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Rapid and sensitive enumeration of total coliforms and *Escherichia coli* in water and foods by most-probable-number loop-mediated isothermal amplification (MPN-LAMP) method

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ABSTRACT: Total coliforms and *Escherichia coli* are bacterial indicators used to assess the microbiological qualities of water and foods. In this study, we developed a most-probable-number loop-mediated isothermal amplification (most probable number (MPN-LAMP)) method for the enumeration of total coliforms and *E. coli* in drinking water and vegetables. The LAMP primers were designed based on *lacZ* (*lacZ*-LAMP) and *uidA* (*uidA*-LAMP) genes for the detection of total coliforms and *E. coli*, respectively. In pure culture, the *lacZ*-LAMP and the *uidA*-LAMP were able to detect all of the 37 coliforms and 30 *E. coli* strains, respectively. In the artificially contaminated drinking water and vegetables, the combination of MPN and LAMP techniques (MPN-LAMP) was able to detect *E. coli* at 1 colony-forming unit (CFU)/100 ml and 5 CFU/g, respectively. From analysis of 33 drinking water and 46 vegetable samples, the total coliform detection obtained by the MPN-LAMP method was in agreement with the MPN detection technique. However, MPN-LAMP was more sensitive than the MPN technique for *E. coli* detection. Our findings revealed that the MPN-LAMP assay was more rapid and highly sensitive than MPN method. Thus this method could be considered for detection and enumeration of total coliforms and *E. coli* in the food industry.

KEYWORDS: loop-mediated isothermal amplification, microbial quality

INTRODUCTION

Coliforms are present predominantly in the faeces of warm-blooded animals, including humans, and are detected in various environments such as water, soil, and vegetation[1,2]. Total coliforms and *Escherichia coli* are used as the indicator bacteria for the monitoring of the microbial qualities of water and food. It has been demonstrated that the commonest coliforms isolated from rural drinking water are *Klebsiella* spp., *Citrobacter* spp., and *E. coli*, accounting for 51%, 20%, and 12% of the total coliforms, respectively[3]. In several countries, the presence of *E. coli* is not acceptable in drinking water, fresh produce, and any processed food[4,5].

A standard method recommended for the detection and enumeration of total coliforms and *E. coli* is the most probable number (MPN) technique[6]. However, this technique is time consuming, and 4–6 days are required before the presence of coliforms can be confirmed. Rapid culture methods for coliforms based on specific enzymatic activity (β-galactosidase and β-glucuronidase) have been developed to examine water and food samples, but they are expensive and time consuming[7].

Polymerase chain reaction (PCR)-based methods have been developed to increase the rapidity and specificity of coliform detection[8,9]. Primers specific to *lacZ* and *malB* genes have been developed for the detection of coliforms and *E. coli*, respectively; however, cross reaction has been demonstrated with *Shigella* and *Salmonella* species[10]. Hence primers targeted to the *uidA* gene encoding β-D-glucuronidase (GUD) have been developed for the specific detection of *E. coli*[11,12]. However, PCR requires expensive equipment, and the presence of inhibitors in food samples can decrease PCR effi-

Table 1 Bacteria used in this study and specificity of LAMP primers.

Bacteria	No†	*lacZ*-LAMP	*uidA*-LAMP
Coliforms ($n = 37$)			
Escherichia coli	30	+[a]	+
C. freundii	1	+	−
Enterobacter aerogenes	1	+	−
Enterobacter cloacae	1	+	−
K. pneumoniae	3	+	−
Serratia marcescens	1	+	−
Non-coliforms ($n = 15$)			
Proteus spp.	3	−	−
Salmonella enterica	4	−	−
Shigella spp.	4	+	+
Aeromonas spp.	2	−	−
Plesiomonas shigelloides	1	−	−
Pseudomonas aeruginosa	1	−	−

† Number of tested strains.
+, positive result; −, negative result.

ciency[13].

A novel nucleic acid-based method known as loop-mediated isothermal amplification (LAMP) has been developed[14]. The LAMP technique is rapid and easy to perform. Bacterial DNA amplification can be achieved under isothermal conditions by using a simple heating block or water bath, and a positive LAMP reaction can be detected by the naked eye[15]. In addition, LAMP showed higher tolerance to some biological substances and to inhibitory substances found in a culture medium and some biological substances than did PCR[16]. LAMP commercial kits have been developed to detect many foodborne pathogens, including *Salmonella*, *Listeria*, Shiga-toxin-producing *E. coli*, and *Campylobacter*[15]. Diagnosis of *E. coli* infection with this technique reveals high sensitivity and specificity[13,17]. For food safety and for microbial detection in food, the concentrations of some bacteria must be inspected and reported as MPN value; however, the LAMP technique is not applicable in this context. Thus combining the MPN and LAMP techniques (MPN-LAMP) would benefit the food industry by allowing the rapid detection and enumeration of foodborne pathogens. MPN-LAMP has been reported as a potential approach to replace conventional MPN method for detection of many foodborne pathogens, including *Vibrio parahaemolyticus*, *E. coli*, and *Enterococcus faecalis*[18,19]. In this work, the MPN-LAMP technique was evaluated for the detection and enumeration of total coliforms and *E. coli* in drinking water and vegetables.

Table 2 LAMP target genes and primers used in this study.

Target gene	Primer	Sequence (5′to 3′)
lacZ	F3	ACCATCGTCTGCTCATCCA
	B3	TTAAACTGCACACCGCCG
	FIP	CAAGCCGTTGCTGATTCGCGTTTT
		TGACCTGACCATGCAGAGG
	BIP	CCGTTCAGCAGCAGCAGACCTTTT
		ACGCTGATTGAAGCAGAAGC
uidA	F3	CCAGAGGTGCGGATTCAC
	B3	CGATATCACCGTGGTGACG
	FIP	ACTGCGTGATGCGGATCAACAGTT
		TTCACTTGCAAAGTCCCGCTAG
	BIP	CTGACATCACCATTGGCCACCATT
		TTGTCGCGCAAGACTGTAACC

MATERIALS AND METHODS

Bacterial strains

A total of 52 coliform and non-coliform bacteria used in this study (Table 1) were obtained from the culture collection of the Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

LAMP assay

In this study, primers specific to *lacZ* and *uidA* for the detection of total coliforms and *E. coli*, respectively, were designed using Primer Explorer V4 (Eiken Chemical, Co. Ltd., Japan) (Table 2). The *lacZ*-LAMP or *uidA*-LAMP assay was performed in a 25-µl reaction mixture consisting of a 1× ThermoPol reaction buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% TritonX-20, pH8.8 at 25 °C), 1.6 µM of each inner primer (FIP and BIP), 0.2 µM each of the outer primer (F3 and B3), 1.4 mM of dNTPs, 8 mM of $MgSO_4$, 8 U of *Bst* DNA polymerase (New England Biolabs, USA), and 1.5 µl of DNA template. The reaction mixture was incubated in a conventional heating block (Major Science, USA) for 60 min at 65 °C for *lacZ* detection and at 64 °C for *uidA* detection. No DNA-containing reaction mixture was used as a negative control. In this work, for rapid visualization of the LAMP reaction, Pico Green (Sigma-Aldrich, USA) was added to the reaction mixture after the amplification, and green fluorescence was detected for a positive LAMP reaction[20]. Separate areas were used for LAMP reaction setup and analysis to prevent carry-over contamination. Conventional PCR was performed using the LAMP outer primers F3 and B3 (Table 2); the amplified products were then purified and sequenced (Macrogen, Korea) to verify the specificity

of the primers.

Specificity and sensitivity of the LAMP assay

To determine the specificity of the LAMP assay, 37 and 15 strains of coliforms and non-coliforms were evaluated, respectively (Table 1). The sensitivity of the *lacZ*-LAMP and the *uidA*-LAMP were determined using *E. coli* ATCC25922. Briefly, the bacterium was grown overnight at 35 °C in tryptic soy broth and adjusted to 10^8 CFU/ml in normal saline solution using a densitometer (Biosan, Latvia). Then 10-fold serial dilution of bacteria was performed and the number of bacteria was confirmed by the spread plate technique[24]. Bacterial DNA template for the LAMP assay was obtained by boiling. For comparison, the conventional PCR was also performed on the serially diluted DNA templates using the LAMP outer primers F3 and B3.

Artificial inoculation of water and a vegetable

E. coli is a member of the coliform group. In this work, *E. coli* ATCC25922 was used to determine both total coliform and *E. coli* contamination in samples. A drinking water sample was obtained from a public water dispenser, and lettuce was purchased from a local market. Natural bacterial contamination in water was removed by filtration through a 0.22 μm membrane filter (Sartorius Stedim Biotech, Germany). For artificial contamination, 1 ml of *E. coli* at a concentration of between 1 and 10^2 CFU/ml was inoculated into 200 ml of water. For a vegetable sample, 100 g of lettuce was decontaminated using 200 ppm of sodium hypochlorite[21]. A concentration of *E. coli* between 1 and 10^2 CFU/ml was inoculated into a sample containing 25 g of vegetable and 225 ml of phosphate buffer, and the sample was homogenized for 2 min.

MPN and the MPN-LAMP assays in artificially contaminated samples

To enumerate total coliforms and *E. coli* in water, the MPN technique was performed using the 10-tube MPN test with lauryl tryptose broth as described by the U.S. Environmental Protection Agency[22]. For lettuce, the three-tube MPN test was performed as described in the Bacteriological Analytical Manual (BAM)[23].

For MPN-LAMP evaluation, positive presumptive MPN tubes that exhibited gas formation were subjected to the *lacZ*-LAMP and the *uidA*-LAMP for the detection of total coliforms and *E. coli*, respectively. Briefly, 1 ml of culture taken from each positive presumptive tube was boiled, and 2 μl of supernatant was used as a DNA template for each LAMP assay as described above.

MPN-LAMP assay in naturally contaminated water and vegetable samples

A total of 33 drinking water samples were collected from various public water dispensers in Hat Yai, Songkhla province, southern Thailand. The samples were transported and stored in strict accordance with the guidelines described for standard methods[22]. Forty-six vegetable samples were purchased from local markets and were analysed immediately upon their arrival at the laboratory. For the enumeration of total coliforms and *E. coli* in water and vegetables, the 10-tube MPN test and the 3-tube MPN test were performed, respectively, and MPN tubes that exhibited gas formation were subjected to the *lacZ*-LAMP and the *uidA*-LAMP as described above.

The specificity and sensitivity of MPN and MPN-LAMP were evaluated. Specificity was defined as the (number of true negatives)/(number true negatives + number of false positives), and sensitivity was defined as the (number of true positives)/(number of true positives + number of false negatives)[24].

RESULTS

Specificity and sensitivity of the LAMP assay

Regarding the specificity of the *lacZ*-LAMP for total coliform detection, all tested coliform bacteria, including *E. coli*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Serratia marcescens*, were positive (Table 1). All strains of non-coliform bacteria, including *Proteus*, *Salmonella*, and others were negative except for *Shigella* spp. Regarding the specificity of *uidA*-LAMP for *E. coli* detection, all 30 *E. coli* and *Shigella* spp. strains were positive, whereas other tested bacteria were negative (Table 1). Confirmation of LAMP amplification products by sequencing revealed no false positives detected in both the *lacZ*-LAMP and the *uidA*-LAMP assays. In this study, the sensitivity for *E. coli* by both assays was 1 CFU per reaction, which was 1000 times more sensitive than the sensitivity obtained by conventional PCR (Fig. 1).

MPN-LAMP assays in artificially contaminated samples

The sensitivities of the MPN and MPN-LAMP techniques to detect total coliforms and *E. coli* were compared. In the artificially contaminated water,

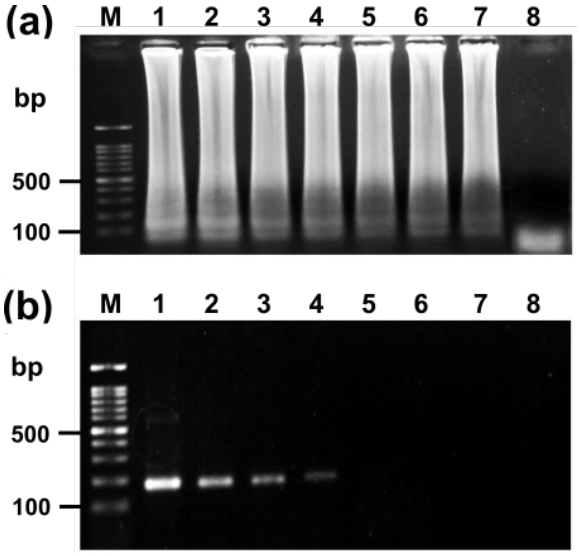

Fig. 1 Sensitivity of LAMP (a) and PCR (b) assays for detection of *Escherichia coli*. The 10-fold serial dilutions of *E. coli* ATCC25922 starting from 10^6 CFU/reaction (lane 1) down to 1 CFU/reaction (lane 7) were tested. Lane 8, non-template control; lane M, DNA marker.

the MPN-LAMP method was able to detect total coliforms and *E. coli* at the lowest concentration of 1 CFU/100 ml, which correlated with the results obtained from the MPN technique. In the artificially contaminated vegetable, the sensitivity of both methods for total coliforms and *E. coli* was lower (5 CFU/g).

MPN-LAMP assay in naturally contaminated water and vegetable samples

The efficiency of the MPN-LAMP method was determined in 33 drinking water and 46 vegetable samples to evaluate its application in water and food analysis. For all tested samples, MPN-LAMP was capable of detecting the numbers of total coliforms positive sample equally to the MPN technique. The total coliforms concentrations in water and vegetable samples were between <1.1–10^2 MPN/100 ml and 3.6–4.6×10^4 MPN/g, respectively (data not shown). However, for the detection of *E. coli* at the same concentrations, the numbers of positive samples obtained by the MPN-LAMP assay were different from those of the MPN technique. In this study, a discrepancy between the MPN technique and the MPN-LAMP method for the detection of *E. coli* in 11 samples (14%) was observed (Table 3).

The sensitivity and specificity of the MPN-LAMP method for the detection of total coliforms and *E. coli* in 79 samples of water and vegetable were compared to the MPN technique. The results obtained from MPN-LAMP correlated to those obtained from MPN for total coliform detection; thus both the specificity and sensitivity of the MPN-LAMP technique for coliform detection were 100% (Table 4). However, for *E. coli* detection, 54 samples were positive by MPN-LAMP, whereas only 43 samples were positive by MPN; thus the sensitivity of MPN-LAMP was superior to MPN for *E. coli* detection (Table 4).

Table 3 Comparison of MPN and MPN-LAMP techniques for enumeration of total coliforms and *Escherichia coli* in drinking water and vegetables.

Sample	Total coliforms		*E. coli*	
	MPN	MPN-LAMP	MPN	MPN-LAMP
water	1.1[a]	1.1	<1.1	1.1
water	1.1	1.1	<1.1	1.1
water	2.2	2.2	<1.1	2.2
water	2.2	2.2	<1.1	2.2
water	9.2	9.2	<1.1	3.6
water	16	16	<1.1	1.1
water	23	23	<1.1	9.2
vegetable	23	23	<3.0	9.2
vegetable	$>1.1\times10^3$	$>1.1\times10^3$	<3.0	35
vegetable	1.1×10^4	1.1×10^4	<3.0	3.0
vegetable	1.5×10^4	1.5×10^4	<3.0	9.2

[a] MPN values (MPN/100 ml of water or MPN/g of vegetable).

DISCUSSION

The MPN technique, the conventional culture-based method for the detection and enumeration of total coliforms and *E. coli* in water and food, is laborious and time consuming. Recently, the LAMP assay, which is 10 times more sensitive than PCR[25], has been developed for the detection of many foodborne pathogens. For the examination of the microbiological qualities of drinking water and food, bacterial count must be evaluated to determine whether they are below the permissible limits. MPN is a standard method used to estimate the concentration of viable bacterial cells. However, LAMP technique detects the amounts of DNA in a reaction tube, rather than bacterial cells as requested in food safety standard and guidelines[13]. Hence a combination of this technique and the MPN method has been developed in this study for the detection of bacterial indicators in water and food.

Table 4 Specificity and sensitivity of MPN technique compared to MPN-LAMP method for total coliforms and *Escherichia coli* detection.

Bacteria	MPN		% specificity	% sensitivity	MPN-LAMP		% sensitivity
	+[a]	−			+	−	
Total coliforms	59	20	100%	100%	59	20	100%
E. coli	43	36[b]	100%	80%	54	25	100%

[a] Number of positive samples; [b]*E. coli* was detected from 11 Lauryl tryptose broth MPN tubes by direct cultivation on Eosin Methylene Blue agar and was confirmed by 16S rRNA sequencing.

In this study, the *lacZ*-LAMP (total coliforms) and the *uidA*-LAMP (*E. coli*) assays were found to be highly specific. It has been reported that *E. coli* and *Shigella* share a high level of DNA similarity and cannot be differentiated by PCR-based methods[8]. In this study, the presence of coliforms and *E. coli* can be confirmed on the basis of the ability to ferment lactose during the MPN step of MPN-LAMP assay, which is not the case for *Shigella* spp.

Our data demonstrate that the sensitivity of MPN-LAMP for the detection of total coliforms or *E. coli* was superior to that of the LAMP assay. A previous report demonstrated that the sensitivity of the LAMP assay for Shiga-toxin-producing *E. coli* (based on Shiga toxin genes, other virulence genes, and O-antigen gene clusters) was between 1 and 20 cells per reaction in pure culture and 10^3 and 10^4 CFU/g in vegetables[26]. The sensitivity of LAMP for the detection of enterotoxigenic *E. coli* in raw milk was 547 CFU/ml[27].

Results from the experiments to detect total coliforms and *E. coli* in drinking water and vegetable samples have shown that MPN-LAMP was more sensitive than the MPN technique for *E. coli* detection. The false-negative results of MPN alone might be due to some strains of *E. coli* being unable to grow in *Escherichia coli* broth (EC broth) during the confirmed phase of conventional MPN method. When the initial density of *E. coli* was low, the presence of bile salts in the EC medium and incubation at 45.5 °C were inhibitory to many of the strains tested[28]. However, the MPN-LAMP assay can overcome the problems occurred by MPN method. To confirm this, an MPN tube that exhibited gas formation at presumptive phase was directly streaked on eosin methylene blue agar, and a lactose-fermenting colony was subjected to sequencing based on the 16S rRNA gene, and *E. coli* was identified. The failure to detect *E. coli* could lead to a significant error in the examination of water and food quality. The *uidA*-PCR has been demonstrated to be more sensitive for *E. coli* detection than culture-based detection methods[9]. This study also demonstrated that the MPN-LAMP based on *uidA* for *E. coli* detection is more sensitive than the conventional MPN technique. Thus the MPN-LAMP assay is effective for application in coliform detection in water and food.

This study demonstrates that the MPN-LAMP method is a rapid and reliable technique for the detection and enumeration of total coliforms in drinking water and vegetables and may be considered as a useful tool for application in the food industry.

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