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A Rapid Colorimetric Loop-Mediated Isothermal Amplification Assay for the Detection of *Lawsonia intracellularis* in Pigs

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Abstract: A colorimetric Loop-mediated isothermal Amplification (LAMP) assay with hydroxy naphthol blue was designed for the detection of *Lawsonia* (L.) *intracellularis* which is an important pathogenic bacteria that causes animal diseases. A set of six primers was designed to target the aspartate ammonia-lyase (*asp*A) gene. Serial 10 fold dilutions of cultured *L. intracellularis* and spiked feces were used for LAMP optimization. The lower limit of the linear range of the assay in *L. intracellularis* was 10^1 *L. intracellularis* which is ten times more sensitive than conventional PCR. Based on testing in 210 porcine fecal samples using LAMP and conventional PCR, the agreement quotients between LAMP and conventional PCR was 0.92. These results suggest that colorimetric LAMP is a simple method for *L. intracellularis* detection in porcine fecal samples.

Key words: *Lawsonia intracellularis*, loop-mediated isothermal amplification, LAMP, porcine proliferative enteropathy, pigs

INTRODUCTION

Lawsonia (L.) *intracellularis* is a well-established pathogen in pigs (Lawson and Gehart, 2000). It is the cause of proliferative enteropathy, a condition characterized by a thickening of the mucous membrane of the intestine with proliferation and immaturity of the intestinal epithelium. The disease has a worldwide distribution and typically causes diarrhea and growth retardation (McOrist and Lawson, 1993). In addition to pigs, proliferative enteritis associated with *L. intracellularis* has been described in numerous animal species but not in humans (Lawson and Gehart, 2000). Therefore, rapid and accurate diagnostic methods are a prerequisite for effective treatment, supporting prevention programs and minimizing losses.

Lawsonia (L.) *intracellularis*, the causative agent of Porcine Proliferative Enteropathy (PPE) is a fastidious organism that can be cultivated and maintained only in cell cultures (McOrist and Lawson, 1993). Thus, isolation is not routinely used to detect *L. intracellularis* for diagnostic purposes in clinical settings (Jordan *et al*., 1999). The identification of specific DNA using PCR and immunohistochemistry of tissue samples has been suggested as the gold standard (Jordan *et al*., 1999). More reliable and sensitive diagnostic methods, namely PCR-based assays have been developed for the detection of *L. intracellularis* including conventional PCR (Jordan *et al*., 1999) and real-time PCR (Drozd *et al*., 2010; Wattanaphansak *et al*., 2010). Although, these assays perform well for sensitive detection of *L. intracellularis*, they are not readily transferable to low-technology settings where there is limited access to expensive fluorescence detector-based thermocyclers.

As an alternative to PCR-based assays, Loop-mediated isothermal Amplification (LAMP), auto-cycling and strand displacement DNA synthesis have been reported as possible replacements for PCR (Notomi *et al*., 2000). The LAMP technique is carried out with a set of four oligonucleotide primers which recognize six distinct sequences on the target DNA at a constant temperature ranging from 60-65°C (Nagamine *et al*., 2002). Nagamine *et al*. (2002) reported that the addition of one or two loop primers accelerates the reaction. In LAMP assay, a large amount of DNA is synthesized, yielding a large pyrophosphate ion by-product. These ions react with Mg^{2+} concentration. Thus, the amplified target DNA can be easily visualized with the naked eye by addition of a metal ion-binding indicator dye such as hydroxy naphthol blue (Goto *et al*., 2010).

The aim of the present study was to develop and validate the LAMP assay for rapid and simple detection

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of *L. intracellularis* in porcine fecal samples. The results of this assay were compared to those of PCR assays currently used to detect *L. intracellularis* nucleic acids in fecal samples of clinically ill pigs.

MATERIALS AND METHODS

Bacterial strains and DNA extraction: The bacterial strains used in this study were *L. intracellularis* B3903 (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) and 11 non-*L. intracellularis* bacteria (*Brachyspira hyodysenteriae* American Type Culture Collection [ATCC] 35218, *Brachyspira pilosicoli* [ATCC 51139], *Campylobacter coli* [field isolate], *Campylobacter jejuni* [field isolate], *Clostridium perfringens* [field isolate], *Escherichia coli* [ATCC 25922], hemolytic *Escherichia coli* [ATCC 35218], *Salmonella typhimurium* [ATCC 14028], *S. cholerasuis* [field isolate], *Shigella flexneri* [ATCC 12022] and *Yersinia enterocolitica* [ATCC 9610]).

Chromosomal DNA was extracted and purified from these adjusted bacterial suspensions using a silica-membrane-based spin kit (DNeasy® Tissue kit; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA samples were stored at -20°C.

Spiking of fecal samples and DNA extraction: Sterile saline suspensions (100 μL) with known concentrations of *L. intracellularis* in the range of 10^0-10^3 $TCID_{50}$ were added to fresh fecal samples. Bacterial DNA for the spiked fecal samples was extracted from the pellets of centrifuged samples using the AccuPrep Stool DNA extraction kit (Bioneer Co., Daejeon, Korea) according to the manufacturer's instructions. The isolated DNA was used as the template for LAMP and PCR.

Colorimetric LAMP assay: The colorimetric LAMP assay was conducted as described earlier (Goto *et al*., 2010). Briefly, LAMP requires a set of six primers (B3, F3, BIP, FIP, LF and LB) within the target DNA. LAMP primers for *L. intracellularis* were designed against the aspartate ammonia-lyase (*aspA*) gene using Primer Explorer V4 Software (FUJITSU, Tokyo, Japan) and are described in Table 1. LAMP assays were carried out in a 25 μL reaction mixture containing 1 μL (40 pM) of diluted DNA extracted from cultured bacteria or DNA extracted from fecal samples, 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.4 mM of each dNTP, 8U of the large fragment of Bst DNA polymerase (New England BioLabs, Tokyo, Japan) and 120 μM of Hydroxy Naphthol Blue trisodium salt (HNB, CAS No. 63451-35-4; Dojindo, Kumamoto, Japan) in LAMP buffer (20 mM Tris-HCl [pH 8.8], 8 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2$ SO_4, 0.1% Tween 20 and 0.8 M betaine). The DNA extracts from fecal samples were boiled for 5 min before use. Distilled water was used instead of DNA extract for the negative control. All of the reaction mixtures were incubated at 63°C for 1 h and were then heated at 80°C for 2 min in a water bath to terminate the reaction. The results were considered to be negative when the color of the solution was violet and positive when the color was sky blue.

Determination of sensitivity and specificity of LAMP assay: To determine the specificity of the method, the real-time LAMP assay was performed under conditions described above with DNA templates from *L. intracellularis* and 11 non-*L. intracellularis* bacteria. Each bacterium was examined three times.

The sensitivity of the real-time LAMP assay was determined using feces spiked with *L. intracellularis* B3903 as a positive control. A 100 μL aliquot of each dilution was used to extract the DNA as described earlier.

Application of the LAMP assay to clinical samples: The suitability of the assay for detection of *L. intracellularis* was evaluated by comparing the detection results of the LAMP assay using 210 fecal samples obtained from 210 pigs from 11 pig farms. All 210 pigs had been diagnosed clinically with *L. intracellularis* infections. Fecal samples were collected with a sterile swab, immersed immediately in a 1.5 mL tube containing 400 μL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and stored at

Table 1: Primer sequences of colorimetric Loop-mediated isothermal Amplification (LAMP) and conventional PCR used in this study

Methods	Primer	Position*	Sequence (5'-3')	References
Real-time	F3	596-614	GCACCCGTATCTTGTGTAG	This study
LAMP	B3	809-830	TGACATTAGGTCAAGAATTTCG	
	FIP(F1c+F2)	674-698	TCCCCCAGTATAAAGAACTTGCTAC+	
		618-642	CTATTAAATCTGGAGAACTAGTGAG	
	BIP(B1c+B2)	711-732	CTGTACCAATAGCTGTAGCCCC+	
		770-792	ATGAGAGAAGATATTGAACGAAT	
	LF	643-662	CAGAAATTACAGAATTAACC	
	LB	747-765	GCATAAGTTTTGATGTTTC	
PCR	LIF	1248-1226	GCAGCACTTGCAAACAATAAACT	Suh and Song (2005)
	LIR	1039-1061	TTCTCCTTTCTCATGTCCCATAA	

*Genome position according to genbank accession number AM180252

-80°C until used. *L. intracellularis* B3903 was used as a positive control. DNA was extracted from fecal samples and from *L. intracellularis*-infected (positive control) and non-infected (negative control) fecal samples using the AccuPrep Genomic DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The isolated DNA was used as the template for LAMP and PCR. The sensitivities and specificities of these three assays were compared and agreement among the assays was examined using kappa statistics (Cho *et al*., 2006).

PCR assay: PCR was conducted as described earlier (Suh and Song, 2005). Primers used in this study are described in Table 1.

RESULTS AND DISCUSSION

Specificity of the LAMP assay for *L. intracellularis*: The specificity of the LAMP assay was examined with genomic DNA from various species of bacteria. The results of the LAMP assay were positive for the *L. intracellularis* strain whereas they were negative for the 11 non-*L. intracellularis* bacteria tested (Table 2).

Sensitivity of the LAMP assay for *L. intracellularis*: Using a 10 fold dilution series of spiked fecal samples (10^0-10^5 *L. intracellularis* of template DNA), researchers determined that the detection limit of LAMP was 10^1 *L. intracellularis* bacteria, compared to 10^2 *L. intracellularis* for PCR (Fig. 1).

Evaluation of the LAMP assay using fecal samples: Results of the LAMP and PCR assays of 210 fecal samples from 11 pig farms are shown in Table 2. Of the 210 fecal samples tested, 161 (76.7%) were positive by both LAMP and PCR, 6 (2.9%) were positive by LAMP and negative by PCR, 0 (0%) were positive by PCR and negative by LAMP and 43 (20.5%) were negative by both tests. The agreement quotients (kappa) which measure the levels of agreement beyond that of random chance were 0.92 (Table 3). There were six discordant samples that were positive according to LAMP but negative by PCR. Together, these results indicate very high sensitivity of LAMP for *L. intracellularis* detection.

Recently, the LAMP assay has gained popularity for its usefulness in the detection of pathogens because of its sensitivity, specificity and ease of use compared to conventional methods such as PCR and real-time PCR (Guedes *et al*., 2002; Sun *et al*., 2010). In addition, real-time PCR requires up to 4 h to complete therefore this method is slower and more costly than LAMP.

Table 2: Specificity of real-time loop-mediated isothermal amplification assay

Species	No. of positive strains/total[a]
Brachyspira sp.	0/2
B. hyodysenteriae	0/1
B. pilosicoli	0/1
Campylobacter sp.	0/2
Campylobacter coli	0/1
Campylobacter jejuni	0/1
Clostridium perfringens	0/1
Escherichia coli	0/2
Salmonella sp.	0/2
S. typhimurium	0/1
S. cholerasuis	0/1
Shigella flexneri	0/1
Yersinia enterocolitica	0/1

[a]Number of strains giving positive results in the real-time LAMP assay per total number tested

Table 3: Comparison of the sensitivity and specificity of Loop-mediated isothermal Amplification (LAMP) and Polymerase Chain Reaction (PCR) for detection of *Lawsonia intracellularis* in 210 fecal samples of pigs

		Conventional PCR		
Methods	Symbols	+	-	Kappa index
Colorimetric	+	161	6	0.92
LAMP	-	0	43	-

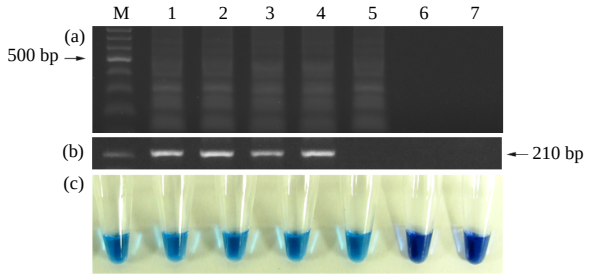

Fig. 1: Comparison of the analytical sensitivities of colorimetric LAMP and PCR assays using porcine fecal samples spiked with *Lawsonia intracellularis*. Amplification by colorimetric LAMP; a) shows a ladder-like pattern whereas PCR; b) shows a 210 bp amplification product. c) Color change of LAMP products (violet to light blue). Lanes (tubes) M: 100 bp DNA ladder (Bioneer, Korea); 1: 10^5 cells; 2: 10^4 cells; 3: 10^3 cells; 4: 10^2 cells; 5: 10^1 cells; 6: 100 cells; 7: negative control without target DNA

In the present study, a novel method utilizing the LAMP assay with HNB to detect *L. intracellularis* DNA in pig fecal samples was developed. This method was superior to other assays because:

- Opening the reaction tube was not required to determine whether the reaction was positive or negative (this reduces the risk of cross-contamination)

- Detection sensitivity was equivalent to that of the assay using HNB (a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions)
- The positive/negative result of the LAMP reaction could be easily assessed by the naked eye (Goto *et al*., 2010)

The LAMP assay offers several advantages over PCR for the detection of *L. intracellularis* in the feces of pigs. The whole assay requires only 2 h to complete and it is a sensitive method that can amplify a few copies of DNA to a magnitude of 10^9 copies of target in <1 h under isothermal conditions (Notomi *et al*., 2000; Nagamine *et al*., 2002).

An earlier study (Cho *et al*., 2006) found that the detection limit of the LAMP assay for canine parvovirus was 10^0 $TCID_{50}$/0.1 mL which is 100 times more sensitive than PCR. In the current study, the LAMP Method used for detecting *L. intracellularis* was highly sensitive and it detects *L. intracellularis*-DNA template at concentrations as low as 10^1 *L. intracellularis*, 10 times more sensitive than conventional PCR.

In Korea, the reported prevalence of PPE ranges from 44-69% in individual pigs as determined by indirect immunofluorescence antibody testing (Lee *et al*., 2001). However, the detection rate of *L. intracellularis* using PCR is 7.9% (15 of 191 samples) (Chu *et al*., 2010). Although, it is somewhat difficult to directly compare the prevalence of PPE as determined by different methods, the prevalence of PPE has decreased due to the use of antibiotics as feed additives in Korea. Specifically, approximately 600 tons of veterinary antimicrobials were used as feed additives in Korea in 2004 which is 20 fold higher than the amount of growth promoters used in the United Kingdom in the same year (Kim *et al*., 2008). The Korea food and drug administration banned the use of all types of antibiotics in feed for livestock and farm-raised fish beginning in the second half of 2011 which increased the prevalence of PPE. Therefore, it is an ideal time to develop a highly sensitive and useful LAMP assay for the detection of *L. intracellularis*.

CONCLUSION

The high sensitivity and rapidity of the LAMP assay for detection of *L. intracellularis* detection makes this technique a useful tool for PPE diagnosis. In addition, the LAMP assay is advantageous over PCR because it can be carried out with simple and cost-effective equipment in a short amount of time through the use of visual detection.

The LAMP assay developed in the current study should provide a valuable alternative to PCR for use in veterinary field clinics.

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