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RESEARCH ARTICLE

Rapid Detection of *Vibrio parahaemolyticus* Strains and Virulent Factors by Loop-mediated Isothermal Amplification Assays

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Abstract A loop-mediated isothermal amplification (LAMP) method for rapid detection of the foodborne *Vibrio parahaemolyticus* strains and related virulent factors had been developed and evaluated in this study. Six primers, including outer primers, inner primers, and loop primers, were specially designed for recognizing 8 distinct sequences on 3 target genes, which were *tlh*, *tdh*, and *trh*. The detection limits were found to be 100, 100 fg, and 1 pg DNA/tube for *tlh*, *tdh*, and *trh*, respectively. Application of LAMP assays were performed on 368 foodborne *V. parahaemolyticus* strains, the sensitivities of LAMP assays for the *tlh*, *tdh*, and *trh* were 100, 95.6, and 96.4%, and the negative predictive values (NPV) were 100, 84.7, and 93.1%, respectively; with a 100% specificity and positive predictive value (PPV) for all 3 target genes.

Keywords: loop-mediated isothermal amplification (LAMP), *Vibrio parahaemolyticus*, rapid detection

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Introduction

Food safety remained one of the most important global health issues and foodborne diseases caused by microbes were widespread public health problem. It had been reported that approximately 1.8 million people died from diarrheal diseases in 2005, while the estimated global incidence of foodborne disease still remained unknown (1).

Vibrio parahaemolyticus is widely distributed in coastal and estuarine environments throughout the world (2). This organism has been recognized as the causative agent in 50-70% of all cases of diarrhea and one of the major forms of foodborne gastroenteritis, mostly associated with the consumption of raw or undercooked seafood. It can be classified into 13 O serotypes and 71 K serotypes, of which O3:K6 is regarded as the predominant serovar responsible for most outbreaks worldwide and raises great significance. Thermostable direct haemolysin (TDH), an inducing factor of Kanagawa phenomenon, has been considered the major virulence factor of *V. parahaemolyticus*. Studies on clinical strains that were Kanagawa-negative led to the discovery of another important virulence factor, TDH-related

haemolysin (TRH), which is also involved in the production of gastroenteritis as well as TDH. As a species-specific marker for *V. parahaemolyticus*, the thermolabile haemolysin gene (*tlh*), together with the gastroenteritis related pathogenicity genes *tdh* and *trh*, is regarded as useful markers for the detection of this pathogenic organism.

Traditionally, culture-based procedure with enrichment in liquid media and subsequent isolation of colonies on selective culture media was used as diagnostic test for detection of *V. parahaemolyticus* (3,4). While, time consumption as positive identification requiring long as 7 days and false negative results cause by viable but non-cultivable state due to starvation and physical stress, are considered to be two major concerns for this procedure (5). Polymerase chain reaction (PCR)-based assays have been employed and developed for the past decades (6,7). However, despite its high sensitivity and specificity, these methods require post detection procedures, increasing the risk of contamination. Furthermore, the ability of PCR assays to detect low levels of pathogenic *V. parahaemolyticus* bacteria in the presence of a high background of nonpathogenic *V. parahaemolyticus* organism still remained unclear (8). Recently, a number of real-time PCR assays are proposed (5,8) and applied to outbreak investigation for the detection of total and pathogenic *V. parahaemolyticus*. Nevertheless, the requirement for trained personnel, operating space, expensive equipment, and reagents proved to be an obstacle for its broad application. Therefore, development of a rapid, low-cost, easy operating, highly sensitive, and specific detection method is of urgent necessity. Recently, a novel nucleic acid amplification method, designated loop-mediated isothermal amplification (LAMP), had been reported. This method relies on an auto-cycling strand displacement DNA synthesis performed by the Bst DNA polymerase large fragment (9-11), which is different from PCR in that 4 or 6 primers perform the amplification of the target gene (Fig. 1). The amplification uses isothermal conditions between 60-65ºC, and the amplification products are mixtures of many different sizes of stem-loop DNAs with several inverted repeats of the target sequence and cauliflower-like structures with multiple loops (12-14). LAMP constituted a potentially valuable tool for rapid diagnosis of foodborne pathogens.

The current study aimed at developing and evaluating simple and rapid testing methods based on LAMP assays for detection of *V. parahaemolyticus* and related virulent factors, and applying these assays to detection of a large scale of *V. parahaemolyticus* strains from various samples.

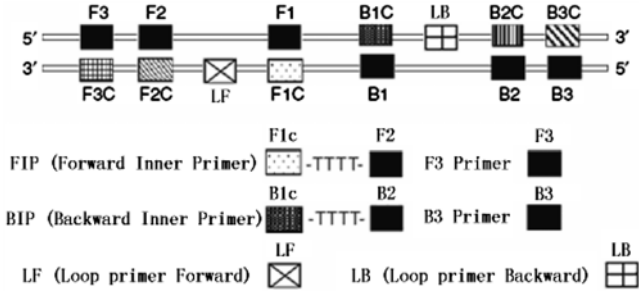

Fig. 1. Schematic diagram of LAMP primers. In detail, 8 distinct regions on every strand were used to design LAMP primers. For inner primers, forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c), a T-T-T-T linker and F2; backward inner primer (BIP) consisted of complementary sequence of B1 (B1c), a T-T-T-T linker and B2. Outer primers F3 and B3 located outside of the F2 and B2 regions, with loop primers LF and LB located between F2 and F1 or B1 and B2, respectively.

Materials and Methods

Bacterial strains Forty-five reference strains, including various species of Gram-negative and Gram-positive isolates, were included in this study to develop and evaluate the specificity and sensitivity of LAMP assays (Table 1). Application of the LAMP assays was performed on a total of 368 *V. parahaemolyticus* strains. These strains were isolated from various food samples during 2003-2007, and had been preliminarily identified in the Lab of Clinical Microbiology.

Primer design Three target genes were selected to differentiate strains. The protocol was designed to detect the *V. parahaemolyticus*- specific *tlh* gene (coding for thermolabile haemolysin), virulence genes *tdh* (coding for thermostable direct haemolysin), and *trh* (coding for TDH-related haemolysin). For each target gene, a set of inner primers (including forward and backward inner primer), outer primers (including F3 and B3), and loop primers (including LF and LB, to accelerate reaction) were designed for LAMP to target 8 distinct regions (Table 2). Forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c), a T-T-T-T linker and F2; backward inner primer (BIP) consisted of complementary sequence of B1 (B1c), a T-T-T-T linker and B2. The outer primers F3 and B3 located outside of the F2 and B2 regions, while loop primers LF and LB located between F2 and F1 or B1 and B2, respectively.

Establishment of LAMP assays Forty-five reference strains were used to develop and evaluate the specificity and sensitivity of LAMP assays. Cultural conditions and DNA extraction of Gram-negative and Gram-positive strains were performed as described previously (15-19). To ascertain the detection limits of LAMP and PCR assays, template DNA from *V. parahaemolyticus* WF04232 was

Table 1. Reference strains used and the results of LAMP assays

Reference strains	No. of strain	LAMP assay		
		tlh	*tdh*	*trh*
Gram-negative organisms				
Vibrio parahaemolyticus O1: K25 WF 04213	1	+	+	-
Vibrio parahaemolyticus O1: K56 WF 02314	1	+	-	+
Vibrio parahaemolyticus O3: K6 WF 04232	1	+	+	+
Vibrio parahaemolyticus O3: K6 WF 01031, WF 04506, WF 06215	3	+	+	-
Vibrio parahaemolyticus O3: K6 WF 02321, WF 03218, WF 05612	3	+	-	+
Vibrio parahaemolyticus O3: K12 WF 02108	1	+	-	-
Vibrio parahaemolyticus O3: K72 WF 02471	1	+	+	-
Vibrio parahaemolyticus O4: K37 WF 01309, WF 04238	2	+	-	+
Vibrio parahaemolyticus O4: K55 WF 03256, WF 07521	2	+	-	+
Vibrio parahaemolyticus O4: K68 WF 07926, WF 03422, WF 02613	3	+	+	-
Vibrio parahaemolyticus O10: K28 WF 06438	1	+	+	-
Vibrio parahaemolyticus ATCC 17802	1	+	-	-
Vibrio cholerae SK10	1	-	-	-
Vibrio vulnificus ATCC 27562	1	-	-	-
Vibrio mimicus ATCC 33653	1	-	-	-
Psuedomonas aeruginosa ATCC 27853	1	-	-	-
Salmonella typhimurium ATCC 14028	1	-	-	-
Salmonella choleraesuis ATCC 13312	1	-	-	-
Klebsiella pneumoniae ATCC 13883	1	-	-	-
Enterobacter cloacae ATCC 23355	1	-	-	-
Acinetobacter baumannii GH31	1	-	-	-
Escherichia coli O157: H7 WF01201 ATCC43889, NCTC12900	3	-	-	-
Escherichia coli O127: H6 WF073522	1	-	-	-
Escherichia coli C600 ATCC 25922, ATCC8739, C600, DH5á	4	-	-	-
Gram-positive organisms				
Staphylococcus aureus ATCC 25923	1	-	-	-
Staphylococcus epidermidis ATCC 29887	1	-	-	-
Enterococcus faecalis GH152	1	-	-	-
Enterococcus faecium GH148	1	-	-	-
Stretococcus pyogenes GH126	1	-	-	-
Stretococcus mitis GH185	1	-	-	-
Stretococcus pnuemoniae GH165	1	-	-	-
Stretococcus hemolyticus GH177	1	-	-	-
Total	45			

diluted for serial 10 fold. LAMP assays was carried out in a total of 25 μL reaction mixture containing 1.6 μM (each) of the primers FIP and BIP, 0.2 μM (each) of the primers F3 and B3, 0.8 μM (each) of primers LF and LB, 1.6 mM of deoxynucleoside triphosphates, 6 mM $MgSO_4$, 1 M betain (Sigma-Aldrich, St. Louis, MO, USA), 1× thermopol buffer (New England Biolabs, Ipswich, MA, USA), and the specified amounts of target genomic DNA. The reaction was heated at 95°C for 3 min, then chilled on ice, 1 μL (8 U) of Bst DNA polymerase (New England Biolabs) was added, after incubation at 65°C for 45 min, the reaction was terminated by heating at 80°C for 2 min. PCR amplification was carried out in a 50 L reaction volume, using the 2 outer primers F3 and B3. The thermal profile for PCR was 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and a final extension cycle at 72°C for 7 min. The amplified products (5 μL/well) were analyzed by gel electrophoresis in 2% agarose gels and stained with ethidium bromide for 10 min. For LAMP assays, the lowest bands from amplicons were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and cloned into the pMD18-T vector (TaKaRa, Shiga, Japan). The sequencing was done by an ABI PRISM 310 genetic analyzer (PE Biosystems, Foster City, CA, USA).

Application of LAMP assays on a large scale of *V. parahaemolyticus* strains Three-hundred and sixty-eight

Table 2. List of oligonucleotide primer sequences

Target	Sequence (5' to 3')	Size	Position
tlh			
F3	CGCTGACAATCGCTTCTCAT	20	486-505
B3	GTTCTTCGCTTTGGCAATGT	20	686-705
FIP	CTGTCACCGAGTGCAACCACTT-AACCACACGATCTGGAGCA	41	508-526, 560-581
BIP	GCATCACAATGGCGCTTCCC-ACCGTTGGAGAAGTGACCTA	40	610-629, 650-669
LF	GTTGATTTGATCTGGCTGCATTG	23	536-558
LB	AACCCGAACAGCTGGTTCT	19	631-649
tdh			
F3	GTTCGAGATACAACTTTTAATACCA	25	130-154
B3	CGTGCTTATAGCCAGACAC	19	322-340
FIP	TGACCATAAACATCTTTGTACGGTT-CACCGGTCAATGTAGAGG	43	158-175, 212-236
BIP	ATCAGTATTCACAACGTCAGGTACT-CGCTGCCATTGTATAGTCTT	45	237-261, 302-321
LF	TTACGGTTTGTCCAAAAGTCAGAGA	25	176-200
LB	AAATGGCTGACATCCTACATGAC	23	262-284
trh			
F3	ACAACAATAAAAACTGAATCACC	23	139-161
B3	GACCGTTGAAAGGCCATC	18	337-354
FIP	ACCGTGAACGCTTTTATATGGT-TCGTTGATGACTACTGGAC	41	173-191, 213-234
BIP	TCACGACTTCAGGCTCAAAATG-AGAGCAGCCATTGTGTAG	40	245-266, 306-323
LF	AAACCGAAACATAAACGA	19	192-210
LB	AGCGCCTATATGACGGTAAACATTA	25	271-295

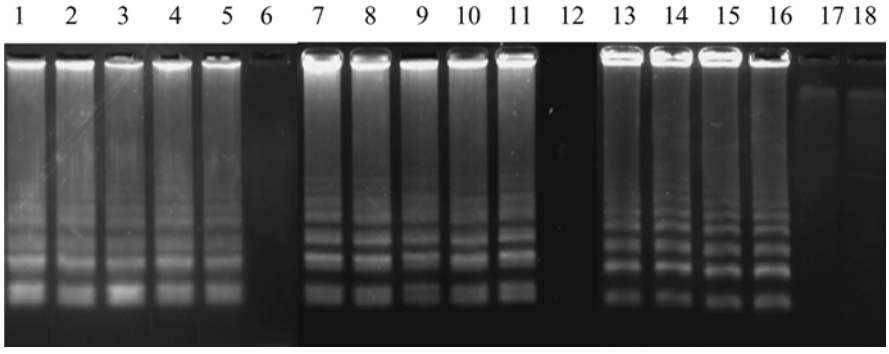

Fig. 2. Sensitivity of LAMP assays for detection of *V. parahaemolyticus* strain WF21201. Lane 1-6, 7-12, and 13-18 referring to LAMP assays of *tlh*, *tdh*, and *trh,* respectively. Lane 1, 7, 13: 1 ng template DNA; lane 2, 8, 14: 100 pg template DNA; lane 3, 9, 15: 10 pg DNA; lane 4, 10, 16: 1 pg DNA; lane 5, 11, 17: 100 fg DNA; lane 6, 12, 18: 10 fg DNA

strains were subjected to detection by LAMP and PCR assays as described above. Template DNA was prepared through a rapid procedure. In detail, overnight Luria-Bertani (LB) broth cultures was diluted 10 fold in 10 mM Tris-HCl (pH 8.0) containing 1 mM ethylenediamine tetraacetic acid (EDTA) and the suspension was boiled for 10 min and kept on ice. After centrifugation at 12,000×g for 3 min, the resulting supernatant was used as templates for LAMP and PCR assays. Heating and isothermal amplification were performed on water bath and heating block. Amplification products of LAMP assay were dyed with Sybr Green, positive or negative were determined through both visually observation of the color change by naked eye and a fluorescence assay under ultraviolet (UV). PCR amplicons were determined as mentioned above. This experiment was performed twice to ensure reproducibility.

Results and Discussion

Optimization of the conditions of LAMP assays In order to determine the optimal conditions of LAMP, DNA from *V. parahaemolyticus* strain WF04232 was used as target template. The specific amplification generated many

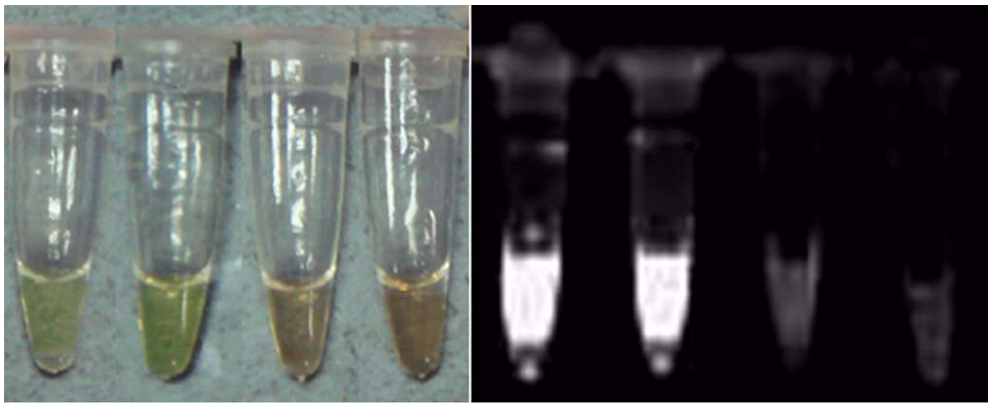

Fig. 3. Amplification products of LAMP assays were visually detected both by observation at the color change by naked eye and by a fluorescence assay under UV.

ladder-like pattern bands on agarose gel due to its characteristic structure, sizes ranges from 220 bp for *tlh*, 211 bp for *tdh*, and 216 bp for *trh*, respectively, up to the loading wells. LAMP assays were under isothermal condition between 60 and 65ºC. No significant difference were observed, however, the LAMP product amplified at 65ºC showed slightly larger amount of DNA when compared to other temperatures (data not shown), which was consistent with studies previously . Reaction lengths of LAMP assays were varied in 15, 30, 45, 60, 75, 90, 105, and 120 min, under 65ºC, with 10 ng template DNA. Without loop primers, amplification products could not be observed until 90 min. While with loop primers, the amplification was initially detected at 30 min, and reached maximal at 45 min. LAMP assays were performed with omission of 1 or 2 of the primers, under 65ºC for 45 min. However, no amplification could be observed in the absence of FIP, BIP, F3, or B3 primer. For each of the primers plays an indispensable role in auto-cycling strand placement and forming the loop out structure. The LAMP was only performed in the existence of both inner primers and outer primers.

Sensitivity and specificity of LAMP assays The detection limits of LAMP and PCR assays were found to be 100 fg DNA/tube and 10 pg DNA/tube for *tlh*, 100 fg DNA/tube and 10 pg DNA/tube for *tdh*, and 1 pg DNA/tube and 100 pg DNA/tube for *trh,* respectively, indicating that LAMP was at least 100 fold more sensitive than PCR (Fig. 2). Sizes ranges from 220 bp for *tlh*, 211 bp for *tdh*, and 216 bp for *trh*, respectively, up to the loading wells. Sequences of the bottoms bands were identical to those PCR amplified with F3 and B3. LAMP assays were under isothermal condition between 60 and 65ºC. To determine specificity of the primers, LAMP assays were also subjected to 37 Gram-negative and 8 Gram-positive isolates. No false positive amplification was observed, indicating high specificity of the established LAMP assays.

Application of LAMP assays on *V. parahaemolyticus* isolates The established LAMP assays were applied to detect 368 *V. parahaemolyticus* strains using simple equipment and observed directly by naked eye and under UV light (Fig. 3). Of these strains, 368, 283, and 238 strains were detected to be positive for *tlh*, *tdh*, and *trh* by LAMP respectively, with 342, 245, and 208 were detected by PCR. The sensitivity of LAMP assays for the *tlh*, *tdh*, and *trh* was 100, 95.6, and 96.4%, and the negative predictive value (NPV) was 100, 84.7, and 93.1%, respectively; with a 100% specificity and positive predictive value (PPV) for all 3 targets. Comparing with PCR method, LAMP was advantageous on sensitivity and NPV, while high specificity and PPV were obtained by both assays (Table 3).

V. parahaemolyticus is a foodborne pathogen throughout the world. Rapid and accurate enumeration methods are highly needed for evaluation of post harvest treatments to reduce risk of *V. parahaemolyticus* caused by infection and contamination (1). This study aimed to establish simple and rapid testing methods based on LAMP assays for detection of *V. parahaemolyticus* strains and its related virulent factors. Until recently, conventional PCR and real-time PCR approaches had been found to be useful techniques for detection of *V. parahaemolyticus* strains. However, PCR was technically demanding and requires several hours for a complete diagnosis. It was even known that some inhibitors in samples reduce the sensitivity of PCR when attempting to detect a target gene (20). Real-time PCR assay had many advantages over conventional PCR, including rapidity, lower contamination rate, higher sensitivity, and easy standardization. However, demanding operation and expensive kits and equipment restricted its application to clinical laboratories (21). Other isothermal amplification techniques, such as nucleic acid sequence-based amplification (NASBA) and the self-sustained sequence reaction (SSR), were reported to be less specific owing to the low stringency (40ºC) and, thus, requiring either a precision instrument for amplification or an

Table 3. Comparison of LAMP and PCR assays on *V. Parahaemolyticus* strains isolated from food samples

	V. parahaemolyticus [1)]	LAMP vs PCR assay (in percentage)			
		Sensitivity	Specificity	PPV	NPV
No. of isolate	368				
tlh	368/368/342	100/92.9	100/100	100/100	-/-
tdh	296/283/245	95.6/82.8	100/100	100/100	84.7/58.5
trh	247/238/208	96.4/84.2	100/100	100/100	93.1/75.6

[1)]The 3 number referring to preliminary identified data using standard culturing method, results detected by LAMP assays and PCR assays, respectively.

elaborate method for detection of the amplified products due to the poor specificity of target sequence selection. As a novel nucleic acid amplification method, LAMP was known as a rapid, specific, sensitive, cost-effective, and easy-operating alternative for detection of clinical pathogens. With inner and outer primers recognizing 6 distinct regions, and with the reaction under isothermal without thermal cycler, LAMP was bestowed by high sensitivity, specificity, and amplification efficiency. Significance and interest in *V. parahaemolyticus* had been rising with the emergence of pandemic strains that resulted in outbreaks of unprecedented magnitude and the geographical expansion of *V. parahaemolyticus* illnesses (8). Pathogenic *V. parahaemolyticus* from various food samples usually occur at low levels with a background of nonpathogenic *V. parahaemolyticus* strains and related species, proposing a great challenge for the detection of PCR, which may not allow the detection of low numbers of pathogenic strains. In this study, detection limits of LAMP assay were found to be 100 fg (*tlh*, *tdh*) or 1 pg (*trh*) of template DNA, showing 100 times more sensitive than PCR assay, which may expectably fulfill the requirement of low level detection of pathogenic *V. parahaemolyticus* strains. In addition, LAMP assay was less affected by various components of clinical samples (22), as consequence, LAMP assays showed advantage on sensitivity especially when simple and rapid DNA purification methods were applied. For the application experiments, sensitivities of 100, 95.6, and 96.4% for *tlh*, *tdh*, and *trh* were found for LAMP, comparing with 92.9, 82.8, and 84.2% for PCR assay, which demonstrated LAMP assays developed in the current study were also optimized for the detection of low levels of *tlh*, *tdh*, and *trh*. Furthermore, LAMP had advantages on easiness and rapidness in operation of reaction and determination of result. The total detection time in the application assays, including DNA preparation, LAMP reaction and results observation, was approximately 60 min. As aforementioned, the obstacle for the abroad application of real-time PCR, NASBA, and SSR was requirement for expensive reagents and precise equipment. However, only simple equipment as water bath was required for the operation of LAMP assay, which was also costless. In conclusion, LAMP assays were demonstrated to be useful and powerful methods for detection of *V. parahaemolyticus* strains and its related virulent factors, which also constituted potentially valuable tools for rapid diagnosis of foodborne pathogens in both commercial and clinical fields.

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