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Full Length Research Paper

Application of an improved loop-mediated isothermal amplification detection of *Vibrio parahaemolyticus* from various seafood samples

Li Wang[1], Xihong Zhao[2], Jin Chu[3], Yue Li[1], Yanyan Li[4], Chunhou Li[5*], Zhenbo Xu[2,6*] and Qingping Zhong[1]

[1]Food Safety Key Laboratory of Guangdong Province, College of Food Science, South China Agricultural University, Guangzhou 510642, China.
[2]College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China.
[3]School of Food Science and Nutrition, Faculty of Mathematics and Physical Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom.
[4]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
[5]South China Sea Fisheries Research Institute, CAFS; Key Laboratory of South China Sea Fishery Resources Exploitation, Ministry of Agriculture, Guangzhou 510300, China.
[6]Department of Microbial Pathogenesis, Dental School, University of Maryland-Baltimore, Baltimore, MD 21201, USA.

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The specificity and sensitivity of an improved loop-mediated isothermal amplification (LAMP) method for rapid detection of *Vibrio parahaemolyticus* strains from various seafood samples had been developed and evaluated in this study. Six primers, including outer primers and inner primers, were specially designed for recognizing six distinct sequences on the target gene of *tlh*. The optimal reaction condition was found to be 65°C for 45 min, with the detection limit as 10 CFU/ml. Application of LAMP assays was performed on 416 food borne *V. parahaemolyticus* strains isolated from various seafood samples, and the total detection rate for LAMP and polymerase chain reaction (PCR) assay was found to be 96.2% (400/416) and 85.6% (356/416). This is the first report of an improved and simple LAMP detection assay on *V. parahaemolyticus* employed procedures of simple template deoxyribonucleic acid (DNA) preparation, equipment for LAMP reaction (water bath) and direct result determination via observation of color change. In addition, this is also the first application of LAMP detection on this considerable amount of *V. parahaemolyticus* isolates (416 strains for application together with 105 reference strains for establishment) with the total identification rate as 96.2%, as well as its extensive application to marine fish, shrimp, oyster, mussel, jellyfish, cuttlefish and seaweed samples, with detection rate ranging from 89.5 to 100%.

Key words: Loop-mediated isothermal amplification (LAMP), *Vibrio parahaemolyticus*, seafood samples.

INTRODUCTION

Food safety remained one of the most important global health issues and food-borne 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 food-borne disease still remained unknown (Rasekh et al., 2005). As a major concern in food-borne microbes, *Vibrio parahaemolyticus* is a halophilic, Gram-negative bacteria, and has been recognized as the causative agent in 50 to 70% of all cases of diarrhea and one of the major forms of food-borne gastroenteritis, mostly associated with the

*Corresponding author. E-mail: scslch@vip.163.com zhenbo.xu@hotmail.com. Tel: +862087113843. Fax: +862087113843.

consumption of raw or undercooked seafood (Ono et al., 2006). *V. parahaemolyticus* is generally found in marine environment, and 60 to 100% of seafood samples in the USA had been reported to be contaminated with the bacteria. In recent years, global increasing incidence of *V. parahaemolyticus* infection had been reported, which prompted interest in developing fast and sensitive methods for detection of this pathogenic microorganism from seafood samples (Deepanjali et al., 2005; Matsumota et al., 2000). Conventionally, 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*. In addition, this organism exists in food samples at levels of around 10^2 to 10^3 CFU/g. Time consumption as positive identification requiring long as 7 days, as well as 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.

PCR and real-time PCR-based assays have been employed and developed for the past decades (Zhao et al., 2010). However, required post detection procedures, high risk of contamination, low levels detection limit for PCR, and requirement of trained personnel and operating space, expensive equipment and reagents for real-time PCR proved to be an obstacle for their broad application. To overcome these obstacles, rapid and sensitive detection methods are needed. Recently, a novel nucleic acid amplification method, designated LAMP, had been reported. This method relies on an auto-cycling strand displacement DNA synthesis performed by the Bst DNA polymerase large fragment (Wang et al., 2008a, b; 2011), which is different from PCR in that four or six primers perform the amplification of the target gene. The amplification uses isothermal conditions between 60 to 65°C, 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. LAMP constituted a potentially valuable tool for rapid diagnosis of food-borne pathogens (Xu et al., 2011c). In the current study, we develop a simple and rapid LAMP assay and apply it to detection on a large scale of *V. parahaemolyticus* strains from 7 common seafood samples, in which only visual observation of color changes was employed for the confirmation of results and approximately 60 min was needed for the whole process.

MATERIALS AND METHODS

Bacterial strains

105 Reference strains, including various species of Gram-negative and -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 416 *V. parahaemolyticus* strains, the strains of which were isolated from various food samples during 2003 to 2010.

Primer design

The thermolable haemolysin gene (*tlh*), regarded as useful markers for the differentiation of *V. parahaemolyticus* and non- *V. parahaemolyticus* strains, had been selected as the genus specific target in this study. A set of 6 primers was designed for LAMP to target 8 distinct regions. Forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c), a T–T–T–T linker and F2 (5'-CCACCAGTAGCCGTCAATGGTGGCGACCGATTGGGAATGG-3'); backward inner primer (BIP) consisted of complementary sequence of B1 (B1c), a T–T–T–T linker and B2 (5'-ACACCAACACGTCGCAAAACGTGCGTTCTCGTTCGCCAAAT-3'). The outer primers F3 (5'-CGCACCAGCTACTCGAAAG-3') and B3 (5'-CGGCGAAGAACGTAATGTCT-3') located outside of the F2 and B2 regions, while loop primers LF (5'-AGTAGCTACCTACTTCGT-3') and LB (5'-CGTCAGCGTTGTGAAGCAAC-3') located between F2 and F1 or B1 and B2, respectively.

Establishment of Lamp assay

105 Reference strains, including 68 Gram-negative and 37 -positive strains were subjected to develop and evaluate the specificity and sensitivity of LAMP assay. Cultural conditions and DNA extraction of Gram-negative and Gram-positive strains were performed as described previously (Xu et al., 2007, 2008a, b, 2009, 2010, 2011a, b). To ascertain the detection limits of LAMP and PCR assays, template DNA from *V. parahaemolyticus* V16 was diluted for serial 10-fold. The detection limits of LAMP and PCR assays were ascertained by both minimal CFU of bacterial and template DNA amount. LAMP and PCR assays was carried out as described previously (Wang et al., 2008a, b, 2011d; Yamazaki et al., 2008, 2010). 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, St. Louis, MO, USA), 1 X 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, Ipswich, MA, USA) 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 µl reaction volume, using the two 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 s, 50°C for 30 s, and 72°C for 30 s 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 assay, the lowest band from amplicons was cut out from the agarose gel, purified by the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and determined by BigDye Terminator Cycle Sequencing FS Ready Reaction Kit on ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Japan Applied Biosystems, Tokyo, Japan). Nucleotide sequence homology searches were performed against all sequences in the GenBank database by using the basic local alignment search tool (BLAST) algorithm, which is available through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Application of LAMP assay on various seafood samples

Application of the optimized LAMP and PCR assays was performed

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

Reference strain	No	Culture	PCR	LAMP
Gram-negative organisms				
Vibrio parahaemolyticus O1: K25 WF 04213	1	+	+	+
Vibrio parahaemolyticus O1: K56 WF 02314	1	+	+	+
Vibrio parahaemolyticus O3: K6 WF 04232, WF 01031, WF 04506, WF 06215, WF 02321, WF 03218 and WF 05612	7	+	+	+
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 and WF 02613	3	+	+	+
Vibrio parahaemolyticus O10: K28 WF 06438	1	+	+	+
Vibrio parahaemolyticus ATCC 17802, V16, CGMCC1.1614	3	+	+	+
Vibrio harveyi ATCC 14126	1	-	-	-
Vibrio cholerae SK10	1	-	-	-
Vibrio vulinficus ATCC 27562	1	-	-	-
Vibrio mimicus ATCC 33653	1	-	-	-
Vibrio alginolyticus ZJ51	1	-	-	-
Psuedomonas aeruginosa ATCC 27853, P81	2	-	-	-
Salmonella typhimurium ATCC 14028, WF 04313	2	-	-	-
Salmonella choleraesuis ATCC 13312	1	-	-	-
Salmonella enteritidis WF 05148, WF 07086, HB009, HB215, HB371, HB143, HB069	2	-	-	-
Salmonella typhi WF 03201, WF 05026 and WF 08138	3	-	-	-
Salmonella paratyphi WF 06426	1	-	-	-
Salmonella aberdeen WF 04542	1	-	-	-
Salmonella gallinarum WF 05938	1	-	-	-
Klebsiella pneumoniae ATCC 13883	1	-	-	-
Enterobacter cloacae ATCC 23355	1	-	-	-
Acinetobacter baumannii GH31	1	-	-	-
Escherichia coli O157: H7 WF01201 ATCC43889, NCTC12900, WF07803, WF06544, WF05395, WF04402, WF05311, WF06837 WF04587, WF08385, WF06349	12	-	-	-
Escherichia coli O26: H11 WF054489	1	-	-	-
Escherichia coli O127: H6 WF073522	1	-	-	-
Escherichia coli O148: H28 WF063224	1	-	-	-
Escherichia coli C600 ATCC25922, ATCC8739, C600, DH5α, 240	5	-	-	-
Gram-positive organisms		-	-	-
Listeria monocytogenes WF 03213, WF 06410, WF 08211, 112	4	-	-	-
Listeria invanovii WF 06319	1	-	-	-
Listeria welshimeri WF 05086	1	-	-	-
Listeria seeligeri WF 04426	1	-	-	-
Staphylococcus aureus ATCC25923, ATCC29213, 10442, COL, N315, 85/2082, CA05, JCSC 1978, JCSC 4469, MR108, M03-68, WIS, 200	13	-	-	-
Staphylococcus epidermidis ATCC29887, ATCC700586, 042219, ATCC12228, 012217	5	-	-	-
Staphylococcus hominis 032315, 042306, 012307, 80	4	-	-	-
Staphylococcus warneri 012501, 012502	2	-	-	-
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	105			

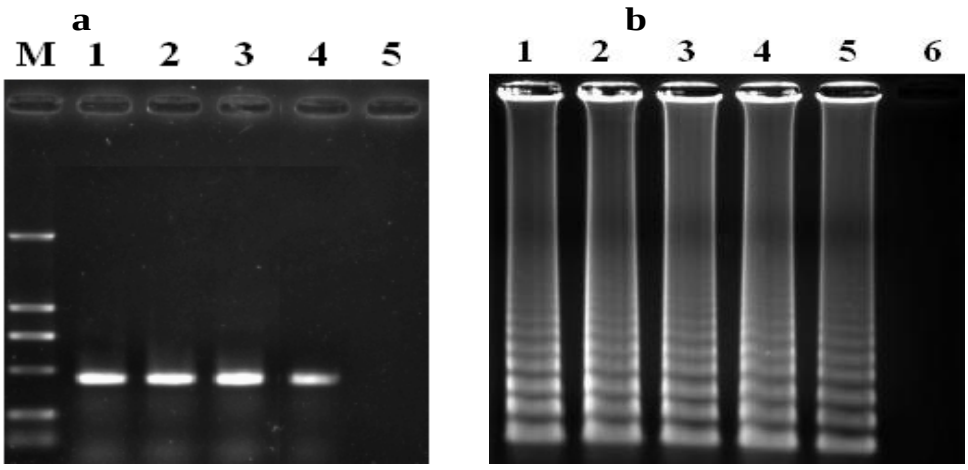

Figure 1. Comparison of detection sensitivity between LAMP; **a** and PCR **b**; M, 2-kb DNA ladder as size markers; lanes 1 to 6 correspond to 10^6 CFU/ml, 10^5 CFU/ml, 10^4 CFU/ml, 10^3 CFU/ml, 10^2 CFU/ml, 10 CFU/ml, respectively.

on 416 food borne *V. parahaemolyticus*, which had been isolated from various seafood samples including 160 marine fish (38.5%), 92 shrimp (22.1%), 66 oyster (15.9%), 38 mussel (9.1%), 26 jellyfish (6.3%), 19 cuttlefish (4.6%) and 12 seaweed (2.9%) samples. Sample processing and template DNA preparation had been performed through a rapid procedure. In detail, 25 g sample were added to 225 ml of 3% alkaline peptone water (10 g peptone, 10 g NaCl and distilled water to 1000 ml; pH 8.4) and grinded, then 10 ml meat emulsion were inoculated into 100 ml 7.5% sodium chloride (NaCl) enrichment medium (20 g peptone; 75 g NaCl; 5 ml 0.01% crystal violet solution and distilled water to 1000 ml) at 37°C for 12 h with shaking at 180 rpm. Following centrifugation at 1,500 rpm for 2 min, the supernatant was suspended in Luria-Bertani (LB) broth cultures diluted 10 fold in 10 mM Tris-HCl (pH 8.0) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and was then 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 visual observation of the color change by naked eye and a fluorescence assay under ultraviolet (UV). This experiment was performed twice to ensure reproducibility. Bacterial were identified to the species level using regular "gold standard" procedure. In brief, the enrichment broth was then sub-cultured onto thiosulfate-citrate-bilesalt-sucrose (TCBS) agar (OXOID, England) and CHROMagar Vibrio (CV) agar (CHROMagar, France) plates. After incubation at 37°C overnight, green or blue-green colonies, 2 to 3 mm in diameter on TCBS agar plates and violet colonies on CV agar plates, were presumptively selected as *V. parahaemolyticus* (Matsumota et al., 2000; Ono et al., 2006). The selected colonies were confirmed by their colonial characteristics after transferring the same colony to fresh TCBS and CV agar plates using sterile toothpicks. After incubation overnight at 37°C, characteristic colonies of *V. parahaemolyticus* were selected. This experiment was performed twice to ensure reproducibility.

RESULTS

Optimization of the conditions of LAMP assay

In order to determine the optimal conditions of LAMP, DNA from *V. parahaemolyticus* strain V16 was used as target template. The specific amplification generated many ladder-like pattern bands on agarose gel due to its characteristic structure up to the loading wells, with a 211-bp size amplicon obtained. LAMP assays were under isothermal condition between 60 and 65°C. No significant difference were observed, however, the LAMP product amplified at 65°C showed slightly larger amount of DNA when compared to other temperatures (data not shown), which was consistent with studies previously (Wang et al., 2008a, b, 2011; Yamazaki et al., 2008, 2010). Reaction lengths of LAMP assays were varied in 15, 30, 45, 60, 75, 90, 105 and 120 min, under 65°C, with 10 ng template DNA. Without loop primers, amplification products could not be observed until 60 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 one or two of the primers, under 65°C 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 assay

The detection limits of LAMP assay was found to be 1 pg DNA/tube and 10 CFU/reaction (LAMP was positive for sample containing 10^4 CFU/ml, with 1 µl was included in the reaction system), and PCR was 100 pg DNA/tube and 10^3 CFU/reaction, respectively, indicating that LAMP was 100-fold and is more sensitive than PCR (Figure 1). Simultaneously, high specificity was acquired when LAMP assay was subjected to 105 reference strains, with no false positive amplification observed (Table 1).

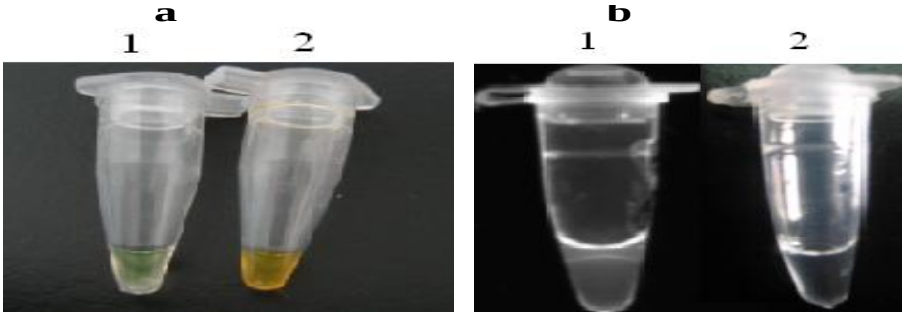

Figure 2. Visual inspections of LAMP products. LAMP reactions detected by white turbidity of the tube caused by magnesium pyrophosphate, a by-product of LAMP; **a**.The original orange color of SYBR Green I turned to be green in the positive reaction mixture; **b**. 1, *V. parahaemolyticus* positive; 2, *V. parahaemolyticus* negative.

Table 2. Evaluation of LAMP and PCR for detection of isolated samples.

Sample	Positive results[a]	LAMP versus PCR assays (in percentage)
		Sensitivity (%)
Marine fish	160, 154 and 136	96.3 and 77.5
Shrimp	92, 91 and 84	98.9 and 91.3
Oyster	66, 62 and 56	93.9 and 84.8
Mussel	38, 34 and 28	89.5 and 73.7
Jellyfish	26, 25 and 22	96.2 and 84.6
Cuttlefish	22, 22 and 20	100 and 91.0
Seaweed	12, 12 and 10	100 and 83.3
total	416 , 400 and 356	96.2 and 85.6

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

Application of the LAMP assay on seafood samples

The established LAMP and PCR assays were applied to detect 416 food borne *V. parahaemolyticus* strains isolated from various seafood samples using a simple DNA preparation process, and results confirmed by observation directly by naked eye and under UV light (Figure 2). The positive results yielded by LAMP and PCR were 154 (96.3%, 154/160) versus 136 (77.5%, 136/160) for marine fish, 91 (98.9%, 91/92) versus 84 (91.3%, 84/92) for shrimp, 62 (93.9%, 62/66) versus 56 (84.8%, 56/66) for oyster, 34 (89.5%, 34/38) versus 28 (73.7%, 28/38) for mussle, 25 (96.2%, 25/26) versus 22 (84.6%, 22/26) for jellyfish, 22 (100%, 22/22) versus 20 (91.0%, 20/22) for cuttlefish and 12 (100%, 12/12) versus 10 (83.3%, 10/12) for seaweed, with the total detection rate as 96.2% (400/416) and 85.6% (356/416), respectively (Table 2).

DISCUSSION

The improved LAMP assay was demonstrated to be a useful and powerful tool for rapid detection of food borne *V. parahaemolyticus* strains from various seafood samples. Up to date, some reports on LAMP detection of *V. parahaemolyticus* are available. In general, Yamazaki et al. (2008) had performed LAMP assay for the detection of the species specific gene and related virulent genes of *V. parahaemolyticus*, as well as determination of this assay using artificially contaminated shrimp samples (Yamazaki et al., 2008, 2010). Nemoto et al. (2009) and Chen and Ge (2010) had reported the LAMP assay detection of *V. parahaemolyticus* from various samples including seafood and clinical samples (Chen and Ge, 2010; Nemoto et al., 2009). However, limitation by the requirement of elaborate reagents for DNA process and expensive equipment for result determination had posed a dilemma for broad application. As application was concerned, evaluation and assessment of a series of attributes should be considered and compared, including cost, rapidity, easiness and sensitivity as well as specificity.

Comparing with previously reported LAMP assays for the detection of *V. parahaemolyticus* strain, this present improved LAMP method offers comparable sensitivity and specificity, as well as particular advantages on cost-effectiveness, easiness in operation, time consumption,

as well as the broadness and flexibility of application. In the current study, 105 reference strains had been included to ensure sensitivity and specificity, and for rapidity, the whole procedure, including DNA preparation, LAMP reaction and results determination, required approximately 60 min with acceleration by loop primers (Zhao et al., 2011, 2010a, b, c, d), which included one of the fastest LAMP reaction system. As far as cost and easiness were concerned, LAMP assay was applied to seafood samples, with high detection rate as 96.2% obtained. Comparing, elaborate equipment as real-time turbid meter (LA320) was required in currently available reports. As far as we can recall, this is the first report of an improved and simple LAMP detection assay on *V. parahaemolyticus* employed procedures of simple template DNA preparation, equipment for LAMP reaction (water bath) and direct result determination via observation of color change, together with acquirement of high sensitivity and specificity (for 105 reference strains), rapidity (30 to 45 min for LAMP reaction and approximately 60 min for the whole procedure), cost-effectiveness and easiness. In addition, as our knowledge can reach, this is also the first application of LAMP detection on this considerable amount of *V. parahaemolyticus* isolates (416 strains for application together with 105 reference strains for establishment) with the total identification rate as 96.2%, as well as its extensive application to marine fish, shrimp, oyster, mussel, jellyfish, cuttlefish and seaweed samples, with detection rate ranging from 89.5 to 100%. Undoubtedly, rapidness, easiness, cost-effectiveness, broadness and flexibility in application of this present LAMP assay will aid in the broad application of bacteriological detection of *V. parahaemolyticus*.

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