia types were isolated based on morphological differences after examining over 20,000 moths under a microscope, as shown in Fig. 2. According to Lim and Cho (1983), Microsporidia K79 is ovo-cylindrical shape and approximately 4.5 μm in size, whereas *N. bombycis* and Microsporidia S80 are oval-shaped, with sizes of 3.9 and 2.9 μm, respectively (Moharrami *et al.*, 2022). Therefore, the Microsporidia isolates in this study were classified by size and shape: Fig. 2A corresponds to K79, Fig. 2B to *N. bombycis*, and Fig. 2C to S80. However, this morphological classification is imprecise and should only be used as an initial step in detecting various microsporidia during diagnostic method development (Jyothi and Patil, 2011). Further research focusing on genetic differences through base sequence analysis is necessary for more accurate classification (Kiani-Azad *et al*., 2022).

Fig. 1. Normal silkworm moth (A) and pebrine-infected silkworm moth (B). Scales readily fall off the body, leading to a naked appearance. Low fecundity and laying eggs in irregular heaps are indications of pebrine infection in moths.

Primer production

The primer sequences used in this study are listed in Table 1. The primer sets ID1, ID6, ID18, ID30, and SA45 each comprised four pairs of primers, while ID5, ID45, ID76, and ID82 comprised six pairs. Notomi *et al*. (2000) reported that specificity and reactivity in LAMP assays could be enhanced by using primers that attach to loops formed during the

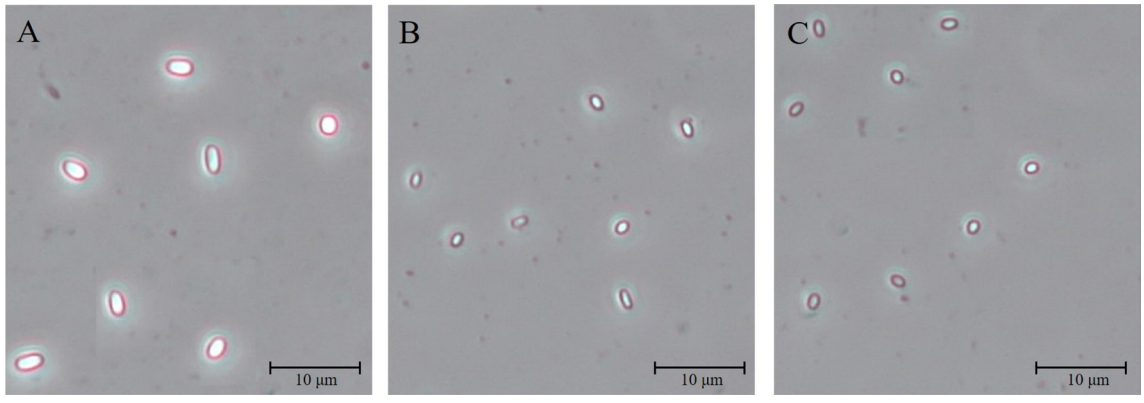

Fig. 2. Isolated microsporidia spores under 40X magnification using a light microscope. (A) Spores with an average length of approximately 3.5 × 1.8 μm and an oblong shape, (B) spores with an average length of 2.3 × 1.0 μm and an elliptical shape, and (C) spores with an average length of 2.0 × 1.2 μm and an elliptical shape.

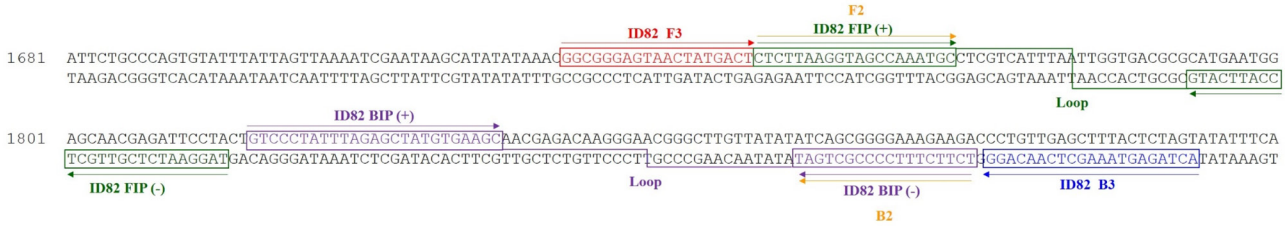

Fig. 3. The 400 bp contiguous sequences of *N. bombycis* (accession No. AY259631.1) was used to design the six primers. The sequences of the LAMP primer sites are indicated by rectangles. Right and left arrows indicate sense and complementary sequences used for the primers. F3, forward primer; FIP, forward inner primer; F2, forward loop primer; B2, backward loop primer; BIP, backward inner primer; B3 , backward primer.

amplification process. Accordingly, four types of four- and six-pair primers were produced. Fig. 3 illustrates the binding sites of each primer using ID82 as an example. The primer set for ID82 included six pairs of primers: F3 and B3 at the amplification site ends, FIB and BIP primers forming loops on either side, and F2 and B2 that binding near the loop.

Specificity of LAMP assay

To verify the specificity of the primers, template DNA was extracted using a crude DNA extraction method from the presumptive K79 and S80 samples, followed by analysis of the reactions with each primer, as shown in Fig. 4A. Out of the nine primers tested, ID5, ID18, and ID76 exhibited nonspecific reactions, as evidenced by the appearance of bands on electrophoresis, even in the absence of template DNA. Conversely, primers ID30 and SA45, which were used in the study by Dai *et al.* (2019), showed low nonspecific reactions, but also exhibited low reactivity, even when template DNA was present. Primers ID1, ID6, ID45, and ID82 demonstrated low nonspecific reactions and good amplification, with no observed difference between Microsporidia K79 and S80. However, repeated experiments revealed frequent primer dimer formation with ID1, ID6, and ID45 under the same conditions, while ID82 consistently produced excellent amplification results. The frequent occurrence of primer dimers is characteristic of the LAMP assay, which uses four or more long primers, and does not pose a problem unless it interferes with the amplification reaction (Notomi *et al*., 2000). Nonetheless, caution is warranted, as excessive primer dimer formation can lead to false-positive results, especially when detected using a colorimetric assay (Dai *et al*., 2019. In a colorimetric comparison (Fig. 4B), the color change resulting from the amplification reaction was easily observable with primers ID1, ID6, ID45, and ID82. According

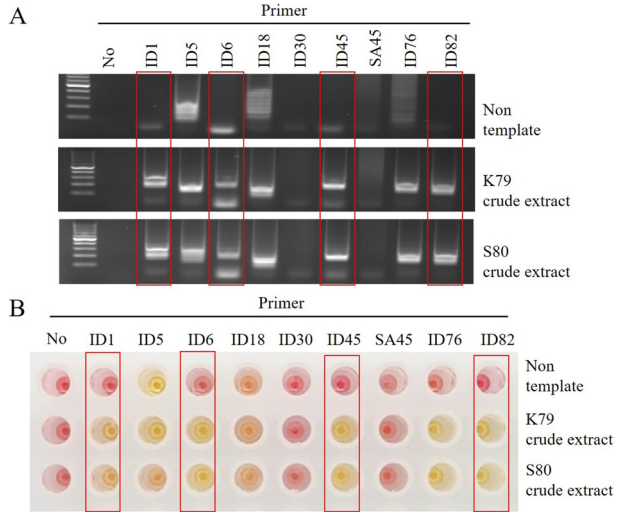

Fig. 4. Reaction specificity of LAMP primer set. (A) Electrophoresis and (B) color development reaction for the selection of suitable primers for LAMP-PCR. Crude nucleic acid, an appropriately concentrated primer mixture, and Warmstart colorimetric LAMP 2x Master Mix were combined and incubated at 65°C for 40 min, followed by electrophoresis on a 2% agarose gel.

to Notomi *et al*. (2015), specificity is critical for LAMP assays due to their high sensitivity and rapid reaction times. Therefore, the ID82 primer, which minimizes primer dimer formation while maintaining high reactivity, is more suitable for detecting Microsporidia compared to primers that frequently form dimers under the same conditions.

Detection limit of LAMP assay

From the collected samples, 10^5 spores were counted and nucleic acids were extracted using a fungal gDNA extraction kit, yielding an average DNA concentration of 30 ng/ul. The extracted DNA served as the template for both PCR and LAMP assays, as shown in Fig. 5. When the template DNA was diluted from 0.175 ng/ul to 2.5 pg/ul, amplification product bands

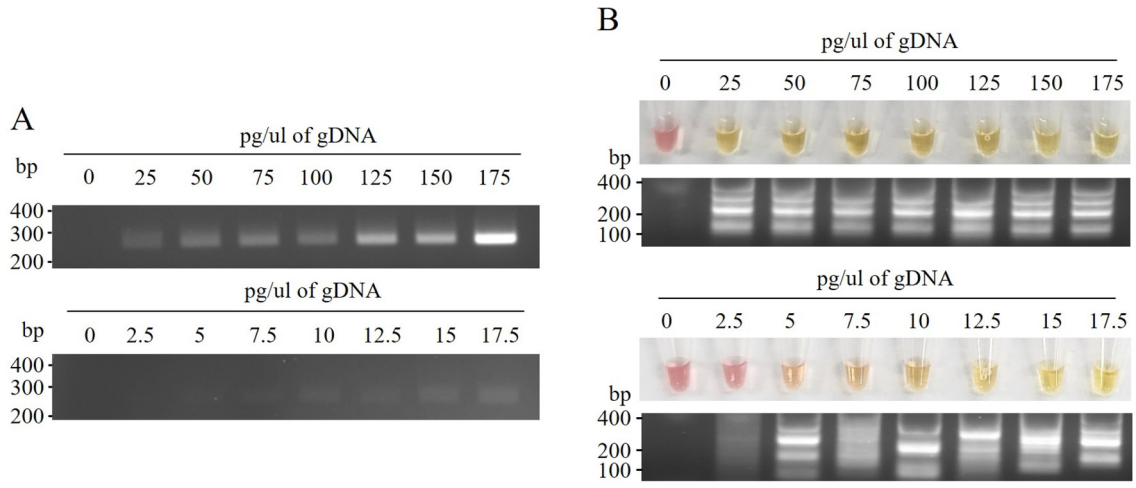

Fig. 5. Detection limit of the *N. bombycis* large subunit ribosomal RNA gene using conventional PCR (A) and the LAMP assay (B). Reactions were conducted using varying dilutions of *N. bombycis* genomic DNA, ranging from 175 to 2.5 pg/ul. Electrophoresis was subsequently performed on a 2% agarose gel.

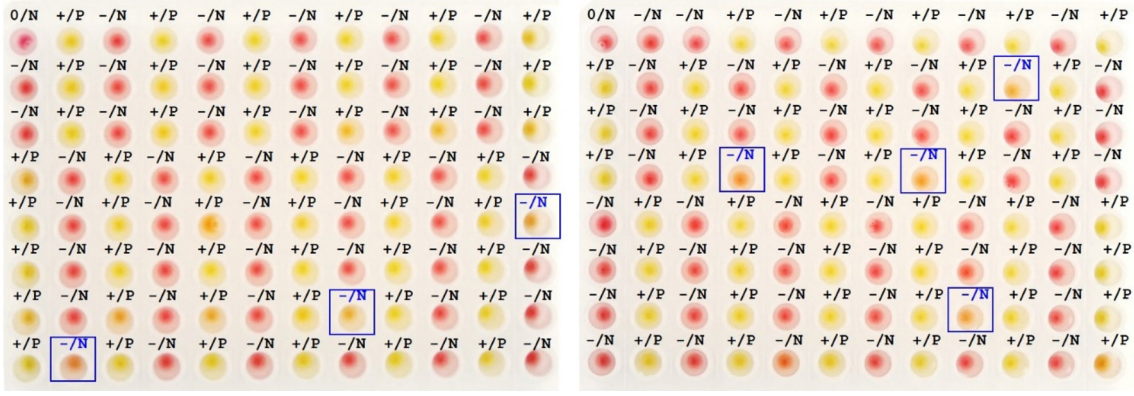

Fig. 6. Diagnosis of microspores using the colorimetric reaction of the LAMP assay. The LAMP assay was conducted using crude DNA from 190 microspore-infected or normal moths and the ID82 primer set. O, Non-template control; +, crude DNA template extracted from microspore-positive moths; -, crude DNA template extracted from normal moths; N, negative reaction; P, positive reaction. Boxes indicate samples with false positive reactions.

were observed on electrophoresis for template concentrations as low as 10 pg/ul in the PCR reaction (Fig. 5A). In contrast, the LAMP assay could detect amplification even at a template DNA concentration of 5 pg/ul, with the color change clearly indicating a positive reaction, and amplification products were confirmed at concentrations as low as 2.5 pg/ul (Fig. 5B). These results surpass the lowest detection concentration of 10 fg/ul reported for the LAMP assay by An *et al*. (2008). However, when compared to electrophoresis, the LAMP assay's detection limit was more than three times lower than that of PCR, and the LAMP assay detecting concentrations as low as 7.5 pg/ul, using a colorimetric reaction, compared to the 10 pg/ul detectable by PCR electrophoresis, demonstrating its high sensitivity.

Pebrine diagnosis using LAMP assay

To evaluate the practical application of the LAMP assay for detecting microsporidia using the ID82 primer, crude nucleic acids were extracted from microscopically confirmed infected samples and normal samples, followed by LAMP assay testing, as shown in Fig. 6. Among 95 normal and 95 infected samples, 102 samples exhibited a color change from red to green, with all 95 positive samples demonstrating this change, resulting in a sensitivity of 100%. However, seven of the 95 negative samples also showed a positive reaction, leading to a specificity of 92.6%. These results suggest that the LAMP assay can microspore infection with a high probability, although the specificity is low, and can replace the particle test using the microscope. However,

in order to use it in the actual silkworm egg production and virulence test process, research is needed to further simplify the process of obtaining crude DNA from samples so that samples can be processed in large quantities. In addition, for a more accurate diagnosis, it is judged that research is necessary to classify various types of microparticles through genetic research on microspores found in as suggested by Kiani-Azad *et al*. (2022). This study has several limitations. Firstly, the classification of microsporidia was primarily based on morphological differences, which may not fully capture the genetic diversity among the species. While the LAMP assay demonstrated high sensitivity and potential for practical application in detecting microsporidia, its specificity was somewhat limited, as indicated by the false-positive reactions observed. Additionally, the study relied on crude DNA extraction methods, which may not be optimal for large-scale diagnostic use in silkworm egg production. Future research should focus on refining the LAMP assay to enhance its specificity and reduce false positives. This could include exploring more advanced DNA extraction techniques that are both efficient and scalable. Moreover, a more comprehensive genetic analysis of microsporidia in Korea is necessary to accurately classify the different species, which could improve diagnostic precision and contribute to better management practices. Policy implications include the potential integration of the LAMP assay into routine testing protocols, provided its specificity is improved. Further studies should also evaluate the cost-effectiveness and practicality of implementing the LAMP assay in large-scale operations.

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