RAPID AND SIMPLE DETECTION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) O157:H7 AND STEC NON-O157:H7 SEROTYPES USING COLORIMETRIC LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY

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Abstract. Shiga toxin-producing *Escherichia coli* (STEC) detection in food is crucial for foodborne pathogen assessment, control and prevention. A colorimetric malachite green loop-mediated isothermal amplification (LAMP-MG) assay was applied to detect STEC O157:H7 and non-O157:H7. LAMP assay primers targeted *E. coli* O157 *wzy*, O157:H7 *yliE* and virulence genes *eae*, *stx1* and *stx2*, and assay results were visualized using the naked eye. Detection limit of LAMP-MG assay was 10-100 copies/reaction, with sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 95, 99, 80, 100, and 99%, respectively compared to reference standard. STEC contamination in vegetable and fruit samples (n = 300) from fresh food markets in Bangkok, Thailand is significantly higher than that of supermarket samples (24.7 *vs* 11.3%; p-value = 0.0001; odds ratio = 2.18, 95% confidence interval: 1.28-3.70), with *E. coli* O157, O157:H7 and non-O157:H7 harboring *stx2* found more often in fresh food market samples. In conclusion, LAMP-MG assay provides a simple, sensitive and rapid technique for detection of Shiga toxin-producing *E. coli* O157:H7 and non-O157:H7 contamination in fresh fruits and vegetables.

Keywords: Bangkok, fruit and vegetable, LAMP, malachite green, Shiga toxin-producing *Escherichia coli*

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INTRODUCTION

Among diarrheagenic *Escherichia coli* strains, Shiga toxin-producing *E. coli* (STEC) is the most frequently associated with foodborne outbreaks (Kim *et al*, 2020). STEC is named according to the presence of chromosomal *stx1* (encoding Shiga toxin 1) or *stx2* (encoding Shiga toxin 2) derived from a lambdoid bacteriophage (Croxen *et al*, 2013). STEC comprises a variety of pathotypes causing from mild to severe diseases (Kim *et al*, 2020), with enterohemorrhagic *E. coli* (EHEC) being the major pathogenic STEC subgroup and responsible for hemorrhagic colitis (HC), a watery-bloody diarrhea, and hemolytic-uremic syndrome (HUS), an acute renal failure in children (Joseph *et al*, 2020).

The most notable pathogenic EHEC is serotype O157:H7 discovered in 1982 (Karmali *et al*, 1983) and has since remained the most common serotype causing diseases in humans (Scallan *et al*, 2011; Sharp *et al*, 2020). However, STEC non-O157:H7 serotype has been increasingly related with STEC outbreaks and sporadic cases in humans (Johnson *et al*, 2006). *E. coli* O104:H4 was responsible for a large outbreak in Germany (WHO, 2011) and O26:H11 in Italy (Germinario *et al*, 2016). In USA, STEC O26, O103, O104, O111, O121, and O145 are among the top six serotypes involved in causing HC and HUS (latter with the exception of O145) (Brooks *et al*, 2005; Hodge, 2012). Given the public health importance of STEC, a simple, rapid and effective method is crucial to detect all STEC serotypes in clinical specimens and food samples for planning appropriate therapy, control and prevention measures.

Every STEC serotype can produce Stx1 or Stx2 (Kim *et al*, 2020) and several STEC serotypes carry enterocyte effacement (LEE) locus responsible for attaching and effacing (A/E) lesions on enterocytes (Croxen *et al*, 2013). LEE locus contains *eae*, responsible for production of intimin, the most important adherence factor involved in enterocyte attachment (Nataro and Kaper, 1998). Epidemiological data reveal STEC strains carrying *stx2* and *eae* are significantly associated with severe human diseases including HC and HUS (Kim *et al*, 2020).

Three main STEC detection methods are in current use, namely, conventional culture method (Jenkins *et al*, 2020), enzyme immunoassay (EIA) (Costa *et al*, 2019), and PCR-based methods (Parsons *et al*, 2016). However, all three methods are time-consuming and PCR-based techniques requires relatively expensive instruments. Since its invention in 2000, the loop-mediated isothermal amplification (LAMP) method has drawn considerable attention as a simple and convenient means of nucleic acid amplification (Notomi *et al*, 2000). LAMP requires incubation at a single temperature (ranging from 60 to 65°C), uses four to six specific primers and *Bst* DNA polymerase with strong strand displacement activity. Being isothermal, LAMP obviates the need for a thermal cycling instrument and has the advantage of allowing direct visual inspection of the amplification products

through turbidity of generated magnesium pyrophosphate (Mori *et al*, 2001). LAMP technique has been employed as a simple and effective diagnostic method of microorganisms in clinical specimens and food samples (Notomi *et al*, 2000; Mori and Notomi, 2020).

However, observation of turbidity with the naked eye can be equivocal, particularly with weakly positive samples, but sensitivity can be improved using a turbidimeter (Wong *et al*, 2018). Other post-amplification detection steps have been introduced, such as gel-electrophoresis and lateral flow dipstick (LFD), but both techniques require opening of reaction tubes thereby risking possible cross-contamination (Wong *et al*, 2018). Colorimetric methods employing DNA-intercalating dyes have been introduced to improve sensitivity of LAMP assay based on turbidity output (Scott *et al*, 2020), among which malachite green (MG) dye has the advantage of being observable with the naked eye under visible light. This so-called LAMP-MG method has been successfully employed to improve standard LAMP detection of *Leishmania* infection (Sriworarat *et al*, 2015) and *Plasmodium* spp (Lucchi *et al*, 2016; Barazorda *et al*, 2020).

Here, LAMP-MG assay was developed to detect STEC O157:H7 and non-O157:H7 serotypes in raw fruit and vegetable samples from fresh markets and supermarkets located in Bangkok, Thailand. The findings should provide information on the prevalence of STEC O157:H7 and non-O157:H7 serotypes in these settings to enable appropriate and timely programs to be launched to control and eliminate health risks arising from these highly pathogenic microorganisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions

E. coli (DMST 4212) and *E. coli* O157:H7 (DMST 12743) reference strains from the Department of Medical science, Ministry of Public Health, Nonthaburi Province, Thailand, were used for LAMP-MG validation and development. Reference strains were grown on tryptic soy agar (TSA) plates (Merck KGaA, Darmstadt, Germany) at 37°C.

Sample collection locations and size number

Sample size calculation was based on a 2.7% prevalence STEC contamination in food samples in Thailand (Chomvarin *et al*, 2005). The formula, $n = Z^2P(1\text{-}P)/d^2$ (Daniel, 1998) at 95% confidence level and 5% confidence interval (CI), was employed to calculate a requirement of 300 fruit and vegetable samples, which were obtained from fresh food markets and supermarkets in Bangkok, Thailand from May to December 2017. Samples constituted products that are consumed uncooked including parsley, morning glory, spring onion, cilantro, cabbage, celery, lettuce, carrot, Chinese chive, Chinese kale, young long bean, sweet basil, bok choy, tomato, cucumber, eggplant, apple, cantaloupe, grape, strawberry, and watermelon. After purchasing, each sample was separately placed in a sterilized plastic bag and kept on an ice foam box during transportation

and were immediately processed in laboratory according to the procedure. The time before the sample being processed was no longer than 3 hours.

Bacteria isolation and enrichment procedures

Reference bacteria strains were cultured as described above for 16 hours. For fruit and vegetable samples, 2.5 g of each sample was soaked in 22.5 ml of modified tryptic soy broth (mTSB) supplemented with 8 mg/l novobiocin (Sigma-Aldrich, St Louis, MO), a selective enrichment medium for EHEC (Feng *et al*, 2020) for 30 minutes at ambient temperature, then removed from culture broth, which was incubated for a further 16 hours at 37°C. For confirmation of LAMP-MG results, one loopful of an overnight enrichment culture was streaked onto a chromogenic CHROMagar™ STEC medium (CHROMagar, Paris, France) and was incubated for 24 hours at 37°C. Colored suspected STEC colonies were confirmed using LAMP-MG and PCR methods.

Standard LAMP assay protocol

Genomic DNA (gDNA) was extracted from 1 ml aliquot of enrichment culture using a boiling method (Feng *et al*, 2020). LAMP primers targeting *wzy* (GenBank accession no. AF061251) were used for STEC O157 detection (Wang *et al*, 2012b), LAMP primers targeting *yliE* (GenBank accession no. CP002185.1) for STEC O157:H7 detection (Lee *et al*, 2016) and LAMP primers targeting *eae*, *stx1* and *stx2* (GenBank accession nos. Z11541, M19473 and X07865, respectively) for STEC virulence genes detection (Wang *et al*, 2012a) (Table 1). For LAMP reaction, 2 types of LAMP buffers, namely, Eiken Loopamp DNA Amplification Kit (Eiken Chemical Co Ltd, Tokyo, Japan) and an in-house buffer, were used for the buffer efficacy comparison. For the in-house LAMP buffer, the 25 µl of LAMP reaction mixture comprised 1X ThermoPol buffer [20 mM Tris-HCl, 10 mM $(NH_4)2SO_4$, 10 mM KCl_2, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 (New England BioLabs, Ipswich, MA)], 0.4 M betaine (USB Corporation, Cleveland, OH), 8 mM $MgSO_4$ (Sigma-Aldrich, St Louis, MO), 1.4 mM dNTP mix (Promega, Madison, WI) and 8 units of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA). Primer concentrations of *wzy*, *stx1* and *stx2* comprised 0.1 µM each of F3 and B3, 1.8 µM each of FIP and BIP, and 1.0 µM each of LF and LB. Primer concentrations for *eae* consisted of 0.3 µM each of F3 and B3, 2.0 µM each of FIP and BIP, and 1.2 µM each of LF and LB. For *yliE* detection, primer concentrations were 0.3 µM each of F3 and B3, 1.6 µM each of FIP and BIP, and 0.8 µM each of LF and LB. LAMP reaction was performed at 60°C for 90 minutes followed by DNA polymerase inactivation at 80°C for 5 minutes. Turbidity was measured at 650 nm in a Loopamp Realtime Turbidimeter LA-320C (Eiken Chemical Co Ltd, Tokyo, Japan), with distilled water as negative control. One µl aliquot of 10^4 copy/µl STEC O157:H7 gDNA was used to compare efficacy of the two LAMP buffers, and one µl aliquot of a 10-fold serial dilution (1-10^4 copy/µl) of STEC O157:H7 gDNA was employed to determine sensitivity (using in-house buffer).

Table 1

Primers used for LAMP assay

Target genes/Primes		Primer sequences (5′ → 3′)	References
wzy	F3	TCCCTTTAGGGATATATATACCTT	Wang *et al*, 2012b
	B3	ATAACTGATATTTTCATTTCGTGAT	
	FIP	TTCCCAGCCACTAAGTATTGCAATA-TGAAAAAAACCCATAGCTCGA	
	BIP	TGCATCGGCCTTCTTTTTTGG-AACGTATCATGCAATAAGATCA	
	LF	ATAATGATATATGAATAGAATGCGC	
	LB	TCCTTTTCTCTCCGTATTGAT	
yliE	F3	ATTGAGTCCAACAATAATCAGT	Lee *et al*, 2016
	B3	CATTTTCCCGACACAGAAG	
	FIP	TGACGATTTTATTGCGTTCATCAACGCTGTGGGGATCATCAAAC	
	BIP	ATTGTGTCAGCGAATTGCCGTAAATAGAGATCGGCATTGAATG	
	LF	ACCAAACACCGCTTCCA	
	LB	TGCGCAATGCTGCGATA	
stx1	F3	TGATTTTTCACATGTTACCTTTC	Wang *et al*, 2012a
	B3	TAACATCGCTCTTGCCAC	
	FIP	CCTGCAACACGCTGTAACGT-CAGGTACAACAGCGGTTA	
	BIP	AGTCGTACGGGGATGCAGAT-AGTGAGGTTCCACTATGC	
	LF	GTATAGCTACTGTCACCAGACAATG	
	LB	AAATCGCCATTCGTTGACTACTTCT	

Table 1 (cont)

Target genes/Primes		Primer sequences (5′ → 3′)	References
stx2	F3	CGCTTCAGGCAGATACAGAG	Wang *et al*, 2012a
	B3	CCCCCTGATGATGGCAATT	
	FIP	TTCGCCCCCAGTTCAGAGTGA-GTCAGGCACTGTCTGAAACT	
	BIP	TGCTTCCGGAGTATCGGGGAG-CAGTCCCCAGTATCGCTGA	
	LF	GCGTCATCGTATACACAGGAGC	
	LB	GATGGTGTCAGAGTGGGGAGAA	
eae	F3	TGACTAAAATGTCCCCGG	Wang *et al*, 2012a
	B3	CGTTCCATAATGTTGTAACCAG	
	FIP	GAAGCTGGCTACCGAGACTC-CCAAAAGCAACATGACCGA	
	BIP	GCGATCTCTGAACGGCGATT-CCTGCAACTGTGACGAAG	
	LF	GCCGCATAATTTAATGCCTTGTCA	
	LB	ACGCGAAAGATACCGCTCT	

Note: There are 6 distinct regions (F1, F2, F3 and B1, B2, B3) needed for the design of LAMP primers. The FIP (Forward Inner Primer) consists of the F2 region at the 3′ end and the F1c region at the 5′ end. The BIP (Backward Inner Primer) consists of the B2 region at the 3′ end and the B1c region at the 5′ end. The underlined sequences correspond to the F2 or B2 region of the FIP or BIP primer, respectively.

Optimization and evaluation of LAMP-MG assay protocols

MG (Sigma-Aldrich, St Louis, MO) at 0.004, 0.006 and 0.008% (w/v) were used for optimization determination of LAMP-MG assay. Efficacy of LAMP-MG assay was determined using in-house buffer with 10 and 100 copies/µl STEC O157:H7 gDNA and *wzy* primers. LAMP reaction mixture was as described above but included MG solution and carried out from 60 to 65°C (at 1°C interval) for 90 minutes followed by reaction termination as described above. Color change of LAMP-MG reaction was noted visually of three independent volunteers blinded to reaction mixture composition. LAMP-MG amplicons were also analyzed by 3% agarose gel-electrophoresis and staining with SYBR™ Safe DNA Gel dye (Invitrogen, Waltham, MA).

LAMP-MG assay conducted on spiked vegetable samples

Three vegetable samples from a supermarket were washed with 70% ethanol to remove surface bacteria, and 2.5 g of each vegetable sample was spiked with 1 ml aliquot of a 10-fold serial dilution of overnight culture of STEC O157:H7 to generate inoculating levels of 1-10^4 CFU/2.5 g sample. Negative control was unspiked sample. Each sample was added to 22.5 ml of mTSB supplemented with 8 mg/l novobiocin (Sigma-Aldrich, St Louis, MO), mixed and incubated at 37°C for 12-14 hours prior to gDNA extraction and LAMP-MG assay as described above.

PCR assay and amplicons analysis methods

PCR assays for identification of STEC O157, STEC O157:H7, *eae*, *stx1*, and *stx2* were carried out using primers targeting *rfbE* (responsible for O157 antigen biosynthesis) (GenBank accession no. S83460) (Paddock *et al*, 2012), γ-*eaeA* (encoding STEC O157:H7 intimin) (No GenBank accession no. indicated in the original paper) (Fratamico and Strobaugh, 1998), *eae* (GenBank accession no. M 58145) (Monday *et al*, 2007), *stx1* and *stx2* (No GenBank accession no. indicated in the original paper) (Cebula *et al*, 1995). PCR primers, reaction mixtures and conditions were as previously described (Cebula *et al*, 1995; Fratamico and Strobaugh, 1998; Monday *et al*, 2007; Paddock *et al*., 2012). Amplicon sizes of *eae, rfbE, stx1, stx2,* and γ-*eaeA* was 482, 296, 348, 584, and 397 base pairs, respectively.

In order to confirm any discordant results between LAMP-MG and PCR assays, *E. coli* O157, *eae* and *stx2* amplicon were digested with *Dde*I, *Alu*I and *BsrD*I (Thermo Fisher Scientific, Waltham, MA) respectively and analyzed by 3% agarose gel-electrophoresis, staining with SYBR™ Safe DNA Gel dye (Invitrogen, Waltham, MA) and visualizing under UV illumination. In some cases, a pGEM®-T Easy Vector (Promega, Madison, WI) was used to clone the amplicon for subsequent sequencing of the recombinant plasmid (Macrogen Inc, Seoul, South Korea).

Statistical analysis

LAMP-MG reaction was categorized as true positive, true negative, false

positive or false negative using positivity in two or more assays, namely, PCR, chromogenic culture assay, restriction enzyme digestion and DNA sequencing. LAMP-MG sensitivity [(number of true positives)/(number of true positives + number of false negatives) × 100], specificity [(number of true negatives)/(number of true negatives + number of false positives) × 100], positive predictive values (PPV) [(number of true positives/(number of true positives + number of false positives) × 100] and negative predictive values (NPV) [(number of true negatives/(number of true negatives + number of false negatives) × 100], and accuracy [(number of true positives + number of true negatives)/(total number of samples) × 100] were calculated based on 1,500 reactions (5 reactions/sample; 300 samples). Student's t-test was applied to compare difference of STEC contamination between fresh food market and supermarket samples and odds ratio with 95% confidence interval (CI) to analyze likelihood of STEC contamination in fruit and vegetables obtained from fresh food markets and that from supermarkets. A p-value <0.05 is regarded as significant. Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM SPSS Inc, Chicago, IL) was utilized for statistical calculations.

RESULTS

LAMP buffer efficacy

In order to reduce LAMP reaction cost, efficacy of in-house buffer was compared with that of Eiken LAMP kit (Eiken Chemical Co Ltd, Tokyo, Japan). Turbidity was measured kinetically in a Loopamp real-time turbidimeter (Eiken Chemical Co Ltd, Tokyo, Japan) and the data was shown as the signal represented the amplified product of each time point at real-time which the turbidity peaked and then dropped with time when the LAMP substrate was run out. Applying primers targeting *stx1*, *stx2* and *wzy*, maximum levels of turbidity using in-house buffer for all targets occurred 1-2 minutes slower and at lower levels than those with Eiken-LAMP kit buffer (Figs 1A and B). However, targeting *eae* and *yilE*, efficacy of the two buffer systems were equivalent (Figs 1B and C). Thus, in experiments to determine LAMP sensitivity, in-house buffer was utilized with primers targeting all five STEC genes and 1-10^4 copy/µl STEC O157:H7 gDNA. Limit of detection targeting STEC *stx1*, *stx2*, and *yliE* was 10 copies/reaction (Figs 1E, F and H), while that targeting *wzy* and *eae* was ten folds higher (Figs D and G).

Optimization of LAMP-MG assay

In order to improve efficacy of LAMP assay by visual detection, MG was included as a color indicator. Targeting STEC *wzy*, optimal temperature, measured between 60 and 65°C, was the latter (data not shown), and optimal MG concentration, tested between 0.004 and 0.008%, was 0.006%, consistent with 3% agarose gel-electrophoresis results (Figs 2A and B).

LAMP-MG sensitivity

LAMP-MG assay sensitivity conducted at 65°C with in-house buffer,

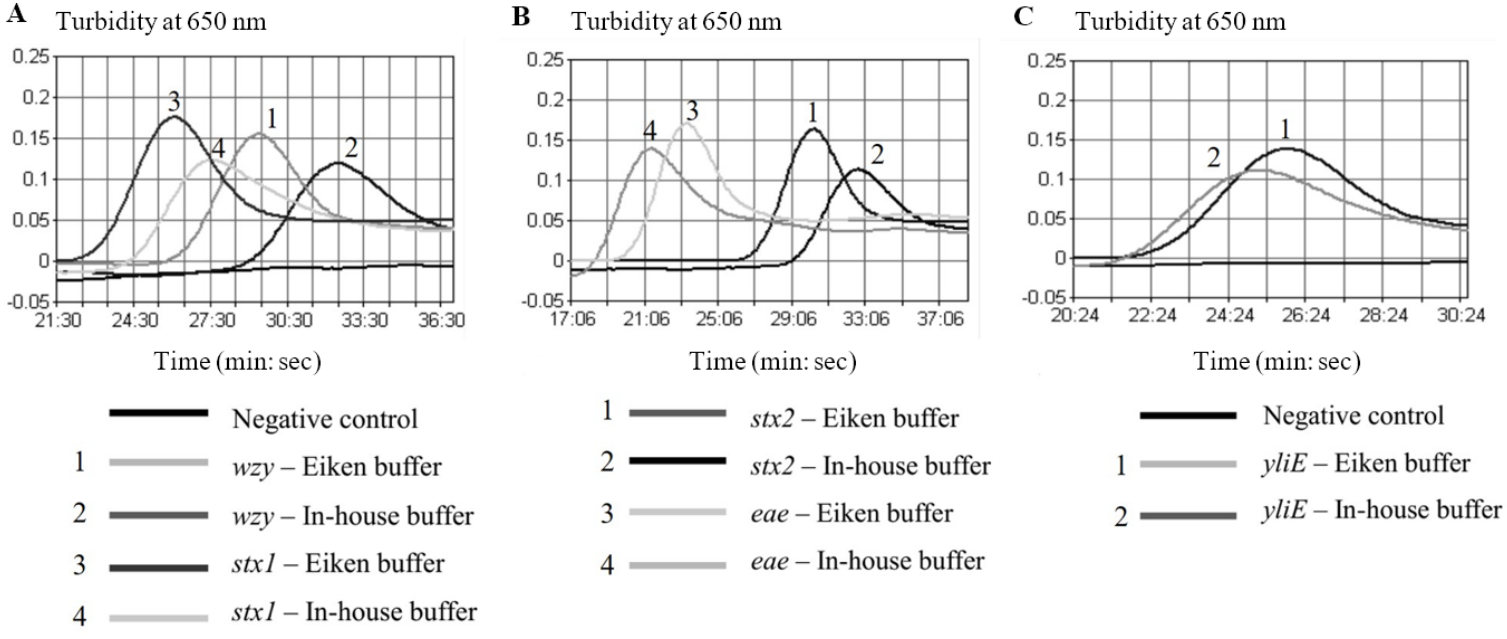

Fig 1 - Kinetics of LAMP turbidity in detection of STEC

LAMP assays were carried out at 60°C for 90 minutes, followed by DNA polymerase inactivation at 80°C for 5 minutes. Turbidity was measured at 650 nm in a Loopamp Realtime Turbidimeter LA-320C (Eiken Chemical Co Ltd, Tokyo, Japan). (A - C) Comparisons of Eiken-LAMP kit buffer (Eiken Chemical Co Ltd, Tokyo, Japan) and an in-house buffer using 10^4 copies/µl Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 gDNA and (A) primers specific for *wzy* and *stx1*, (B) primers specific for *stx2* and *eae*, (C) primers specific for *yliE*. Negative control contained distilled water. LAMP reaction was initiated at different times.

LAMP: loop-mediated isothermal amplification; min: minute; µl: microliter; nm: nanometer; sec: second; STEC: Shiga toxin-producing *Escherichia coli*

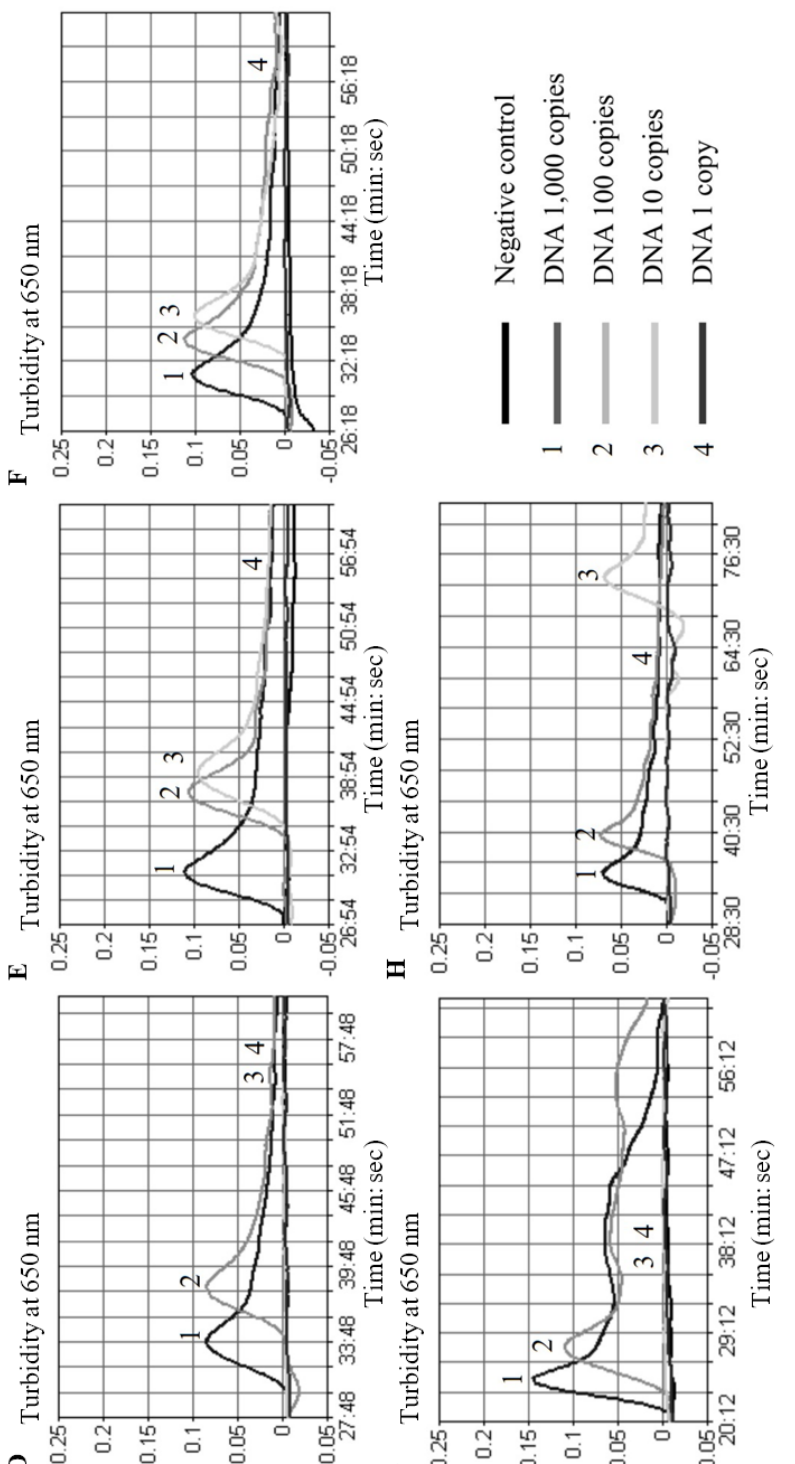

Fig 1 - (cont)

LAMP assays were carried out at 60°C for 90 minutes, followed by DNA polymerase inactivation at 80°C for 5 minutes. Turbidity was measured at 650 nm in a Loopamp Realtime Turbidimeter LA-320C (Eiken Chemical Co Ltd, Tokyo, Japan). (D - H) Comparisons of different amounts of STEC O157:H7 gDNA template using in-house buffer and primers specific for (D) *wzy*, (E) *stx1*, (F) *stx2*, (G) *eae*, (H) *yliE*. Negative control contained distilled water. LAMP reaction was initiated at different times.

LAMP: loop-mediated isothermal amplification; min: minute; µl: microliter; nm: nanometer; sec: second; STEC: Shiga toxin-producing *Escherichia coli*

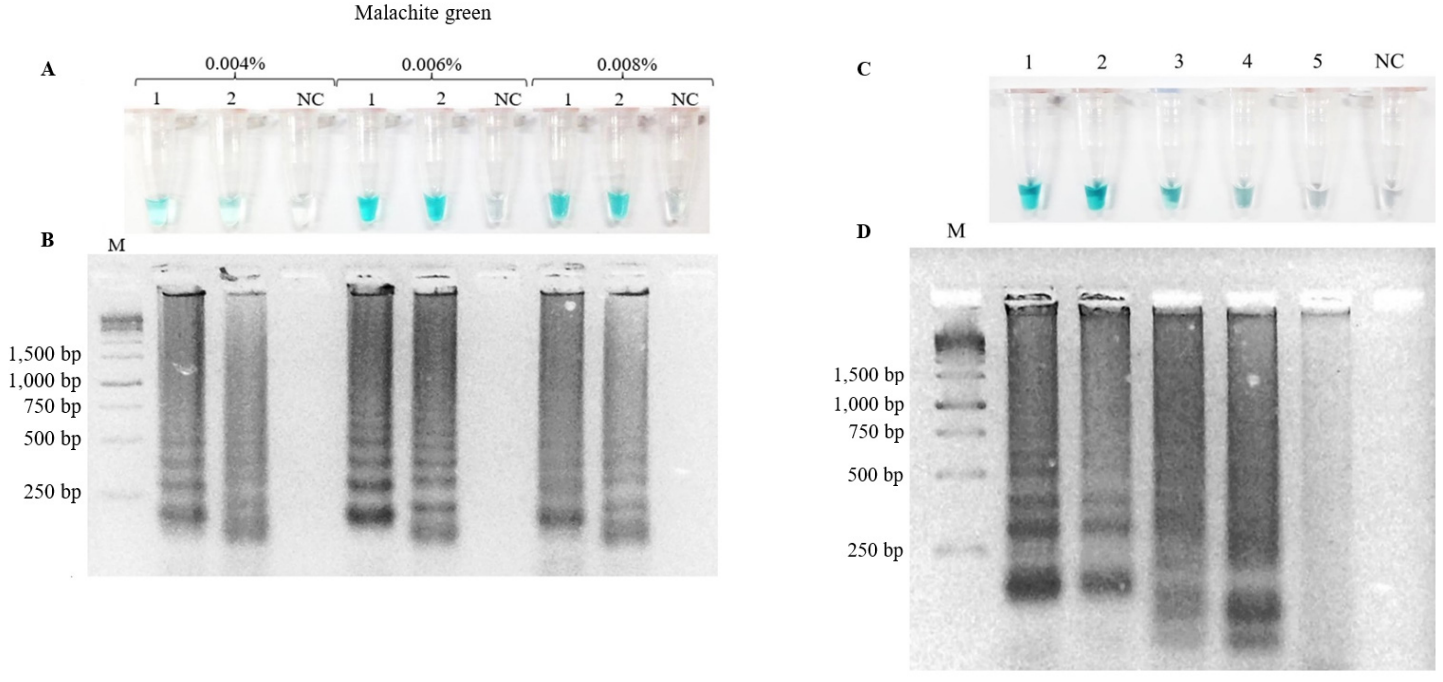

Fig 2 - LAMP-MG detection of Shiga toxin-producing *Escherichia coli* (STEC)

LAMP reaction was conducted as described in legend to Fig 1 with the addition of malachite green (MG) dye (Sigma-Aldrich, St Louis, MO) and results observed visually (A, C, E) and by inspection under UV illumination of 3% agarose gel-electrophoresis of amplicons following staining with SYBR™ Safe DNA Gel dye (Invitrogen, Waltham, MA) (B, D, F).

(A, B): Results using different MG concentrations and (1) 100, (2) 10, (NC) 0 copy/µl STEC O157:H7 gDNA; (C, D): Results using 0.006% MG and (1) 10^4, (2) 10^3, (3) 10^2, (4) 10, (5) 1, (NC) 0 copy/µl STEC O157:H7 gDNA; A 5 µl aliquot of gDNA extracted from an overnight culture was used as template together with 0.006% MG.

µl: microliter; NC: negative control (no DNA template)

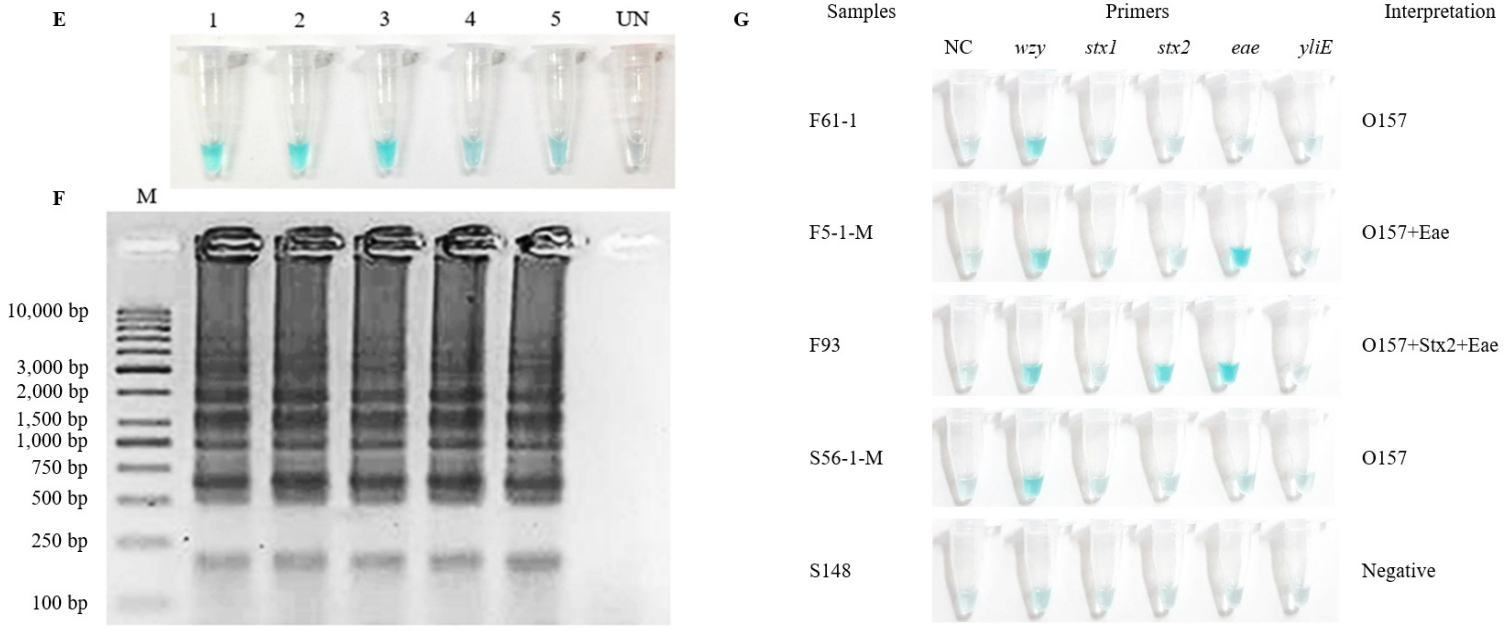

Fig 2 - (cont)

LAMP reaction was conducted as described in legend to Fig 1 with the addition of malachite green (MG) dye (Sigma-Aldrich, St Louis, MO) and results observed visually (A, C, E) and by inspection under UV illumination of 3% agarose gel-electrophoresis of amplicons following staining with SYBR™ Safe DNA Gel dye (Invitrogen, Waltham, MA) (B, D, F).

(E, F): Results of vegetable samples spiked with (1) 10^4, (2) 10^3, (3) 10^2, (4) 10, (5) 1, (UN) 0 CFU/ml STEC O157:H7 [Lane M in Fig 2F: DNA size markers]; (G): Detection of STEC O157 and virulent gene(s) isolated from raw fruit and vegetable samples using primers specific for *eae, stx1, stx2, wzy*, and *yliE*. A 5 µl aliquot of gDNA extracted from an overnight culture was used as template together with 0.006% MG.

µl: microliter; NC: negative control (no DNA template)

0.006% MG and STEC *wzy* primers over a range of 1-10^4 copy/µl STEC O157:H7 gDNA indicated a limit of detection of 10 copies/reaction, both by visual inspection and by 3% agarose gel-electrophoresis results (Figs 2C and D). Employing STEC-spiked (1-10^4 CFU/ml) 2.5 g of vegetable samples, limit of LAMP-MG assay detection tested under the same conditions was 1 CFU/2.5 g of spiked vegetable (Figs 2E and F).

Detection of STEC O157:H7 and STEC non-O157:H7 in raw fruit and vegetable samples

LAMP-MG assay efficacy was evaluated using 300 raw fruit and vegetable samples collected from fresh food markets and supermarkets (Fig 2G). PCR and chromogenic culture assay (CHROMagar STEC plate: CHROMagar, Paris, France) were used to confirm LAMP-MG results. Any discordant results among PCR and chromogenic culture assays were further evaluated by restriction enzyme digestion and DNA sequencing. True positivity is defined as positivity in two or more assays (Table 2). LAMP-MG assay identified 54/300 samples with positive STEC contamination. From 1,500 LAMP-MG reactions (5 reactions/sample of 300 samples) sensitivity, specificity, PNV, NPV, and accuracy was determined to be 95 (70/74), 99 (1,409/1,426), 80 (70/87),

Table 2

Comparison of LAMP-MG and reference assays for detection of *Escherichia coli* O157, *E. coli* O157:H7 and STEC virulence genes in 300 raw fruit and vegetable samples from fresh food markets and supermarkets, Bangkok, Thailand (May - December 2017)

Target	LAMP-MG assay[a]		Reference assay[b]	
	Number of positive results	Number of negative results	Number of positive results	Number of negative results
E. coli O157	26	274	24	276
E. coli O157:H7	16	284	9	291
stx1	17	283	13	287
stx2	12	288	16	284
eae	16	284	12	288
Total	87	1,413	74	1,426

[a]Five assays/sample; [b]Positivity in two or more assays (PCR, chromogenic culture assay, restriction enzyme digestion and DNA sequencing)

LAMP-MG: a colorimetric malachite green loop-mediated isothermal amplification; STEC: Shiga toxin-producing *Escherichia coli*; PCR: Polymerase chain reaction

Table 3

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of LAMP-MG assay relative to reference assay for detection of Shiga toxin-producing *Escherichia coli* (STEC) O157, STEC O157:H7, *eae, stx1*, and *stx2* in 300 raw fruit and vegetable samples from fresh food markets and supermarkets, Bangkok, Thailand (May - December 2017)

Target	Percent Sensitivity	Percent Specificity	Percent PPV	Percent NPV	Percent Accuracy
STEC O157	100 (24/24)	99 (274/276)	92 (24/26)	100 (274/274)	99 (298/300)
STEC O157:H7	100 (9/9)	98 (284/291)	56 (9/16)	100 (284/284)	98 (293/300)
eae	100 (12/12)	99 (284/288)	75 (12/16)	100 (284/284)	99 (296/300)
stx1	100 (13/13)	99 (283/287)	76 (13/17)	100 (283/283)	99 (296/300)
stx2	75 (12/16)	100 (284/284)	100 (12/12)	99 (284/288)	99 (296/300)

Sensitivity = [(number of true positives)/(number of true positives + number of false negatives) × 100]

Specificity = [(number of true negatives)/(number of true negatives + number of false positives) × 100]

PPV = [(number of true positives/(number of true positives + number of false positives) × 100]

NPV = [(number of true negatives/(number of true negatives + number of false negatives) × 100]

Accuracy = [(number of true positives + number of true negatives)/(total number of samples) × 100]

100 (1,409/1,413), and 99 (1,479/1,500) percent, respectively. LAMP-MG assay sensitivity, specificity, PPV, NPV and accuracy of *E. coli* O157, *E. coli* O157:H7, *eae*, *stx1*, and *stx2* detection were also evaluated, resulting in value of 75-100, 98-100, 56-100, 99-100 and 98-99%, respectively (Table 3).

Prevalence of STEC O157:H7 and STEC non-O157:H7 contamination in raw fruit and vegetable samples

Among 150 fruit and vegetable samples collected from fresh food markets, *E. coli* O157, *E. coli* O157:H7 and *E. coli* non-O157:H7 harboring *stx2* were more frequently found while *E. coli* O157 harboring *stx1*, *E. coli* non-O157:H7 harboring *stx2* and *E. coli* non-O157:H7 harboring *eae* were more frequent in supermarket samples (Table 4). Interestingly, one sample from a fresh food market was contaminated with *E. coli* O157:H7 harboring *eae*, *stx1* and *stx2*. Thus, there existed different diversities of STEC contamination of fresh fruits and vegetables between the two market types, with fresh food markets having two folds higher STEC contamination of their fruits and vegetable than supermarkets (p-value = 0.0001, odds ratio = 2.18, 95% CI: 1.28-3.70).

DISCUSSION

Presence of STEC carrying one or more of the three major virulence genes is considered a major concern in food inspection by the US Department of Agriculture's Food Safety and Inspection Service (USDA, 2019). In the present study, LAMP assay was conducted using an in-house buffer rather than an Eiken LAMP kit buffer as the former procedure cost about 15% of that using the commercial buffer (data not shown). Detection limit of LAMP assay using in-house buffer targeting *stx1*, *stx2* or *yliE* was 10 copies/reaction and 100 copies/reaction targeting *eae* or *wzy* compared to 1-20 CFU/reaction for *eae*, *stx1*, *stx2*, and *wzy* amplification reported by Wang *et al* (2012a) and Wang *et al*, (2012b) and 10 cells/reaction for *yliE* by Lee *et al* (2016). However, direct comparison of limit of detection values among the present and other studies should be treated with caution due to differences in samples and methods of DNA extraction.

LAMP-MG was chosen over other colorimetric LAMP assays to improve detection sensitivity. To the best of our knowledge, this is the first report of applying LAMP-MG for STEC detection. Visual detection limit of LAMP-MG (0.006% MG) for STEC *wzy* was 10 folds more sensitive than using a real-time turbidimeter. LAMP-MG using *wzy*-specific primers detected 1 CFU/2.5 g of a spiked vegetable sample while Wang *et al* (2012b) using the conventional LAMP method reported a comparable detection limit of 2 CFU/2.5 g of spiked sample after 6-8 hours of enrichment at 37°C.

LAMP-MG assay of STEC was validated using raw fruit and vegetable samples chosen for their consumption in uncooked state. Of the five STEC genes chosen as targets of LAMP-MG assay, sensitivity of *stx2* was lower than that of the other gene targets, possibly due to more genetic diversity of *stx2* compared to *stx1* (Lee *et al*, 2007).

In order to cover all *stx2* variants, new design of *stx2* LAMP primers will be required. False-positive LAMP-MG results occurred in two, four, four and seven assays of *E. coli* O157, *eae*, *stx1* and O157:H7 respectively. Given LAMP reaction high sensitivity and large amounts of amplicons, there exists a greater risk of carryover contamination.

Currently, people prefer to consume fresh fruits vegetables, thereby increasing exposure to foodborne infection if the produce is not thoroughly cleaned beforehand. Raw fruit and vegetables from fresh food markets are significantly more contaminated with STEC compared to those from supermarkets, as previous noted in South Korea (Tango *et al*, 2018). Supermarkets maintain a cool storage conditions to extend fruit and vegetable freshness and shelf life, a condition not conducive to bacteria growth. In addition, many supermarkets obtain fruits and vegetables from suppliers with Good Agricultural Practice and Good Handling Practices certifications. Nevertheless, *E. coli* O157 harboring *stx1* or *stx2*, O157:H7 harboring *eae* and non-O157:H7 harboring *eae*, *stx1* or *stx2* were detected in fruit and vegetable samples from supermarkets, albeit at low frequencies (Table 4).

In conclusion, the study shows LAMP-MG assay provided a simple, fast and sensitive technique to detect contamination in raw fruits and vegetables from both fresh food markets and supermarkets in Bangkok, and should facilitate implementation of control and prevention measures of food-borne Shiga toxin-producing *Escherichia coli* throughout the country and elsewhere.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest associated with this publication, and no significant financial support for this work that could have influenced its outcome. All authors of this manuscript have read and approved to its submission and are responsible for its content.

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Table 4

LAMP-MG assay-based prevalence of Shiga toxin-producing *Escherichia coli* (STEC) O157, STEC O157:H7, STEC non-O157:H7 and their virulence genes contaminating raw fruit and vegetable samples obtained from fresh food markets and supermarkets Bangkok, Thailand (May - December 2017)

E. coli serogroup, virulent gene(s)	Number of positive samples	
	Supermarket (n = 150)	Fresh food market (n = 150)
O157	2	10
O157, *eae*	0	1
O157, *stx1*	4	4
O157, *stx2*	1	0
O157, *eae, stx2*	0	2
O157:H7	0	6
O157:H7, *stx1*	0	1
O157:H7, *eae, stx1, stx2*	0	1
O157:H7, *eae*	1	0
Non-O157:H7, *stx1*	1	1
Non-O157:H7, *stx2*	4	8
Non-O157:H7, *eae*	4	2
Non-O157:H7, *eae*, *stx1*	0	1
Total number (%)	17 (11)	37 (25)*

*p-value = 0.0001; odds ratio = 2.18, 95% confidence interval: 1.28-3.70

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