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Original research article

Development of the Rapid Test Kit for the Identification of *Campylobacter* spp. Based on Loop-mediated Isothermal Amplification (LAMP) in Combination with a Lateral Flow Dipstick (LFD) and Gold Nano-DNA Probe (AuNPs)

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ABSTRACT

The detection of *Campylobacter* spp. in meat products was developed by using loop-mediated isothermal amplification (LAMP) combined with DNA-based bioassay methods, including a lateral-flow dipstick (LFD) and gold nano-DNA probe (AuNPs) assay. The LAMP primers were designed from the conserved nucleotide regions of *Campylobacter* spp. The analytical sensitivity of the LAMP-LFD and LAMP-AuNPs analysis was 360 fg/µl. The analytical specificity of LAMP-based assays showed no cross-reactions to *Listeria monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Serratia marcescens*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Klebsiella oxytoca* and *Citrobacter diversus*.

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The sensitivity, specificity and accuracy of both LAMP-LFD and LAMP-AuNPs for the detection of pre-enrichment cultures from raw chicken meat samples were 100%, 95% and 96.67%, respectively. Since the processing time of LAMP-based assays is 60-90 minutes, it is applicable as a point-of-care screening test for food safety and as a process control of *Campylobacter* spp. contamination.

Keywords: *Campylobacter*; Loop-mediated isothermal amplification; Lateral flow dipstick; Gold nano-DNA probe.

1. Introduction

Campylobacter species are bacterial foodborne pathogens found in humans worldwide. It is known to cause gastroenteritis and is the most common cause of infectious diarrhea [1]. In 2010, The European Centre for Disease Prevention and Control (ECDC) and The European Food Safety Authority (EFSA) reported that 212,064 cases of campylobacteriosis had been found in the European Union [1, 2]. In 2011, The Centers for Disease Control and Prevention (CDC) reported that 845,000 cases of campylobacteriosis had been observed in the United States [3]. The infection records revealed that 90% of these cases were caused by *Campylobacter jejuni* and, to a lesser extent, *C. coli* [4, 5]. *C. jejuni* and *C. coli* are the most common causes of gastrointestinal illness in humans [6]. Symptoms of *Campylobacter* infections, as observed in a number of developed countries, are characterized by abdominal pain, fever, nausea and bloody stools over a 1-3 week period from the time of infection to incubation [1]. Complications from *Campylobacter* infections can include the development of Guillain-Barre Syndrome (GBS) [7]. The source of *Campylobacter* infections are primarily from the consumption of raw or undercooked poultry meat, as well as milk products and untreated water [8]. The consumption of poultry products that are host to *Campylobacter* account for around 80% of all campylobacteriosis cases [9].

Campylobacter species are gram-negative bacteria, their morphology forms spiral or rod shapes that resemble a bird in flight, often described as a 'gull wing' morphology. They are motile bacteria through the use of either a single polar flagellum or bipolar flagella [10]. The *Campylobacter* species are microaerophilic microorganisms that are microaerobic, growing best in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2, [11] and are thermophilic, growing best at a temperature between 37-42 ºC [10]. The isolated bacteria was taken from several samples, including blood, antibiotics and ferrous iron, and observed for 40-48 hours [10]. The genus of *Campylobacter* contains 25 species and 8 subspecies that have been found in humans, domestic animals, birds, chicken, milk and water [12, 13].

Generally, the conventional methods for identifying *Campylobacter* in food samples are based on enrichment culture, microscopy and biochemical assay, which are time consuming methods and require specialists to identify the bacteria. Currently, molecular techniques such as immunoassays, polymerase chain reactions (PCR), DNA microarrays and DNA probes are used for the rapid detection of *Campylobacter* in food samples. These methods are specific, sensitive and rely on real-time sensing, and are mainly based on sophisticated and costly equipment which may not suitable for field investigation. Hence, the rapid test kit for the identification of *Campylobacter* spp. based on loop-mediated isothermal amplification (LAMP) in combination with lateral flow dipstick (LFD) and gold nano-DNA probe (AuNPs) assays was developed. The

sensitivity and specificity of each assay were compared to those of the conventional methods.

2. Materials and Methods

2.1 Bacterial strains

Campylobacter and other referenced foodborne pathogen strains were obtained from the National Institute of Health, the Department of Medical Sciences Thailand (DMST) and Thailand's Ministry of Public Health. Bacterial strains were identified using PCR and biochemical assays as listed in (Table 1).

2.2 DNA extraction

DNA from all of the bacterial strains was isolated using a QIAamp Tissue Kit (Qiagen, Hilden, Germany). DNA were resuspended in TE buffer and stored in -80°C for use.

2.3 LAMP primers and DNA probe

LAMP primers were designed based on six distinct regions within the 16S rRNA gene by using the PrimerExplorer version 4 (V4)program(http://primerexplorer.jp/elamp 4.0.0/index.html). They were composed of forward outer primers (F3), backward outer primers (B3), forward inner primers (FIP) labeled with the use of biotin at the 5' ends and backward inner primers (BIP), and of loop primers LoopF and LoopB (patent no. 1803000708 and 1803000709). The specific DNA probe for the detection of LAMP products was labeled with FITC at the 5' ends.

2.4 Polymerase chain reaction (PCR)

The PCR was performed in 25 µl containing 2 µM F3 and B3, 2 mM dNTPs, 4.4 mM of $MgCl_2$ (Vivantis, Malaysia), 4 U of *Taq* DNA polymerase (Vivantis, Malaysia), 10X thermopol buffer and 1 µl of DNA template. The PCR conditions contained 35 cycles of denaturation at 95 °C for 30 seconds, annealing temperature at 50-60 °C for 30 seconds and extension at 72 °C for 30 seconds. The PCR product was analyzed by using agarose gel electrophoresis.

2.5 Loop-mediated isothermal amplification (LAMP)

The LAMP was performed in 25 µl reaction containing 2 µM each of forward inner primer (FIP) and backward inner primer (BIP), 2 µM each of primer F3 and B3, 2 µM of LoopF and LoopB primers, 1.6 mM of dNTPs (New England Biolabs, USA), 0.5 M of betaine (Sigma-Aldrich, USA), 0.5 M 10X thermopol buffer, 8 U of the *Bst* 2.0 DNA polymerase (New England Biolabs, USA), 6 mM of $MgSO_4$ (New England Biolabs, USA) and 1 µl of DNA template. The reaction mixture was incubated at 60-65 °C for 60 minutes. The LAMP products were analyzed by using 2% agarose gel electrophoresis [14].

2.6 LAMP-LFD assay

The biotin-labeled LAMP amplification products were hybridized with FITC-labeled DNA probe at 63 °C for 10 minutes prior to the addition of 100 ul buffer. Then, the LFD membrane (Milenia Genline HybriDtect, Germany) was dipped into the tube containing the hybridization products and left for 5-10 minutes at room temperature. The positive indication of *Campylobacter* appeared as a purple color at the control and test lines on the LFD membrane.

2.7 LAMP–AuNPs assay

The preparation of DNA-AuNPs probe solution was prepared by mixing 4 ml of 10 nM colloidal AuNPs (Sigma-Aldrich, USA) and 20 µl of 100 µM thiol-labeled probe DNA in a dark container and incubating it with an agitation of 100 rpm at 50 °C for 24 hours. Then, 0.1 M NaCl and 10 mM phosphate buffer (pH7) were added and further incubated at 45 °C with an agitation of 100 rpm for 48 hours. The unmodified oligonucleotides were removed through centrifugation at 13,000 rpm for 25 minutes,

Table 1. *Campylobacter* spp. and reference foodborne pathogens strains used in this study.

Microorganism	Stain no.	Source
Campylobacter jejuni	ATCC 33291	DMST
Campylobacter coli	NCTC 11353	DMST
Campylobacter lari	ATCC 43675	DMST
Campylobacter fetus	ATCC 27374	DMST
Listeria monocytogenes		DMST
Escherichia coli		DMST
Bacillus cereus		DMST
Pseudomonas aeruginosa		DMST
Staphylococcus aureus		DMST
Enterobacter aerogenes		DMST
Serratia marcescens		DMST
Vibrio parahaemolyticus		DMST
Vibrio cholerae		DMST
Klebsiella oxytoca		DMST
Citrobacter diversus		DMST

* ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures, London, United Kingdom; DMST, Department of Medical Science, Ministry of Public Health.

followed by washing the pellet with 500 µl of resuspension buffer A containing 10 mM PBS (pH7.4), 150 mM NaCl and 0.1% SDS. After centrifugation, the pellet was resuspended in 50 µl of buffer A. This step was repeated three times to completely remove excessive thiol-labeled oligonucleotides. Determination of DNA-AuNPs concentration was achieved by using UV/vis absorption spectrometry. Finally, the DNA-AuNPs probe solution was stored in a refrigerator before use.

Detection of LAMP products was accomplished by denaturation at 95 °C for 10 minutes prior to hybridization with AuNPs probe at 53 °C for 10 minutes. Then, 5-500 mM $MgSO_4$ was added to induce the aggregation of AuNPs before being left at room temperature for another 10 minutes. In the presence of $MgSO_4$, the positive reaction solution appeared as a red color since LAMP products-AuNPs probe complex had dispersed. In contrast, the free AuNPs probe in the negative reaction solution was induced to aggregation at the high salt concentration, with the reaction solution appearing as a pale purple color. The LAMP products-AuNPs probe complex solution color change was observed by the naked eye.

2.8 Analytical sensitivity of LAMP-based assays

To determine the analytical sensitivity of each LAMP-based assay, the 10-fold serial dilutions of purified *C. jejuni* genomic DNA were used as the templates for LAMP assays prior to each detection method, in comparison to 2% agarose gel electrophoresis.

2.9 Analytical specificity of LAMP-based assays

The analytical specificity of LAMP based assays for the detection of *Campylobacter* spp. were tested against 12 reference strains. After LAMP amplification, the products were subsequently detected using LFD and AuNP methods in comparison to 2% agarose gel electrophoresis. *C. jejuni* was used as a positive control in all LAMP reactions.

2.10 Sensitivity for the detection of chicken meat samples spiked with *C. coli* and *C. Jejuni*

100 g samples of sterile chicken meat were spiked with 10-fold serial dilutions of *C. coli and C. jejuni* and suspended in 100 ml of PBS buffer. Each culture suspension concentration was determined by using a total plate count method. The suspensions were centrifuged at 15,000 rpm for 10 minutes prior to the collection of bacterial cells and DNA extraction before proceeding with the LAMP-based assays.

2.11 Raw chicken meat samples

Thirty samples of raw chicken meat were collected from various markets in Nonthaburi province of Thailand. Each 50 g sample was rinsed with 100 µl of 1M Tris buffer for 30 minutes in a Stomacher bag prior to centrifugation at 15,000 rpm for 10 minutes. Sediment was collected and enriched by using the standard culture method. Enrichment culture was accomplished in Preston broth (Thermo Scientific, USA). Rapid DNA extraction was performed by boiling the sample at 100 °C for 10 minutes. The DNA extractions were determined by LAMP-LFD and LAMP-AuNPs compared to the standard culture assay.

3. Results and Discussion

3.1 PCR and LAMP

The identification of standard *Campylobacter* purified DNA through the use of PCR revealed that PCR products 206 bp in size were observed (Fig. 1a.).

Temperature optimization for the LAMP amplification of *Campylobacter* purified DNA indicated that 63 °C generated the highest LAMP products (Fig. 1b.).

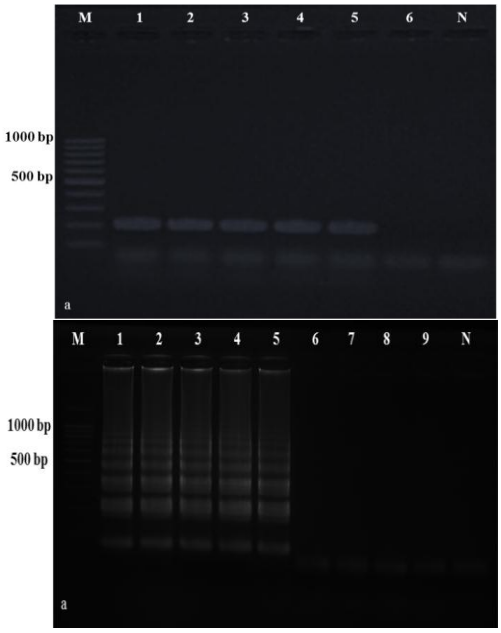


Fig. 1. Temperature optimization for amplifying purified *Campylobacter* DNA by using (1a.) PCR- agarose gel electrophoresis (AGE). Lanes 1-6 represent temperatures of 50 °C, 52 °C, 54 °C, 56 °C, 58 °C and 60 °C, respectively. PCR products 206 bp in size were observed (1b.) LAMP-AGE. Lanes 1-6 represent temperatures of 60 °C, 61 °C, 62 °C, 63 °C, 64 °C and 65 °C, respectively. Lane M represents the 100 bp DNA marker. Lane N represents negative control (no-DNA template.

3.2 Analytical sensitivity of LAMP-based assays

The analytical sensitivity or limits of detection of PCR, LAMP-LFD and LAMP-AuNP for the detection of *Campylobacter* spp. were 3.6 pg/ul, 0.36 pg/µl and 0.36 pg/µl, respectively (Fig. 2.).

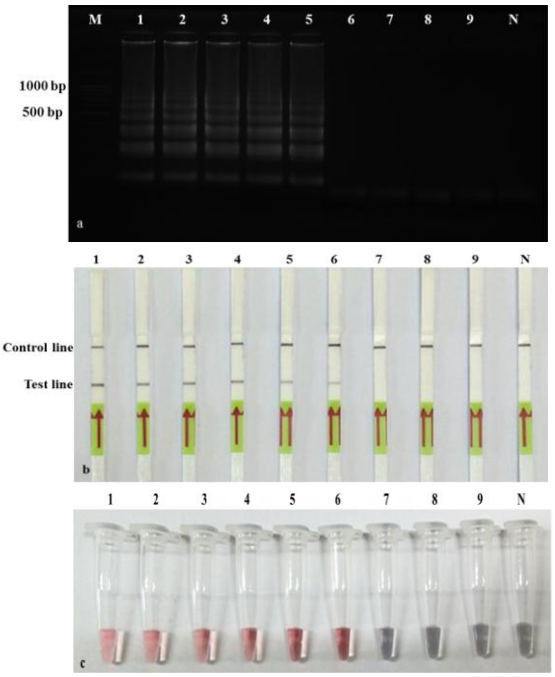


Fig. 2. Analytical sensitivity test for detecting purified *C. jejuni* DNA by using (2a.) LAMP-AGE, (2b.) LAMP-LFD and (2c.) LAMP-AuNPs. Lanes 1-9 represent 36 ng/µl, 3.6 ng/µl, 360 pg/µl, 36 pg/µl, 3.6 pg/µl, 0.36 pg/µl, 36 fg/µl, 3.6 fg/µl and 360 ag/µl, respectively. Lane M and N represent the 100 bp DNA ladder marker and negative control (no-DNA template), respectively.

3.3 Analytical Specificity of LAMP-based assays

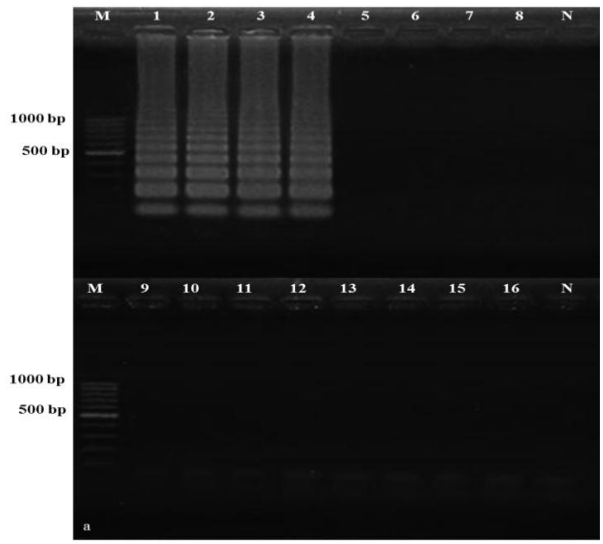
Analytical specificity of the LAMP-based assays for the detection of *Campylobacter* spp. was tested against 12 strains of foodborne pathogens. No cross-reactions were observed for all assays (Fig. 3.).

3.4 Analytical sensitivities for the detection of *C. coli* and *C. jejuni* in spiked chicken samples

Analytical sensitivities of LAMP-based assays for the detection of *C. coli* and *C. jejuni* in spiked chicken samples were $1x10^2$ and $1x10^3$ CFU/ml, respectively. LAMP-based assays were 10 times more sensitive than that of PCR methods (Table 2).

3.5 Raw chicken meat samples

DNA of 30 pre-enrichment raw chicken meat samples were tested using LAMP-based assays in comparison to standard culture methods. The positive and negative predictive values of LAMP-LFD and LAMP-AuNPs were 90.91% and 100%, respectively. The data revealed that the sensitivity, specificity and accuracy of both LAMP-LFD and LAMP-AuNP for the detection of pre-enriched *Campylobacter* from raw chicken meat samples were 100 %, 95% and 96.67%, respectively (Table 3).



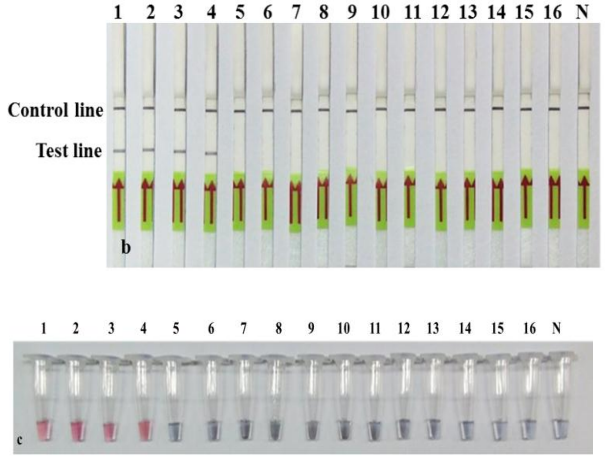


Fig. 3. Analytical specificity test for the detection of *Campylobacter* species and foodborne pathogens using (3a.) LAMP- AGE, (3b.) LAMP-LFD and (3c.) LAMP- AuNPs. Lanes 1-16 represent *C. jejuni*, *C. coli*, *C. lari*, *C.fetus*, *S. typhimurium*, *L. monocytogenes*, *E. coli*, *C. diversus*, *E. aerogenes*, *K. oxytoca*, *V. paraheamolyticus*, *V. cholera*, *B. cereus*, *S. aureus*, *P. aeruginosa* and *S. marcescens*, respectively. Lane M and N represent the 100 bp DNA ladder marker and negative control (no-DNA template), respectively.

Loop-mediated isothermal amplification (LAMP) assays have been applied as molecular detection tools for various microaerobic bacteria. Herein, specific primers and DNA probes for LAMP-LFD and LAMP-AuNPs have been designed based on conserved regions of the 16S RNA gene of *Campylobacter* spp. The 16S RNA has been recommended as a species-specific gene and an application was submitted for its identification or organism classification. The overall assay time was less than 90 minutes, which included DNA extraction, amplification, hybridization and interpretation. Our study has demonstrated that the process times of LAMP-LFD and LAMP-AuNP were comparable to those of [15] and that the assays took markedly less than 40 minutes and 80 minutes respectively, to detect single colonies in spiked samples. According to [16], LAMP-

based assays are simple and convenient when compared to PCR and conventional culture techniques.

With regards to LAMP-LFD, the results can be easily observed by the naked eye within 5-10 minutes. Our data demonstrated that the analytical sensitivity of LAMP-LFD and LAMP-AuNPs was 10 times better than that of PCR assays. According to [15], the sensitivity of LAMP assays for detection was 10 times higher than that of PCR assays. However, both LAMP-LFD and LAMP-AuNPs showed no cross-reactions to other foodborne pathogens.

The sensitivity, specificity and accuracy of both LAMP-LFD and LAMP-AuNPs were observed as 100%, 95% and 96.67%, respectively, in detecting pre-enriched *Campylobacter* from 30 raw chicken meat samples in comparison to conventional culture methods. One sample that was positively identified as being contaminated when tested with LAMP-LFD and LAMP-AuNPs was given a negative reading when tested with conventional culture methods. This discrepancy may be due to both LAMP-LFD and LAMP-AuNPs assays focusing on bacterial DNA, whereas conventional culture methods are only achieved with living bacterial cells. Therefore, the sample may have been contaminated with the dead cells of *Campylobacter,* but because the DNA still existed, it made detection by the LAMP-based assays possible. In conclusion, the LAMP-based assays could be applicable as a screening test for detecting *Campylobacter* contamination in chicken samples, which would prove beneficial for quality control in food spoilage and consumption.

4. Conclusion

The analyses to detect *Campylobacter* spp. by using LAMP-LFD and LAMP-AuNPs methods were faster than PCR method and culture techniques. LAMP assay is less than 90 minutes from the beginning of DNA extraction to final detection.

The results of LAMP-LFD can be easily observed using the naked eye within 5-10 minutes [17]. LAMP-LFD and PCR assay detected at 0.36 pg/µl and 3.6 pg/µl, respectively. Sensitivity was 10 times higher than PCR. LAMP-LFD and PCR had no cross to the reactions to other foodborne pathogens.

Measurement of LAMP-AuNPs depends on the size, shape and level of inclusion of the gold nanoparticles. LAMP-AuNPs is a fast and easy method to detect ion exchange detector [18]. The analytical DNA color changes showed that it could be used to colorimetrically detect the complementary oligonucleotide [19]. The detection limits of *Campylobacter* spp. using LAMP-AuNPs assay and PCR method were 0.36 pg/µl and 3.6 pg/µl, respectively. The detection sensitivity was 10 times higher than PCR. According to the specificity test, there were no cross-reactions to *Campylobacter* spp. The LAMP and PCR method were specific for *Campylobacter* spp.

The sensitivity of the LAMP-based assay to detect *Campylobacter* spp. showed that LAMP-LFD and LAMP-AuNPs were 360 fg/µl.

The LAMP products were hybridized with a probe and detected by LFD and AuNPs assays compared with the standard culture. The LAMP assay can be adapted easily for detection of *Campylobacter* spp. However, LAMP-LFD and AuNPs methods were rapid when compared to the traditional culture. Our data corresponded to the previous report of [14]. LAMP-based assays for detection of *Campylobacter* spp. pre-enrichment raw chicken samples LAMP-LFD and AuNPs assay were 100 % and 95% of sensitivity and specificity, respectively.

Table 2. Sensitivities for detection of chicken meat samples spiked with *C. coli* and *C. Jejuni*.

Methods	Total Cells (CFU/ml)								
	$1x10^0$	$1x10^1$	$1x10^2$	$1x10^3$	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
C. coli									
PCR	-	-	-	+	+	+	+	+	+
LAMP-LFD	-	-	+	+	+	+	+	+	+
LAMP-AuNPs	-	-	+	+	+	+	+	+	+
C. jejuni									
PCR	-	-	-	+	+	+	+	+	+
LAMP-LFD	-	-	-	+	+	+	+	+	+
LAMP-AuNPs	-	-	-	+	+	+	+	+	+

Table 3. Validity of LAMP-based assays for detection of *Campylobacter* spp. in pre-enrichment raw chicken meat samples.

	No. of food samples		
	Standard culture	**LAMP-LFD**	**LAMP-AuNPs**
True positive	10	10	10
True negative	20	19	19
False positive	0	1	1
False negative	0	0	0
Positive predictive value	100.00	90.91	90.91
Negative predictive value	100.00	100.00	100.00
Sensitivity %	100.00	100.00	100.00
Specificity %	100.00	95.00	95.00
Accuracy %	100.00	96.67	96.67

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