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Original Article

Development of a multiplex loop-mediated isothermal amplification assay to detect shiga toxin-producing *Escherichia coli* in cattle

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A multiplex loop-mediated isothermal amplification (mLAMP) assay was developed for simultaneous detection of the *stx*1 and *stx*2 genes and applied for detection of shiga toxin-producing *Escherichia coli* (STEC) in cattle farm samples. Two target genes were distinguished based on T_m values of 85.03 ± 0.54°C for *stx*1 and 87.47 ± 0.35°C for *stx*2. The mLAMP assay was specific (100% inclusivity and exclusivity), sensitive (with a detection limit as low as 10 fg/μL), and quantifiable (R^2 = 0.9313). The efficacy and sensitivity were measured to evaluate applicability of the mLAMP assay to cattle farm samples. A total of 12 (12/253; 4.7%) and 17 (17/253; 6.7%) STEC O157, and 11 (11/236; 4.7%) non-O157 STEC strains were isolated from cattle farm samples by conventional selective culture, immunomagnetic separation, and PCR-based culture methods, respectively. The coinciding multiplex PCR and mLAMP results for the types of shiga toxin revealed the value of the mLAMP assay in terms of accuracy and rapidity for characterizing shiga toxin genes. Furthermore, the high detection rate of specific genes from enrichment broth samples indicates the potential utility of this assay as a screening method for detecting STEC in cattle farm samples.

Keywords: cattle farm, *E. coli* O157, LAMP, shiga toxin, *stx*

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne zoonotic pathogen that frequently causes human illness ranging from mild gastrointestinal problems to serious fatal complications [20,21]. It is estimated that approximately 176,000 cases of foodborne illnesses caused by STEC occur annually in the United States [18]. Among the 2,400 hospitalizations and 20 deaths caused annually by STEC, serotype O157 is implicated in roughly 35% of illnesses, 89% of hospitalizations, and 100% of deaths [18]. Because cattle are known to be a natural reservoir of STEC, asymptomatically infected cattle can transmit STEC to humans [14,22]. Therefore, investigating the prevalence of STEC in cattle and their environment is important for control and prevention of STEC transmission to humans.

STEC harbors essential virulence genes *stx*1 and/or *stx*2 [17]. STEC O157 is traditionally detected by a selective culture method based on the inability to ferment sorbitol [17]; however, detection of non-O157 STEC is difficult due to the lack of phenotypic characterization. Fast, sensitive, and specific nucleic acid-based amplification methods have recently been adopted to supplement the conventional culture method. The standard manuals established by the United States Department of Agriculture (USDA) and the United States Food and Drug Administration (FDA) recommend polymerase chain reaction (PCR) and real-time PCR (qPCR) as screening methods for detection of STEC by targeting various genes, including *stx*1 and *stx*2 [5,6].

The loop-mediated isothermal amplification (LAMP) assay is a promising novel nucleic acid amplification method in terms of its accuracy, simplicity, and rapidity [16]. The use of 4 ~ 6 specially designed primers (FIP, BIP, F3, B3, LF, and LB) results in high efficiency of the assay, which can amplify low titers of DNA templates [15]. The LAMP assay recognizes 6 ~ 8 distinct regions of the DNA template, thereby providing high specificity and sensitivity [15,16]. In addition, a special DNA polymerase, *Bst*

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polymerase or *Gsp* polymerase, reacts under isothermal conditions at 60°C to 65°C within 1 h [8,16]. The amplified product can be detected by simple inspection of insoluble magnesium pyrophosphate, gel electrophoresis, or measuring the turbidity of the product [7,16,23].

Several groups have used LAMP assays targeting *stx*1, *stx*2 [7,13,23], *eae* [23], *rfbE* [11], and serogroup-specific genes *wzx* and *wzy* [24] to detect STEC, and have shown that the LAMP assay is more sensitive than PCR or qPCR.

Despite the many advantages of the LAMP assay, multiplex LAMP (mLAMP) approaches are limited due to difficulty of differentiating more than two specific genes. Visual observation of LAMP products is indirect and cannot be used to distinguish multiple target genes. Gel electrophoresis after restriction enzyme digestion has been attempted [9,10,19], but is laborious and time-consuming. Moreover, the high sensitivity of the LAMP assay raises the concern of carry-over contamination during post-amplification analysis [12]. Probe-based detection is also not applicable due to the various types of cauliflower-like structures of LAMP products [12].

In this study, we developed a real-time mLAMP assay to detect *stx*1 and *stx*2 simultaneously based on different annealing temperatures determined by annealing curve

Table 1. Bacterial strains used for inclusivity and exclusivity test

Species	Number of strains	Shiga toxin type	mLAMP assay	
			*stx*1	*stx*2
Bacterial strains used for the inclusivity test				
Escherichia coli O157:H7 (ATCC 43890)	1	*stx*1	+	−
Escherichia coli O157:H7 (ATCC 43889, 00-2, 00-10, 00-12, 00-16)	5	*stx*2	−	+
Escherichia coli O157:H7 (ATCC 43894, ATCC 35150, 00-11, 00-13, 00-14, 00-15)	6	*stx*1, 2	+	+
Escherichia coli O91:H21 (ATCC 51434)	1	*stx*2	−	+
Escherichia coli O111 (NCCP 13935, NCCP 14540)	2	*stx*1	+	−
Escherichia coli O84 (wild type)	1	*stx*1	+	−
Escherichia coli O108 (wild type)	1	*stx*1	+	−
Escherichia coli O185 (wild type)	1	*stx*2	−	+
Escherichia coli O119 (wild type)	1	*stx*2	−	+
Bacterial strains used for the exclusivity test				
Escherichia coli (ATCC 43888, ATCC 19853, NCCP 15661, NCCP 15659, NCCP 15663, NCCP 15660)	6	None	−	−
Salmonella enterica serovar (Typhimurium; ATCC 43971, Enteritidis; ATCC 13076, Hadar, Montevideo, Schwarzen-grund, Senftenberg, Lagos)	7	None	−	−
Shigella flexneri (ATCC 29903)	1	None	−	−
Shigella sonnei (ATCC 25931)	1	None	−	−
Campylobacter jejuni (ATCC 33560, ATCC 33291)	2	None	−	−
Campylobacter coli (ATCC 33559)	1	None	−	−
Bacillus cereus (ATCC 11778)	1	None	−	−
Staphylococcus aureus (ATCC 33586)	1	None	−	−
Listeria monocytogenes (ATCC 15313)	1	None	−	−
Listeria innocua (ATCC 33090)	1	None	−	−
Listeria ivanovii (ATCC 19119)	1	None	−	−
Listeria seeligeri (ATCC 35967)	1	None	−	−
Listeria welshimeri (ATCC 35897)	1	None	−	−
Listeria grayi (ATCC 25401)	1	None	−	−
Clostridium perfringens (NCCP 10347)	1	None	−	−
Enterobacter cloacae (wild type)	1	None	−	−
Seratia spp. (wild type)	1	None	−	−
Total	48			

analysis. To evaluate the applicability of the mLAMP assay to cattle farm samples, we assessed assay efficacy and sensitivity using cattle feces and environmental samples.

Materials and Methods

Bacterial strains

Bacterial strains used for inclusivity and exclusivity testing are listed in Table 1. Three STEC strains, ATCC 43984 (harboring both *stx*1 and *stx*2 genes), ATCC 43890 (*stx*1), and ATCC 43889 (*stx*2), were tested to evaluate the mLAMP assay. STEC ATCC 43894 was also tested to optimize the mLAMP assay and determine the detection limit.

Sample collection

A total of 253 cattle farm samples (237 cattle feces, 13 farm soil samples, one raw milk, one water, and one forage sample) were collected from 15 cattle farms located in Gyeonggi-do, South Korea, from August 2012 to May 2013. Cow fecal samples were collected directly by rectal retrieval, and at least one environmental sample from each farm was collected. All samples were transported to the laboratory at 4°C for microbiological tests and analyzed immediately upon arrival.

Isolation of STEC O157 strains

STEC O157 was isolated by conventional selective culture (conventional) and immunomagnetic separation (IMS) methods. For the conventional method, approximately 1 g of each sample was homogenized in 9 mL modified EC broth (mEC; Becton, Dickinson and Company, USA) supplemented with novobiocin (20 mg/L, Oxoid, UK) and incubated overnight at 37°C. Following incubation, one loop of mEC broth culture was streaked onto sorbitol MacConkey agar (Becton, Dickinson and Company, USA) supplemented with potassium tellurite (T-SMAC; 2.5 mg/L, Sigma-Aldrich, Canada) and incubated at 37°C overnight. A maximum of six typical colonies were subcultured onto MacConkey agar (MAC; Becton, Dickinson and Company, USA) and CHROMagar O157 (CHROM; CHROMagar Microbiology, France) and then incubated overnight at 37°C. Typical colonies, pink in MAC and mauve in CHROM, were selected for testing using the *E. coli* O157 latex test kit (Oxoid, UK).

For the IMS method, Dynabeads MAX anti-*E. coli* O157 (Dynal; Invitrogen, USA) was used according to the manufacturer's instructions. The suspension of immunomagnetic beads was spread onto T-SMAC and incubated at 37°C overnight. Up to four typical colonies were selected and identified by applying the same criteria as described in the conventional method. If the four colonies yielded no STEC O157 strain, up to four additional colonies were tested.

Isolation of non-O157 STEC strains

Non-O157 STEC strains were isolated from STEC O157-negative samples by PCR-based culture assay as previously described, with minor modification [3]. Briefly, one loop of enriched mEC broth culture was streaked onto T-SMAC and incubated overnight at 37°C. DNA was extracted from randomly swiped areas of heavy bacterial growth and tested for the presence of shiga toxin genes by multiplex PCR (mPCR) as described in the PCR assay section below. Plates possessing shiga toxin genes were subcultured onto MAC and incubated overnight at 37°C. The presumptive colonies were tested for the presence of shiga toxin genes by mPCR. Colonies possessing shiga toxin genes were assessed by conventional agglutination tests to determine the serotype of the isolates using commercial antiserum (Joongkyeom, Korea).

DNA extraction

The boiling method was used to extract DNA of gram-negative bacteria and enrichment broth culture. Briefly, a loopful of bacterial cells grown on tryptic soy agar (Becton, Dickinson and Company, USA) plates or a pellet of 1 mL enrichment broth culture was suspended in 1 mL normal saline and centrifuged at 6,000 × g for 3 min. The pellet was then resuspended with 200 μL sterile water, boiled for 10 min, and centrifuged at 6,000 × g for 3 min. The supernatant was used as a template for the PCR and LAMP assays. Instagene matrix (Bio-Rad Laboratories, USA) was used to extract DNA from gram-positive bacteria. The genomic DNA of three STEC strains (ATCC 43890, 43889, and 43894) was extracted with DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

Primer design and the LAMP assay

To design primer sets targeting shiga toxins genes, the genomic sequences of *stx* genes from various serotypes of STEC were collected from GenBank. For the *stx*1 gene, the genomic sequences of STEC O26, O79, O103, O111, and O157 (GenBank Accession No. AP010953, FR875154, FE94195, AP010958, AP010960, and M19473) were collected and the genomic sequences of STEC O26, O157, and O178 (GenBank Accession No. FR850034, AB048240, X07865, FR850037) were collected for the *stx*2 gene. The consensus sequence of each gene was generated by multiple alignment and comparison using ClustalW method in MegAlign program (Lasergene 10.1.1, USA). Each primer set was designed from the consensus sequence using LAMP designer (Optigene, UK; Table 2).

The mLAMP assay was evaluated using two primer sets targeting *stx*1 and *stx*2. To differentiate between targets,

Table 2. Primer sets used for the polymerase chain reaction (PCR) and the loop-mediated isothermal amplification (LAMP) assay

Assay	Primer name	Nucleotide sequences (5´ to 3´)	Amplicon size	Reference
*stx*1-PCR	mSTX1_F	CAGTTAATGTGGTGGCGAAGG	348 bp	[6]
	mSTX1_R	CACCAGACAATGTAACCGCTG		
*stx*2-PCR	mSTX2_F	ATCCTATTCCCGGGAGTTTACG	584 bp	[6]
	mSTX2_R	GCGTCATCGTATACACAGGAGC		
+93uidA-PCR	mUIDA_F	GCGAAAACTGTGGAATTGGG	252 bp	[6]
	mUIDA_R	TGATGCTCCATCACTTCCTG		
γ-eaeA -PCR	mEAE_F	ATTACCATCCACACAGACGGT	397 bp	[6]
	mEAE_R	ACAGCGTGGTTGGATCAACCT		
*stx*1-LAMP	Stx1_FIP	GCGATTTATCTGCATCCCCGTATGTCTGGTGACAGTAGCTAT		This study
	Stx1_BIP	GGAACCTCACTGACGCAGTCCTTCAGCTGTCACAGTAACA		
	Stx1_LF	ACTGATCCCTGCAACACG		
	Stx1_LB	TGTGGCAAGAGCGATGTT		
	Stx1_F3	ACAACAGCGGTTACATTGT		
	Stx1_B3	GATCATCCAGTGTTGTACGAA		
*stx*2-LAMP	Stx2_FIP	GGCGTCATCGTATACACAGGAGCGCTTCAGGCAGATACAG		This study
	Stx2_BIP	AGACGTGGACCTCACTCTGAAACTCTGACACCATCCTCTC		
	Stx2_LF	CAGACAGTGCCTGACGAA		
	Stx2_LB	GGCGAATCAGCAATGTGC		
	Stx2_F3	GCATCCAGAGCAGTTCTG		
	Stx2_B3	CAGTATAACGGCCACAGTC		

100 ng/μL of DNA from three STEC strains (ATCC 43984, ATCC 43890, and ATCC 43889) were tested in triplicate. The mLAMP assay was performed on a Genie II instrument (Optigene, UK) in a total reaction volume of 25 μL containing 1× Buffer, 1 M Betaine, 4 mM $MgSO_4$, 0.8 mM each deoxynucleoside triphosphate (dNTP), 0.8 μM each of FIPs and BIPs, 0.4 μM each of LFs and LBs, 0.2 μM each of F3s and B3s, 1× EvaGreen (SolGent, Korea), 8 U *Gsp* polymerase (Optigene, UK), and 4 μL template DNA. Distilled water (Invitrogen, USA) was used as a negative control. The LAMP reactions were carried out at 64°C for 30 to 60 min, with annealing curve analysis was conducted from 98°C to 80°C.

To determine the detection limit of the mLAMP assay, 10-fold serially diluted DNA templates with concentrations ranging from 100 ng/μL to 1 fg/μL were tested in triplicate. A standard curve was generated by plotting T_p values versus concentration of template DNA ranging from 100 ng/μL to 1 fg/μL on a log scale for each set, and the correlation coefficient of determination (R^2) was calculated. The detection limit was evaluated and compared to mPCR described in the PCR assay section below.

PCR assay

Multiplex PCR (mPCR) targeting *stx*1 and *stx*2 was performed to determine the detection limit using serially diluted template DNA of STEC ATCC 43894 as for the mLAMP assay, and results of both assays were compared. mPCR was also used to identify STEC in STEC-positive enrichment broth cultures. For genotypic characterization of STEC isolates from cattle farm samples, *stx*1, *stx*2, *eae*, and *uidA* were tested, as described, on BAM (Table 2) [6].

The reaction mixture included 1× Emerald Master Mix (Takara Bio, Japan), 0.5 μM each primer, and 1 μL template. Distilled water was added to a final volume of 20 μL. The optimal reaction conditions were defined as follows: pre-denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 45 sec, and then final extension at 72°C for 5 min. mPCR was performed on a MyCycler thermal cycler (Bio-Rad Laboratories, USA) and the products were electrophoresed in 1.5% agarose gels.

mLAMP assay application in cattle farm samples

The mLAMP assay was used to verify the types of shiga toxin in all STEC isolates relative to the mPCR assay. To evaluate assay sensitivity, the presence of *stx*1 and/or *stx*2 in enrichment broth cultures was assessed by mLAMP and mPCR.

Results

mLAMP evaluation

To evaluate the utility of the mLAMP assay, three STEC strains with different shiga toxin types were tested. The results are shown with the T_p value, which represents the

time taken to detect the amount of fluorescence required to produce a positive peak, and the T_m value (annealing temperature) determined by annealing curve analysis. For well 1 (ATCC 43894; *stx*1 and *stx*2 genes), the mean T_p value was 9:06 (min:sec) and T_m values (mean) were observed at 84.55 ± 0.32°C and 87.22 ± 0.21°C. For well 2 (ATCC 43890; *stx*1), the mean T_p and T_m values were 8:57 and 85.59 ± 0.02°C, respectively. For well 3 (ATCC 43889; *stx*2), the mean T_p and T_m values were 9:23 and 87.71 ± 0.17°C, respectively. There was no product amplification in the negative control (Fig. 1).

Inclusivity and exclusivity of the mLAMP assay

To evaluate the inclusivity and exclusivity of the mLAMP assay, 48 bacterial strains were tested. mLAMP amplified and distinguished the shiga toxin genes in all 19 STEC strains according to their shiga toxin type. The mean T_p value was 10:12 (8:24 to 15:46) and the mean T_m value was 85.32 ± 0.60°C for *stx*1 and 87.77 ± 0.38°C for *stx*2, demonstrating 100% sensitivity of the mLAMP assay. T_m

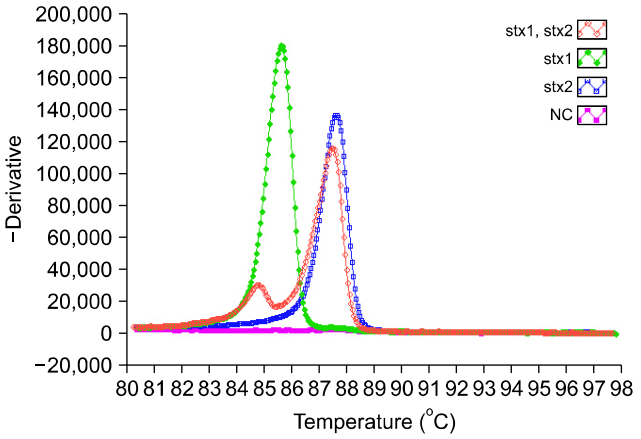

Fig. 1. Discrimination of the *stx*1 and *stx*2 by distinct T_m values generated by annealing curve analysis of the mLAMP assay. The annealing curve shows temperature on the X-axis and fluorescence on the Y-axis. The peaks indicate T_m values generated by annealing curve analysis. Well 1 (red), *stx*1 and *stx*2 (STEC 43894); well 2 (green), *stx*1 (STEC 43890); Well 3 (blue), *stx*2 (STEC 43889); Well 4 (purple), NC (negative control).

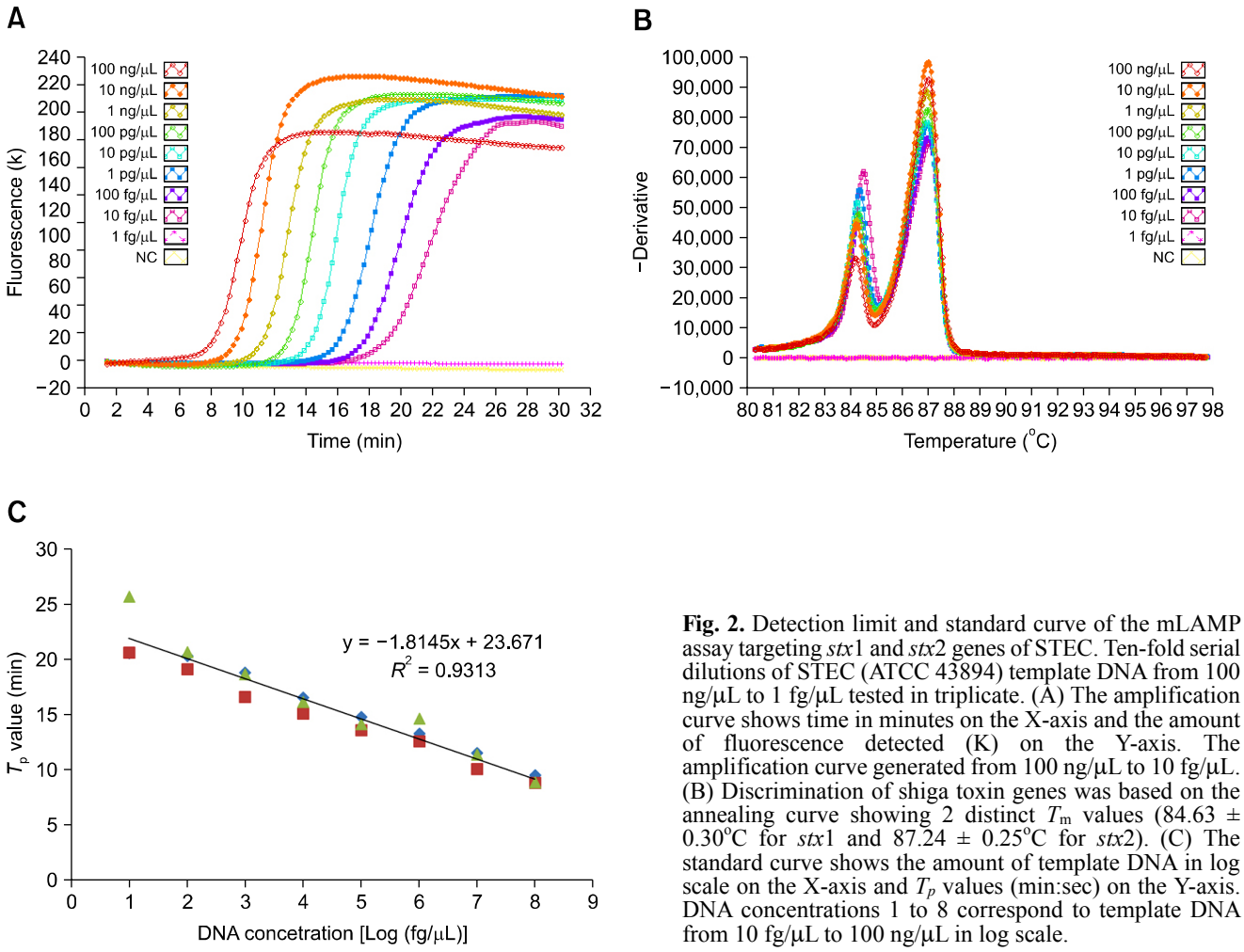

Fig. 2. Detection limit and standard curve of the mLAMP assay targeting *stx*1 and *stx*2 genes of STEC. Ten-fold serial dilutions of STEC (ATCC 43894) template DNA from 100 ng/µL to 1 fg/µL tested in triplicate. (A) The amplification curve shows time in minutes on the X-axis and the amount of fluorescence detected (K) on the Y-axis. The amplification curve generated from 100 ng/µL to 10 fg/µL. (B) Discrimination of shiga toxin genes was based on the annealing curve showing 2 distinct T_m values (84.63 ± 0.30°C for *stx*1 and 87.24 ± 0.25°C for *stx*2). (C) The standard curve shows the amount of template DNA in log scale on the X-axis and T_p values (min:sec) on the Y-axis. DNA concentrations 1 to 8 correspond to template DNA from 10 fg/µL to 100 ng/µL in log scale.

values were not generated for 29 gram-negative and gram-positive strains of non-shiga toxin-producing bacteria, demonstrating 100% specificity.

Detection limit of the mLAMP assay

The detection limit of the mLAMP assay was found to be 10 fg/μL in three independent experiments. At 1 fg/μL, *stx*2 was detected in all three experiments, but *stx*1 was detected in only one of three experiments. Fig. 2 shows a general amplification and annealing curve generated by the LAMP instrument. The DNA templates were amplified with mean T_p values ranging from 9:06 to 22:16 for concentrations ranging from 100 ng/μL to 10 fg/μL. The mean T_m values were 84.63 ± 0.30°C for *stx*1 and 87.24 ± 0.25°C for *stx*2. The quantification equation for the mLAMP assay was determined to be Y = −1.8145X +

Table 3. Characteristics of shiga toxin-producing *Escherichia coli* (STEC) isolated from cattle feces and environmental samples in Gyeonggi-do, Korea

Farm	Isolates	STEC isolated by*			Sample source	Serotype	Shiga toxin type§			LAMP results: *Tm* values (°C)	
		Conventional culture	IMS	PCR-based culture†			*stx1*	*stx2*	*stx1, stx2*	*stx*1	*stx*2
Farm S	0806-3	+	+	UT	Feces	O157	−	−	+	85.8	88.0
	0806-5	+	+	UT	Feces	O157	−	−	+	85.9	88.1
	0806-20	−	+	UT	Soil	O157	−	−	+	85.1	87.7
	1015-9	−	−	+	Feces	O 84	+	−	−	85.7	
	0527-1	−	−	+	Feces	O108	+	−	−	86.1	
	0527-4	−	−	+	Feces	O108	+	−	−	86.1	
	0527-8	−	−	+	Feces	O119	−	+	−		88.1
	0527-15-1‡	−	−	+	Feces	O108	+	−	−	86.1	
	0527-15-2‡	−	−	+	Feces	O119	−	+	−		88.3
	0527-19	−	−	+	Feces	O108	+	−	−	86.2	
	0527-23	−	−	+	Feces	O108	+	−	−	86.1	
	0527-24	−	−	+	Feces	O119	−	+	−		88.1
Farm 4	0823-2	+	+	UT	Feces	O157	−	−	+	85.1	87.5
	0823-4	+	+	UT	Feces	O157	−	−	+	85.1	87.5
	0823-5	−	+	UT	Feces	O157	−	−	+	85.5	87.8
	0823-8	+	+	UT	Feces	O157	−	−	+	86.0	88.0
Farm 7	0827-1	+	+	UT	Feces	O157	−	+	−		87.4
	0827-2	+	+	UT	Feces	O157	−	+	−		87.5
	0827-3	+	+	UT	Feces	O157	−	+	−		87.6
	0827-5	+	+	UT	Feces	O157	−	+	−		87.5
	0827-6	+	+	UT	Feces	O157	−	+	−		87.5
	0827-7	+	+	UT	Feces	O157	−	+	−		87.6
	0827-8	−	+	UT	Feces	O157	−	+	−		87.6
	0827-9	+	+	UT	Feces	O157	−	+	−		88.0
	0827-10	−	+	UT	Soil	O157	−	+	−		87.5
Farm 11	0904-9	−	−	+	Feces	O 84	+	−	−	85.5	
Farm 12	0904-16	−	−	+	Feces	O185	−	+	−		88.1
Farm 13	0905-7	−	+	UT	Feces	O157	−	−	+	86.0	88.2
Total		12/253 (4.7%)	17/253 (6.7%)	11/236 (4.7%)	Soil: 2/28 (7.1%) Feces: 26/28 (92.9%)		7/28 (25.0%)	13/28 (46.4%)	8/28 (28.6%)	85.8 ± 0.4	87.8 ± 0.3

*Twenty-eight STEC strains were isolated from a total of 253 cattle farm samples from 15 enrolled cattle farms by three different methods. For isolation of STEC O157, a conventional culture method based on SMAC agar media was used; while immunomagnetic beads coated with anti-O157 were used for the immunomagnetic separation (IMS) method. †A polymerase chain reaction (PCR)-based culture method was used for isolation of non-O157 STEC strains. From a total of 253 samples, 17 STEC-O157 positive samples were excluded from the PCR assay. ‡Two strains with different serotypic and genotypic characterizations were isolated from the same sample. §Shiga toxin types were determined by mPCR and the mLAMP assay, which were concordant. UT: untested.

23.671, with a correlation coefficient (R^2) of 0.9313. For the mPCR assay, the detection limit was 1 pg/μL in three independent experiments.

mLAMP assay application in cattle farm samples

A total of 253 cattle farm samples (237 cattle feces and 16 farm environmental samples) were tested for STEC. The conventional culture method yielded 12 (4.7%) STEC O157 strains from cattle fecal samples. The IMS method yielded 17 (6.7%) STEC O157 strains from cattle feces and farm soil samples. The IMS method yielded 17 (6.7%) STEC O157 strains from cattle feces and ground samples. Eleven (4.7%) non-O157 STEC strains were isolated from a total of 236 STEC O157-negative cattle fecal samples by the PCR-based culture method. As shown in Table 3, the non-O157 STEC isolates belonged to four different serotypes, O84 (2 isolates), O108 (5 isolates), O185 (1 isolate), and O119 (3 isolates).

Among 28 STEC isolates, seven (25.0%) and 13 (46.4%) harbored *stx*1 and *stx*2, respectively, while eight (28.6%) strains harbored both *stx*1 and *stx*2 (Table 3). All 17 STEC O157 isolates had both *eae* and *uid* genes, while non-O157 STEC did not harbor these genes. The shiga toxin types of 28 STEC isolates were tested by mPCR and mLAMP. The mLAMP determinations were identical to those of the mPCR assay (Table 3). The T_p values of 28 isolates ranged from 8:21 to 12:03 (mean T_p = 10:03), and the mean T_m values were 85.76 ± 0.42°C for *stx*1 and 87.73 ± 0.29°C for *stx*2.

Enrichment broth cultures that were positive for STEC were used to evaluate the sensitivity of the mLAMP assay relative to mPCR. Among the 12 enrichment broth cultures that were positive for STEC O157 by the conventional culture method, shiga toxin genes were detected in three (25.0%) by mPCR and in 12 (100.0%) by mLAMP. For the 17 enrichment broth cultures that were positive for STEC O157 by the IMS method, shiga toxin genes were detected in four (23.5%) and 15 (88.2%) samples by mPCR and mLAMP, respectively. For the ten enrichment broth cultures that were positive for non-O157 STEC, mLAMP detected shiga toxin genes in nine (90.0%) samples, while none were detected by mPCR assay (Table 4).

Discussion

We developed a real-time mLAMP assay for simultaneous detection of the STEC *stx*1 and *stx*2 genes. This assay is highly specific, sensitive, rapid, and quantifiable. When applied to cattle farm samples, the mLAMP assay provided determinations of shiga toxin types within 30 min and provided a high detection rate from enrichment broth culture.

Our mLAMP assay was designed for simultaneous detection of two different types of shiga toxin genes. As shown in Fig. 1, two sets of primers successfully amplified two target genes, which could be distinguished by their different T_m values. The mean T_m values for *stx*1 and *stx*2 in all tested samples were 85.03 ± 0.54°C and 87.47 ± 0.35°C, respectively, and were thus easily distinguished. Similarly, in a recent study of sex determination in the plant *Carica papaya*, two target genes were also differentiated by annealing curve analysis [8].

The limit of detection in our mLAMP assay was 10 fg/μL, which was 100 times more sensitive than mPCR. Kouguchi [12] reported a detection limit of 100 CFU/mL in STEC pure culture. In our study, the detection limit was measured at the DNA concentration level rather than the CFU level, so our results cannot be compared directly. However, in our preliminary tests, 6.2 ± 1.74 × 10^8 CFU/mL corresponded to 140.5 ± 54.7 ng/μL (data not shown). Thus, 10 fg/μL corresponds to 10 CFU/mL, making our assay 10 times more sensitive than the one reported by Kouguchi [12].

In this study, the mean T_p values ranged from 9:06 to 22:16 for the concentration range of 100 ng/μL to 10 fg/μL, which was much faster than previously reported for a LAMP assay conducted using a turbidimeter, in which 21.4 ~ 45.2 min was required to detect the *stx*2 gene from

Table 4. Sensitivity of mLAMP and mPCR assays in STEC-positive enrichment broth cultures

STEC isolated by	Number of positive STEC from enrichment broth*	Number of positive tests† by	
		mPCR (%)	mLAMP (%)
Conventional culture method	12	3 (25.0)	12 (100.0)
IMS method	17	4 (23.5)	15 (88.2)
PCR-based culture method	10	0 (0.0)	9 (90.0)

*Positive STEC from enrichment broth (mEC broth) was categorized according to the method used for STEC isolation. A conventional culture method and immunomagnetic separation method (IMS) were used for isolation of O157 STEC strains, and PCR-based culture method was used for isolation of non-O157 STEC strains. †In enrichment broth culture, detection of *stx*1 and/or *stx*2 was considered positive in both mLAMP and mPCR assay.

a 10 ~ 10^5 CFU/reaction [23]. These results are consistent with those of other studies designed to detect *Vibrio parahaemolyticus* or ammonia-oxidizing bacteria in which the detection time for the fluorescent-based LAMP assay was faster than that of the turbidity-based LAMP assay [1,2]. In previous mLAMP studies based on the post-amplification analysis of two target genes, detection limits were 10 times more sensitive than those of PCR for detecting *Salmonella* or *Shigella* spp. and 10^3 to 10^5 times more sensitive for differentiating *Babesia* spp. [10,19].

The quantitative capability of the LAMP assay was reported previously [2,23,24]. In the present study, a standard curve was generated based on triplicate T_p values (detection time) versus the concentration of template DNA. The standard curve had a linear relationship with R^2 values of 0.9313 within the range of 100 ng/μL and 10 fg/μL, indicating that the mLAMP assay can estimate unknown STEC contamination levels in samples. Indeed, our result is unique in terms of its wide coverage of concentrations. In another study, the R^2 values fell within a relatively narrow range of concentrations (10^2 to 10^5 CFU/mL), possibly due to the delayed detection time observed at lower concentrations [2,23].

Until now, only a few LAMP methods for detecting STEC have been used to analyze samples from beef, beef trimmings, lettuce, spinach, and human stool [23-25]. However, to the best of our knowledge, no LAMP assay has been used to screen for STEC on cattle farms. As a primary source of human STEC infection, cattle and their environment are important to public health and need to be monitored. To the best of our knowledge, this is the first report of use of a real-time mLAMP assay for detection of STEC targeting shiga toxin genes that was applied to cattle farm samples.

To evaluate the efficacy of the mLAMP assay, all STEC strains were examined for the presence of *stx*1 and/or *stx*2. We noted that the shiga toxin types of each isolate were identical when tested by mPCR and mLAMP, indicating high accuracy of the mLAMP assay. Moreover, the mLAMP assay detected shiga toxin genes in 11 non-O157 STEC strains, indicating that the mLAMP assay can be used to detect various serotypes of STEC. In addition, the mLAMP process was completed within 30 min (mean T_p value = 10:03), while a PCR assay generally takes 3 h, including the post-amplification processes. Overall, these findings indicate that the mLAMP assay could be useful for rapid and accurate characterization of various STEC serotypes.

The sensitivity of the mLAMP assay was evaluated in enrichment broth cultures that were found to be positive for STEC by conventional culture, IMS, and PCR-based culture methods. As shown in Table 4, the mLAMP assay detected shiga toxin genes in all 12 broth cultures that were positive for STEC O157 by the conventional culture method, which is regarded as the gold standard [5,6]. In addition, the mLAMP assay was able to detect shiga toxin genes in nine (90.0%) enrichment broth cultures that were positive for non-O157 STEC strains. These results indicate that the detection rate of the mLAMP assay is comparable to the culture-based detection method for STEC strains. Considering that the culture method is labor intensive and takes at least three days to complete, the application of the mLAMP assay to screening enrichment broth cultures would provide a detection rate similar to culture-based methods, but with less time and labor.

The STEC O157 strains were isolated by conventional culture and IMS methods. While 12 strains were isolated by the conventional method, five more strains were isolated by the IMS method. Considering IMS is known to be the most sensitive culture method [4], the contamination of these five samples with STEC O157 seems to be at a low level that may not be detected by conventional culture. The mLAMP assay detected shiga toxin genes in enrichment cultures of three of these five samples, demonstrating high sensitivity. Indeed, the assay could detect even levels of contamination below those that could be detected using the conventional culture method.

Our mLAMP assay may not detect shiga toxin genes in the presence of low amounts of STEC in enrichment broth cultures (under our detection limit, 10 fg/μL), but still showed greater sensitivity than the conventional culture method. In addition, the mLAMP assay was much more sensitive than the mPCR assay in enrichment broth culture, which was expected considering the high sensitivity of the LAMP assay over the PCR assay in pure culture. Conversely, it is possible that the mLAMP assay may be less sensitive than the mPCR assay to inhibitors in the sample matrix. Other studies have compared methods such as PCR, qPCR, or LAMP for detection of target genes from food or clinical samples and shown that the LAMP assay is less sensitive to inhibitors than the PCR or qPCR assay [19,23,25].

In conclusion, the mLAMP assay simultaneously amplifies the *stx*1 and *stx*2 genes, enabling detection of STEC, and enables identification of shiga toxin types more rapidly and accurately than current methods. Moreover, the high detection rate of specific genes from enrichment broth cultures indicates the potential utility of this assay as a primary screening tool for detecting STEC in cattle farm samples.

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Conflict of interest

There is no conflict of interest.

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