DEVELOPMENT HIGH-SENSITIVITY AND RAPID DIAGNOSTIC METHODS FOR IDENTIFYING THE CAUSATIVE PATHOGEN OF EMACIATION DISEASE THREATENING OLIVE FLOUNDER AQUACULTURE

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1. Introduction

The olive flounder (*Paralichthys olivaceus*) is one of the most economically important marine aquaculture species in Korea, particularly on Jeju Island. However, its sustainable production has been increasingly threatened by infectious diseases, among which emaciation disease has become a major cause of economic loss. This disease is characterized by progressive weight loss, a tapering body shape, intestinal inflammation, reduced feed efficiency, and eventual mortality. Several myxosporean parasites have been associated with emaciation disease in olive flounder. *Enteromyxum leei*, an intestinal histozoic parasite, is considered a major causative agent and induces severe epithelial damage, malabsorption, and chronic wasting. In addition, *Parvicapsula anisocaudata*, which mainly infects the urinary system, has also been reported as an emaciation-associated pathogen in Korea. Despite their importance, accurate and timely diagnosis of these pathogens remains challenging. Conventional histopathological and morphological examinations are labor-intensive, require expert knowledge, and may fail to detect early or low-intensity infections. Moreover, morphological similarities among myxosporean species can lead to misidentification. Therefore, molecular diagnostic methods targeting species-specific genetic markers, particularly the 18S rRNA gene, have become increasingly important. PCR-based assays, including one-step PCR, nested PCR, real-time PCR, and multiplex PCR, provide sensitive and specific detection, but their dependence on thermal cycling equipment limits their use in field settings. Therefore, this study aimed to develop and compare molecular diagnostic methods, including one-step PCR, two-step PCR, real-time PCR, multiplex PCR, and LAMP, for the detection of *E. leei* and *P. anisocaudata* in olive flounder. The sensitivity and specificity of each method were evaluated using plasmid DNA and infected tissue samples, and a SYTO-16-based visual LAMP system was incorporated to improve field applicability. These diagnostic methods are expected to support rapid and early detection of myxosporean emaciation disease

2. Materials and methods

Table 1. Primers and PCR condition used in this study

Target	Primer name	Purpose	Oligonucleotide sequences (5'-3')	Expected size (bp)	Reference
E. leei	EL-1F	1-step PCR Multiplex PCR	CTA TTC GAT GGT ATA CAT AG	1,338	Lee et al., 2024
	EL-1R		GTC TAC TCT TTC GAG CAA AC		
	EL-2F	2-step PCR	ACT AGC TAG AAA CCA ACA AG	609	Lee et al., 2024
	EL-2R		AAC ACG GAT TGG CGT CAC CG		
P. anisocaudata	EM-1F	1-step PCR	TTC GTC GAA AGT GCA GAC AGA	678	In this study
	EM-1R		GGA TTA CCC TGA TCG CAG CA		
	EM-2F	2-step PCR Multiplex PCR	CGC CCA GGG AAA CTG AAA GT	381	In this study
	EM-2R		TGC TCG ACT GTT GAC ATT GC		

95°C 5:00 — 95°C 0:30 — 58°C 0:30 — 72°C 0:30 — 72°C 7:00 — 4°C ∞ (35 Cycles)

Component	μL
Templated DNA	1
Foward primer(10 /μL)	1
Reverse primer(10 μM/μL)	1
10X PCR buffer	2
Taq DNA polymerase	0.2
dNTP (each 2.5mM)	1.6
Nulcease-free water	up to final volume 20

Target	Primer name	Purpose	Oligonucleotide sequences (5'-3')	Expected size (bp)	Reference
E. leei	ELNMF	Real-time PCR	CTA TTC GAT GGT ATA CAT AG	201	Shin et al., 2018
	MyNMR		GTC TAC TCT TTC GAG CAA AC		
P. anisocaudata	EM-2F	Real-time PCR	CGC CCA GGG AAA CTG AAA GT	148	In this study
	REM-R		TCA GTC TGT CAA TCC TAC CG		

95°C 10:00 — 95°C 0:20 — 60°C 0:20 — 72°C 0:20 — 4°C ∞ (45 Cycles)

Component	μL
Templated DNA	1
Foward primer(10 μM/μL)	0.5
Reverse primer(10 μM/μL)	0.5
2X PCR buffer	10
Nulcease-free water	up to final volume 20

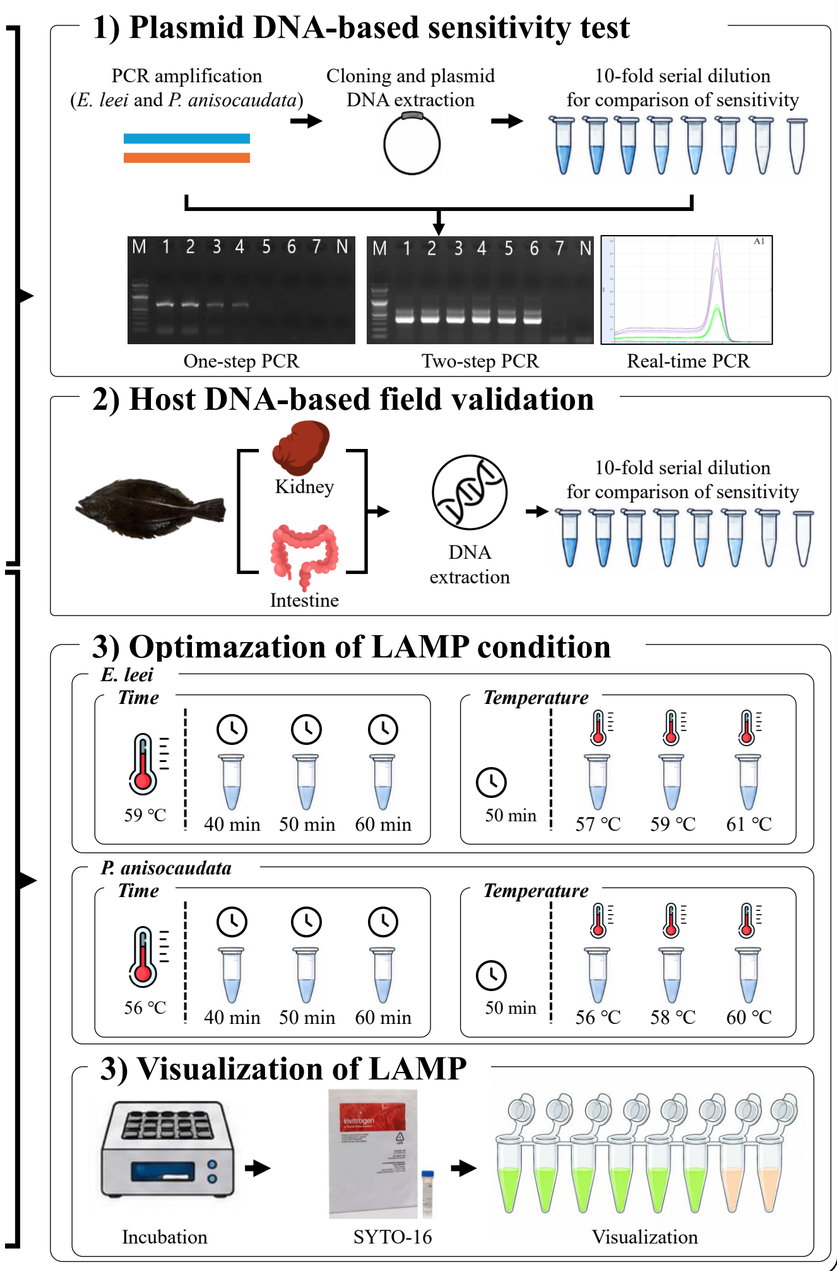
Target	Primer name	Purpose	Oligonucleotide sequences (5'-3')	Expected size (bp)	Reference
E. leei	EL-1F	Multiplex PCR	CTA TTC GAT GGT ATA CAT AG	1,338	Lee et al., 2024
	EL-1R		GTC TAC TCT TTC GAG CAA AC		
P. anisocaudata	EM-2F	Multiplex PCR	CGC CCA GGG AAA CTG AAA GT	381	In this study
	EM-2R		TGC TCG ACT GTT GAC ATT GC		

95°C 5:00 — 95°C 0:30 — 58°C 0:30 — 72°C 0:30 — 72°C 7:00 — 4°C ∞ (35 Cycles)

Component	μL
Templated DNA	1
Foward primer(10 μM/μL)	1
Reverse primer(10 μM/μL)	1
10X PCR buffer	2
Taq DNA polymerase	0.2
dNTP (each 2.5mM)	1.6
Nulcease-free water	up to final volume 20

Target	Purpose	Primer name	Oligonucleotide sequences (5'-3')	Reference
E. leei	LAMP	EL-F3	TTGTTTGCTCGAAAGAGTAGAC	In this study
		EL-B3	ACAAGTACTAGCTCTCCTGAGA	
		EL-FIP	TGCGCTTACGAGGAATTCCACCATTAATTGCTGTCGTGATCGG	
		EL-BIP	TGTGTTGAATACGTCTCTGCCCTCTCGCAGAATCATTCAATTGG	
P. anisocaudata	LAMP	EM-F3	GGAATCGCGATAGCAGAACG	In this study
		EM-B3	CTCAACCGACCCCACCAA	
		EM-FIP	CAAGGCACGCTTAGCAAGCGACTTTTGTGGCAAGTCCCGG	
		EM-BIP	GTGCTCAAAGCAAGCGTAACGCGCAGGCCTGCTCTAACATG	
		EM-LF	AAGCATTGAAGCCCAC	
		EM-LB	GTATGTAATAGCATGGAACG	

Component	Concentration.	Volume
10X Bst DNA Polymerase (Large) Buffer	1 X	2 μL
dNTP mixture (10 mM each)	1.4 mM each	2.8 μL
100 mM MgSO4	6 mM	0.8 μL
FIP Primer	40 μM	0.8 μL
BIP Primer	40 μM	0.8 μL
LF Primer	10 μM	0.8 μL
LB Primer	10 μM	0.8 μL
F3 Primer	5 μM	0.8 μL
B3 Primer	5 μM	0.8 μL
Bst DNA Polymerase (Large fragment) (8 U/μl)	0.4 U/μl	1 μL
Distilled water		7.6/9.4 μL
Template	> 10 copies	1 μL
Total		20 μL



3. Results and discussion

1) PCR amplification

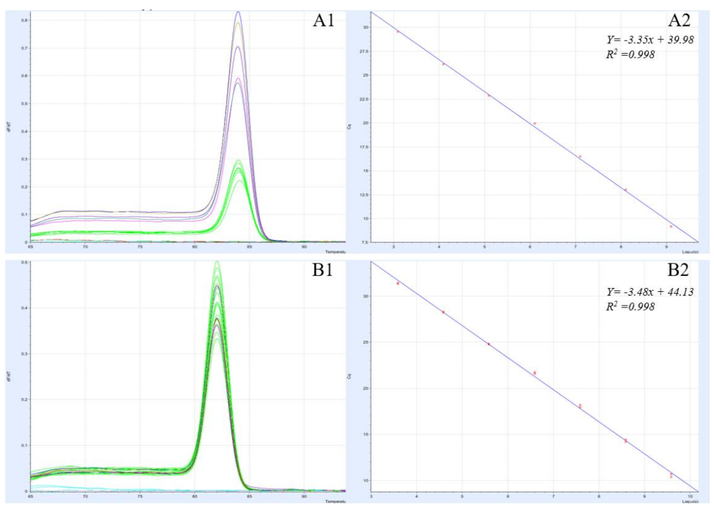


Fig. 1. Melting curve analysis of *P. anisocaudata* (A1) and *E. leei* (B1) transcripts detected by real-time PCR: SSU rDNA are amplified in real-time PCR with specific set of primers and melting curve analysis is performed to confirm the identity of the PCR products. Standard curve derived from 10-fold serially diluted plasmid DNA containing a partial SSU rDNA sequence from *P. anisocaudata* (A2) and *E. leei* (B2). The PCR efficiencies of the real-time PCR assays performed for *P. anisocaudata* and *E. leei* were 98.8% and 93.8%, respectively.

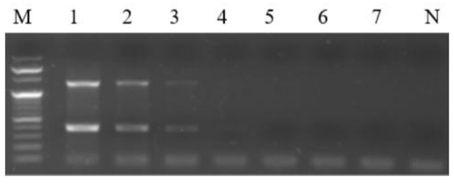


Fig. 4. Sensitivity of multiplex PCR for simultaneous detection of *E. leei* and *P. anisocaudata*. Lane M, DNA size marker; Lanes 1–7, 10-fold serially diluted DNA extracted from *E. leei* and *P. anisocaudata*-infected olive flounder tissues mixed at a 1:1 ratio; Lane N, negative control.

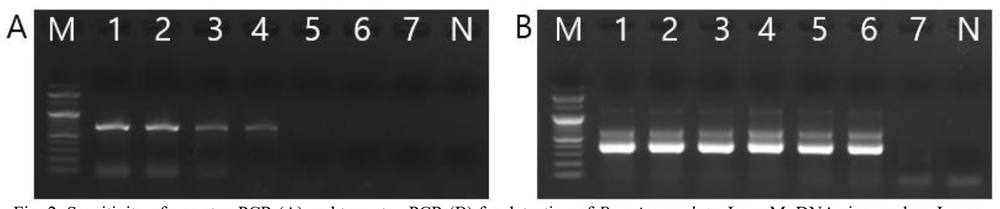


Fig. 2. Sensitivity of one-step PCR (A) and two-step PCR (B) for detection of *P. anisocaudata*. Lane M, DNA size marker; Lanes 1–7, 10-fold serially diluted plasmid DNA from 1.25×10^7 to 1.25×10^1 copies/μL; Lane N, negative control.

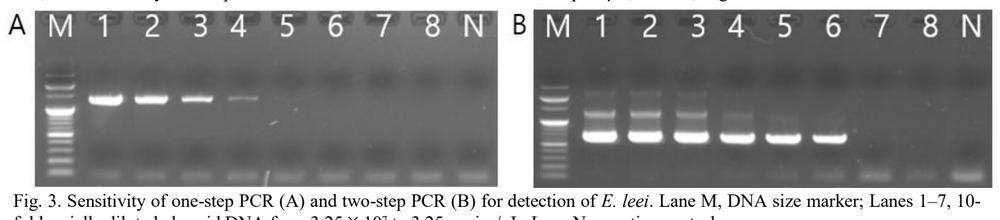


Fig. 3. Sensitivity of one-step PCR (A) and two-step PCR (B) for detection of *E. leei*. Lane M, DNA size marker; Lanes 1–7, 10-fold serially diluted plasmid DNA from 3.25×10^7 to 3.25 copies/μL; Lane N, negative control.

Table 2. Comparison of sensitivity results according to diagnostic method for *E. leei* and *P. anisocaudata*

Concentration of total DNA (Copies/μL)	*P. anisocaudata* Real-time PCR	1-step PCR	2-step PCR
1.25×10^7	P	P	P
1.25×10^6	P	P	P
1.25×10^5	P	P	P
1.25×10^4	P	P	P
1.25×10^3	P	-	P
1.25×10^2	P	-	P
1.25×10^1	-	-	-
Concentration of total DNA (Copies/μL)	*E. leei* Real-time PCR	1-step PCR	2-step PCR
3.25×10^7	P	P	P
3.25×10^6	P	P	P
3.25×10^5	P	P	P
3.25×10^4	P	P	P
3.25×10^3	P	-	P
3.25×10^2	P	-	P
3.25×10^1	-	-	-

The detection limits of the developed assays were evaluated using 10-fold serially diluted plasmid DNA for each target species (Table 2). For *E. leei*, one-step PCR detected the target DNA down to 3.25×10^4 copies/μL, whereas two-step PCR and real-time PCR both extended detection sensitivity to 3.25×10^2 copies/μL, representing a 100-fold improvement over one-step PCR. No amplification was observed at 3.25×10^1 copies/μL for any of the three methods (Fig. 2). For *P. anisocaudata*, one-step PCR achieved a detection limit of 1.25×10^4 copies/μL, while two-step PCR and real-time PCR both detected the target DNA down to 1.25×10^2 copies/μL, also representing a 100-fold improvement over one-step PCR. No amplification was observed at 1.25×10^1 copies/μL for any method (Fig. 3). Overall, real-time PCR and two-step PCR demonstrated equivalent and superior sensitivity compared to one-step PCR for both target species.

Multiplex PCR sensitivity was evaluated using DNA extracted from *E. leei*- and *P. anisocaudata*-infected olive flounder tissues mixed at a 1:1 ratio and serially diluted 10-fold. Specific amplification bands for both species were simultaneously detected down to 10^{-3}. No amplification was observed at the lowest dilution (Fig. 4). These results confirm that the multiplex PCR assay is capable of simultaneously detecting both pathogens in a single reaction without significant loss of sensitivity.

2) LAMP

Fig. 5. Incubation time optimization of LAMP assay for *E. leei*. Reactions were performed at 59 °C for 40, 50, and 60 min. Lane M, 100bp DNA ladder marker; Lanes 1-7, 10-fold serially diluted *E. leei* template DNA; Lane 8, negative control.

Fig. 6. Temperature and incubation time optimization of LAMP assay for *E. leei*. Reactions were performed at 57 °C, 59 °C, and 61 °C at a fixed incubation time of 50 min. Lane M, 100bp DNA ladder marker; Lanes 1–7, 10-fold serially diluted *E. leei* template DNA; Lane 8, negative control.

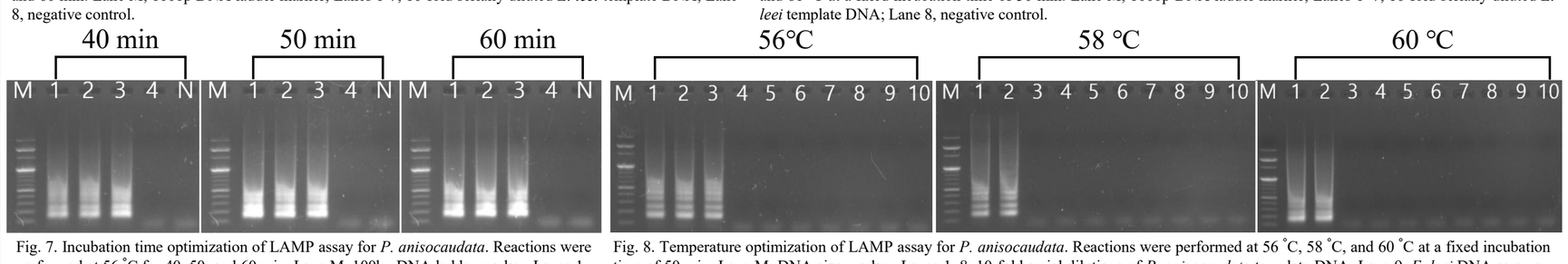


Fig. 7. Incubation time optimization of LAMP assay for *P. anisocaudata*. Reactions were performed at 56 °C for 40, 50, and 60 min. Lane M, 100bp DNA ladder marker; Lanes 1–4, 10-fold serially diluted *P. anisocaudata* template DNA; Lane N, negative control.

Fig. 8. Temperature optimization of LAMP assay for *P. anisocaudata*. Reactions were performed at 56 °C, 58 °C, and 60 °C at a fixed incubation time of 50 min. Lane M, DNA size marker; Lanes 1–8, 10-fold serial dilutions of *P. anisocaudata* template DNA; Lane 9, *E. leei* DNA as a non target control; Lane 10, negative control.

Table 3. Comparison of sensitivity results according to diagnostic method for *E. leei* and *P. anisocaudata*

Concentration of total DNA	*E. leei* Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR	LAMP
6 ng/μL	4.86×10^7	P	P	P
600 pg/μL	5.65×10^6	P	P	P
60 pg/μL	5.50×10^5	P	P	P
6 pg/μL	5.84×10^4	P	P	P
600 fg/μL	5.55×10^3	-	P	P
60 fg/μL	8.65×10^2	-	P	P
6 fg/μL	-	-	-	-

Concentration of total DNA	*P. anisocaudata* Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR	LAMP
6 ng/μL	4.86×10^7	P	P	P
600 pg/μL	5.65×10^6	P	P	P
60 pg/μL	5.50×10^5	P	P	P
6 pg/μL	5.84×10^4	P	P	P
600 fg/μL	5.55×10^3	-	P	-
60 fg/μL	8.65×10^2	-	P	-
6 fg/μL	-	-	-	-

To assess the practical diagnostic utility of the developed LAMP assays, sensitivity testing was performed using serially diluted DNA extracted from infected intestinal tissue samples. This approach provides a more realistic evaluation of assay performance under field conditions where tissue samples would be the primary diagnostic material (Table 2).

For *P. anisocaudata*, the LAMP assay demonstrated a detection limit of 60 pg/μL DNA concentration, corresponding to 5.78×10^2 copies/mg tissue as determined by real-time PCR quantification. This sensitivity was equivalent to both real-time PCR and two-step PCR methods under the same conditions. The 2-step PCR showed the highest sensitivity, detecting down to 60 fg/μL (5.78×10^2 copies/mg tissue), which was 100-fold more sensitive than LAMP. However, the LAMP assay's detection threshold of 2.79×10^4 copies/mg tissue represents a clinically relevant sensitivity level for diagnosing *P. anisocaudata* infections in aquaculture settings.

For *E. leei*, the LAMP assay achieved superior sensitivity compared to *P. anisocaudata*, with a detection limit of 60 fg/μL (8.65×10^2 copies/mg tissue). This performance closely approached that of real-time PCR, which detected down to 60 fg/μL (8.65×10^2 copies/mg tissue). The 2-step PCR method matched real-time PCR sensitivity, while 1-step PCR was significantly less sensitive, detecting only down to 60 pg/μL. The comparable sensitivity between LAMP and real-time PCR for *E. leei* demonstrates the robust performance of the LAMP assay for this target species.

Specificity testing confirmed 100% specificity for both assays, with no cross-reactivity observed against heterologous controls or uninfected tissue samples, ensuring species-specific detection capability.

3. Visualization of LAMP

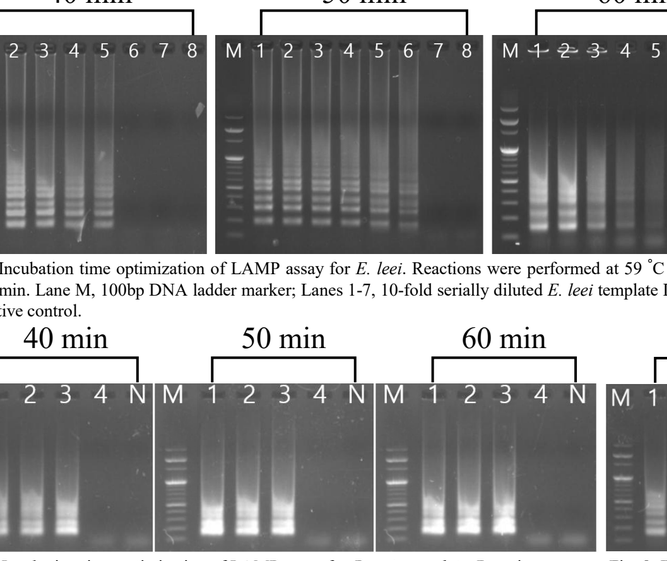
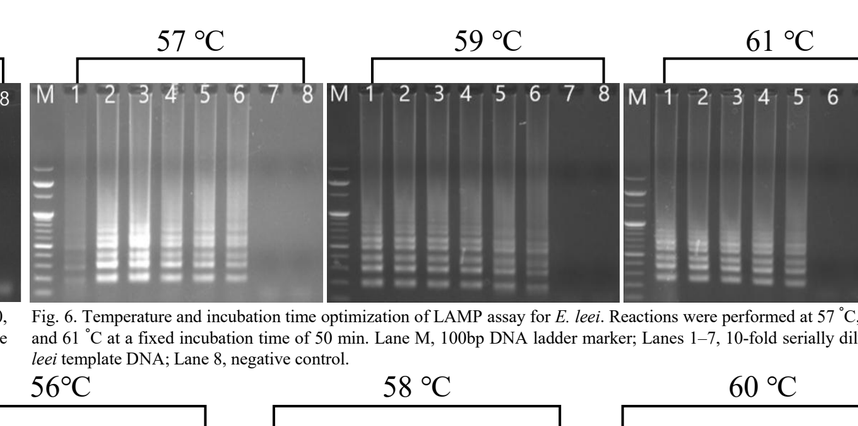


Fig. 9. Visual detection of *E. leei* LAMP amplification at varying temperatures with SYTO-16 fluorescent dye. Reactions were performed at 57 °C (top row), 59 °C (middle row), and 61 °C (bottom row) at a fixed incubation time of 50 min. Lanes 1–7, 10-fold serially diluted *E. leei* template DNA; Lane 8, negative control. Positive reactions are indicated by a color change from orange to fluorescent green.



Field diagnosis can be performed through simple on-site DNA extraction, demonstrating its potential as an effective rapid diagnostic method.

4. Reference

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