Food Sci. Biotechnol. 21(1): 101-106 (2012)
DOI 10.1007/s10068-012-0012-6

RESEARCH ARTICLE

Rapid Detection of Food-borne *Listeria monocytogenes* by Real-time Quantitative Loop-mediated Isothermal Amplification

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Received: 4 August 2011 / Revised: 7 November 2011 / Accepted: 8 November 2011 / Published Online: 29 February 2012

Abstract The purpose of this study was to develop a real-time quantitative loop-mediated isothermal amplication (LAMP) method for the rapid, sensitive, and convenient detection of *Listeria monocytogenes* in food. The LAMP method could amplify the *hlyA* gene of *L. monocytogenes* successfully at 63°C with a loopamp real-time turbidimeter. The detection limits of the LAMP for *hlyA* gene were 6 colony forming units (CFU)/tube. A standard curve was generated for *L. monocytogenes* LAMP by plotting the graph based different log CFU values of *L. monocytogenes* and time of positivity through real-time monitoring of the amplication. Then, the LAMP method was employed to test 94 retail food samples effectively. Sensitivity in detection of *L. monocytogenes* by the LAMP was higher than that of PCR and none of the conventional method-positive samples was missed by the LAMP method.

Keywords: *Listeria monocytogenes*, loop-mediated isothermal amplication (LAMP), real-time turbidimeter, quantitative

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Introduction

Listeria monocytogenes is a food-borne pathogen widely distributed in nature. It may cause a symptom of flu-like illness or severe complications such as meningitis, septicaemia, and spontaneous abortion. Despite its low incidence, food-borne *L. monocytogenes* may manifest severe infection in immunocompromised persons such as pregnant women, newborns, and the elderly (1). Infections are usually caused by ingestion of foods contaminated by *L. monocytogenes*.

Traditional culture-based methods for detecting *L. monocytogenes* are reliable but labor intensive and time-consuming, demanding several days for a definitive result (2,3). Molecular-based methods for identification of *L. monocytogenes* with reduced detection time and cost and improved performance, would be boon to the food testing laboratories. Many articles have been published on the detection of *L. monocytogenes* by PCR, using primers targeting virulence and non-virulence factors, including hemolysin (*hly*), invasion associated protein (*iap*), and 16S rRNA genes (4-6). PCR-based methods, in particular real-time PCR, have increased speed and specificity of identification of food-borne pathogens. Nonetheless, these assays require a dedicated real-time PCR or PCR machine, which is rather expensive and not yet widely available (7,8).

The loop-mediated isothermal amplication (LAMP) method was originally developed by Notomi *et al*. (7). LAMP is simpler than PCR, using only a water bath or heat block, and is highly convenient because the reaction is isothermal and requires no time for thermal changes. It can amplify specific sequences of DNA under isothermal conditions between 63 and 65°C. In addition, a positive result of LAMP can be easily judged by the naked eye through a color change of the reaction mixture with SYBR green I.

Countries including the United States, Australia, New Zealand, and China apply a zero-tolerance policy (absent in 25 g/mL of food) for *L. monocytogenes* in foods (9). In 2006, new EU legislation (EC No2073/2005) was introduced requiring absence of *L. monocytogenes* in 'ready to eat' (RTE) food for certain consumer groups and allowing limits of 100 CFU/g in other categories of RTE foods (10). In most cases, *L. monocytogene*s in food is enumerated using most probable number (MPN) methods, direct enumeration by spread plating on selective agar, or semi quantitative determination. However, an adequate and convenient enumeration method is still lacking, which is essential to provide reliable data for both research studies and routine analysis.

In this study, we developed a real-time quantitative LAMP method for the rapid, sensitive, and convenient detection of *L. monocytogenes* in food. A standard curve was generated for LAMP method by plotting the graph between different concentrations of virus ranging from 2×10^8 to 2×10^3 CFU to time threshold through real-time monitoring of the amplication. The quantication of *L. monocytogenes* load in the positive samples was extrapolated on the basis of their time threshold by employing the standard curve. Then, 94 retail food samples, comprising a large variety of food types, were selected for the detection of *L. monocytogenes* using both the LAMP method established here and the method of ISO11290-1 (3). The result of real-time quantitative LAMP was compared to PCR and the traditional methods.

Materials and Methods

Bacterial culture and growth condition As a control, 107 known bacterial strains, including 96 strains of *Listeria* and 11 non-*Listeria* strains were used in this study (Table 1). All *L. monocytogenes* were kindly donated by Hebei Province Center for Disease Control and Prevention, China. All other bacterial species and strains were laboratory stocks previously obtained from culture collections.

Listeria strains were grown on TSB-Y broth/agar plates [3% tryptone soy broth, 0.6% yeast extract (2% agar for plates)] incubated at 30°C overnight. All non-*Listeria* were grown in liquid Luria-Bertani (LB: 5 g NaCl; 5 g yeast extract; 10 g tryptone and H_2O to 1,000 mL) broth/agar plates at 37°C overnight. Serial 10-fold dilutions of an overnight culture in TSB-Y broth of *L. monocytogenes* were prepared. One-hundred μL of each dilution of *L. monocytogenes* broth series were spread in triplicate on TSB-Y agar plates and colonies on these plates were counted after incubation for 48 h at 37°C. All media and chemicals were purchased from Huankai Co., Ltd., (Guangzhou, China).

Preparation of food samples Ninety-four retail food samples were randomly acquired from local grocery stores and analyzed within the products' shelf life. Four different types of samples including raw meat (fresh grade legs, beef, and pork), deli (boiled salted duck and preserved meat), vegetables (spinach and coleslaw), and seafood (smoked salmon and oysters) were investigated in this study. Twenty-five g food samples were added to 225 mL of half-Fraser broth, in sterile plastic bagmixer filter bag and homogenized in a BagMixer® (400VW; Interscience, Saint Nom, France) for 2 min. After homogenization, the mixture was incubated at 30°C for 22 h on a rotary shaker at 180 rpm. Then, 100 μL of primary enrichment were inoculated into 10 mL Fraser broths and incubated at 37°C for 4 h on a rotary shaker at 180 rpm. In parallel, 100 μL of secondary enrichment culture was spread onto TSB-Y agar plates.

Preparation of positive control food samples One sample served as a 'naturally contaminated' control. Food samples (25 g or mL food) previously shown to be *L. monocytogenes* negative using ISO 11290-1 were added to 225 mL of half-Fraser broth and homogenized as described earlier. To generate the artificially contaminated samples as positive control, 100 μL 10^{-7} dilution (28 CFU/mL) of *L. monocytogenes* strain ATCC19115 were added into the homogenized sample. Then, the articial contaminated samples were treated as described earlier.

DNA extraction DNA was extracted from 1.5 mL aliquots of the secondary enrichment cultures for LAMP assay. Bacterial cells in the culture were pelleted by centrifugation at 12,000×*g* for 3 min, and were resuspended in 100 μL of TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA; Sigma-Aldrich, St. Louis, MO, USA). Then the suspension was heated at 100°C for 10 min to extract DNA (11). After centrifugation at 12,000×*g* for 3 min, the supernatant was saved for use as the DNA template. For the sensitivity test, serial dilutions of an overnight culture of *L. monocytogenes* strain ATCC19115, ranging from 2.8×10^1 to 2.8×10^8 CFU/mL (plate counts were performed to estimate the cell numbers of *L. monocytogenes*) were prepared. The DNA was extracted as described earlier. A negative control reaction was performed using sterile water instead of the DNA template.

PCR reaction In order to compare the sensitivity of LAMP assay, conventional PCR was performed with 2 outer primers F3 and B3 (Table 2). The 25 μL volume reaction mixture contained PCR buffer, 0.2 mM of each dNTP, 12.5 pmol of each PCR primer, 0.25 μL (5 U/μL) of *Taq* DNA polymerase (TaKaRa Biotech, Dalian, China) and 2 μL of DNA template. After a 5 min denaturation at

94°C, the PCR mixtures were subjected to 30 cycles of amplification at 94°C for 30 s, 53°C for 45 s and 72°C for 45 s and a final extension cycle at 72°C for 10 min (12) using a thermal cycler (ABI 2720; Applied Biosystems, Carlsbad, CA, USA). The PCR products were electrophoresed on a 1.5% agarose gel, and the target bands were visualized by staining with ethidium bromide.

Real-time quantitative LAMP reaction The LAMP reaction was conducted as described by Notomi *et al*. (7). A set of LAMP primers, indicated in Table 2, were nominated from the nucleotide sequence of *hlyA* by PrimerExplorer V4 software.

The LAMP reaction was carried out in a total 25 μL reaction volume using the mixture containing 50 pmol each of the primer, FIP and BIP, 5 pmol each of the outer primers F3 and B3, 1.4 mM deoxynucleoside triphosphates, 0.8 M betaine, 0.1% Tween 20, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 10 mM KCl, 20 mM Tris-HCl (pH 8.8, Sigma-Aldrich), 8 units of *Bst* DNA polymerase (New Eanland Biolabs, Beijing, China), and 2 μL of DNA template. The real-time monitoring of the LAMP assay was accomplished by incubating the reaction mixture at 63°C for 60 min in a Loopamp real-time turbidimeter (LA-320C; Eiken Chemical Co., Ltd., Tokyo, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Detection of LAMP products The real-time monitoring of the LAMP amplification of the *L. monocytogenes* DNA template was observed through spectrophotometric analysis by recording the optical density at 650 nm every 6 s with the help of a Loopamp real-time turbidimeter. The cutoff value for positivity by real-time LAMP assay was determined by time threshold (Tt) at which the turbidity increases above the threshold value fixed at 0.1, which is about 2 times more than average turbidity value of the negative controls (14,15).

In order to ensure the application of the LAMP assay, the monitoring of LAMP amplification also could be visualized by the naked eye. LAMP amplification in the reaction mixture was generally observed by eye upon addition to the mixture of 1.0 μL of 10-fold diluted SYBR Green (Biovision, Xiamen, China). In the case of positive amplification, the original orange color of the dye turns to green, which can be judged under natural light (16) by naked eye.

Table 1. Bacterial strains used in this study

Organism	Strain designation[1)]	Number of strain
Listeria monocytogenes	1-90	90
	ATCC 19115	1
	NCTC 7973	1
L. innocua	NCTC 11288	1
L. ivanovii	NCTC 11846	1
L. welshimeri	NCTC 11857	1
L. seeligeri	NCTC 11856	1
Escherichia coli	NCTC 10418	1
Lactobacillus casei	ATCC 393	1
L. delbrueckii	ATCC 12315	1
Salmonella Thompson	ATCC 8391	1
S. Typhimurium	ATCC 13311	1
Serratia liquefaciens	ATCC 27592	1
Staphylococcus aureus	ATCC 29213	1
	NCTC 6571	1
Streptococcus pyogenes	ATCC 12344	1
S. thermophilus	ATCC 19258	1
Vibrio parahaemolyticus	ATCC17802	1

[1)]90 *Listeria monocytogenes* strains were isolated from different food samples previously; ATCC, American Type Culture Collection, Manassas, VA, USA; NCTC, the National Collection of Type Cultures, London, UK

Results and Discussion

Specificity of the LAMP assay In this study, 2 sets of LAMP primers were designed to specifically target the *L. monocytogenes hlyA* gene, a gene previously shown specificity for *L. monocytogenes* detection by PCR (17,18). Specificity of *hlyA* -based LAMP was examined using 96 strains of *Listeria* and 11 non-*Listeria* strains (Table 1). Ninety-two *L. monocytogenes* produced amplification curves in the assay. Four *Listeria species, L. innocua, L. ivanovii, L. welshimeri*, *L. seeligeri*, and all the non-*Listeria* did not yield amplification curves. Among the total of 92 *L. monocytogenes* strains of different serotypes and 15 non-*L. monocytogenes* strains tested, the *hlyA*-based LAMP assay obtained 100% inclusivity and 100% exclusivity. The results of PCR were consistent with the

Table 2. List of LAMP and PCR primers used in this study

Primer	Sequence (5'-3')
hlyA-FIP	TGACGGCCATATGCCACACGAGTGAATGCAGAAAATCCTCCT
hlyA-BIP	GCTGCTTTTGACGCTGCCGTATTTGTCAGTTCTACATCACCTG
hlyA-B3	TGAACTTCATCTTTTGCGGAG
hlyA-F3	CAGATTTTTCGGCAAAGCTGT

Table 3. Detection of *L. monocytogenes* in 17 retail food samples

Food	Number	LAMP assay (Tt)	Concentration estimated by the standard curve (CFU/mL)	Detection method		
				Culture	LAMP	PCR
Pork (*n*=1)	1	25.06	7.54×10^8	+	+	+
Beef (*n*=2)	2	30.02	1.68×10^7	+	+	+
	3	34.02	7.81×10^5	+	+	+
Coleslaw (*n*=5)	4	26.01	3.64×10^8	+	+	+
	5	27.06	1.63×10^8	+	+	+
	6	29.12	3.35×10^7	+	+	+
	7	31.11	7.28×10^6	+	+	+
	8	32.30	2.92×10^6	+	+	+
Smoked salmon (*n*=5)	9	25.12	7.20×10^8	+	+	+
	10	27.48	1.18×10^8	+	+	+
	11	27.30	1.35×10^8	+	+	+
	12	30.04	1.65×10^7	+	+	+
	13	33.09	1.59×10^6	+	+	+
Oysters (*n*=4)	14	27.12	1.55×10^8	+	+	+
	15	28.30	6.28×10^7	+	+	+
	16	30.12	1.56×10^7	+	+	+
	17	32.48	2.55×10^6	+	+	+

results of LAMP (data not shown). These results suggest that it has both high specificity to *L. monocytogenes* and high amplification efficiency in the *hlyA*-based LAMP assay.

In addition, in analyzing food samples, the LAMP demonstrated complete concordance with the conventional method (Table 3). The *hlyA*-based LAMP assay of detecting *L. monocytogenes* in pure culture and in food samples by a real-time turbidimeter is the first report demonstrating the efficacy of a *hlyA*-based LAMP assay for detecting *L. monocytogenes* in food samples.

Sensitivity of the real-time LAMP The detection limit of the real-time LAMP assay was determined by testing serial 10-fold dilutions of *L. monocytogenes* samples that had previously been quantied through plate counts cell number determinations. The LAMP reaction was positive for sample containing 2.8×10^3 CFU/mL or more (Fig. 1), indicating that the sensitivity of LAMP assay was 6 CFU/reaction tube (2 μL of 2.8×10^3 CFU/mL). It also successfully detected the positive sample which was determined to contain 3 CFU *L. monocytogenes* strain ATCC19115 after artificial contamination and 5.0×10^3 CFU/mL (estimated by plate counts) after enrichment. The sensitivity of PCR was 56 CFU/reaction tube (data not shown), up to about 10-fold less sensitive than the LAMP assay. The LAMP methods generally demonstrate higher sensitivity comparing with the PCR methods (19-21). The LAMP reaction can be accelerated, and higher sensitivity would be expected by using additional primer, termed loop primer. We tried to added loop primers to a LAMP reaction

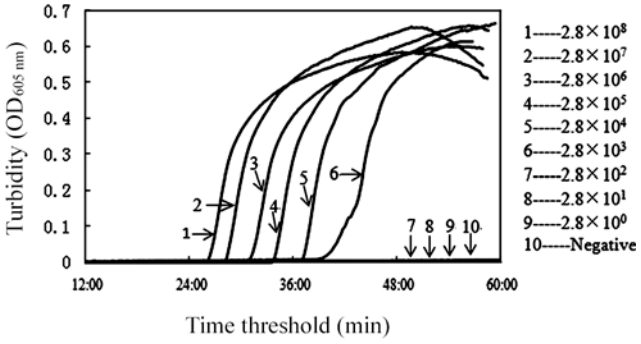

Fig. 1. Sensitivity of LAMP for testing *L. monocytogenes* ATCC 19115 DNA templates is monitored by real-time measurement of turbidity. Shown from left to right are the curves of decreasing concentrations of *L. monocytogenes* from 2.8×10^8 to 2.8×10^0 CFU of the template in a serial 10-fold dilution; No.10 is negative control.

at first. However, the incidence of false positive was increased when using sterile water or non-*L. monocytogenes* DNA as the DNA template. In addition, the complete LAMP detection system presented here was markedly faster than conventional methods. Although the reaction time of LAMP assay with loop primers is reduced by around 30 min (22), it does not significantly reduce the total assay time. In order to ensure the accuracy of the assay, we have to omit the loop primers.

A standard curve was generated for *L. monocytogenes* LAMP by plotting the graph between different log CFU values of *L. monocytogenes* ranging from 2.8×10^3 to 2.8×10^8 CFU/mL to time of positivity through real-time monitoring of the amplication (Fig. 2). For each standard,

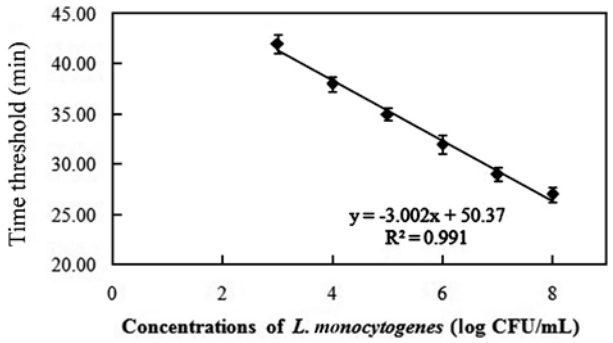

Fig. 2. Standard curve for *L. monocytogenes* LAMP by plotting the graph between different cell numbers of *L. monocytogenes* ranging from 2.8×10^3 to 2.8×10^8 CFU/mL to time of positivity through real-time monitoring of the amplication.

the cell numbers of *L. monocytogenes* was plotted against the time at which the turbidity signal increased above the threshold level (23).

Detection of *L. monocytogenes* in food Ninety-four natural retail food samples and inoculated retail food sample were culture enriched and tested with LAMP assay and the conventional method as described above. The LAMP assay demonstrated the same consequence as conventional method by picking up 17 positive samples (Table 3). None of the conventional method-positive samples were missed by LAMP, thereby indicating a high specificity and sensitivity of the LAMP assay. Following incubation at 63°C for 60 min in a Loopamp real-time turbidimeter, the monitoring of LAMP amplification was also accomplished through visualization by the naked eye with the addition of 1 μL of SYBR Green I (1:1,000) dye to the amplified products. The comparative evaluation of this SYBR Green I-based LAMP assay with 94 samples revealed a very good concordance of 100% with Loopamp real-time turbidimeter and conventional method. The quantication of the bacterium load in the positive samples was extrapolated on the basis of their time of positivity by employing the standard curve.

The quantification of *L. monocytogenes* in foods is generally done by the classical most probable number (MPN) method, which requires replicated dilution series of food in selective enrichment broth followed by plating on selective agar plates and various tests for species identification (1,24). Rapid and sensitive methods for enumeration of *L. monocytogenes* are important for microbiological food safety testing purpose. Recently many DNA amplication methods like real-time PCR technique have been widely used for the detection of *L. monocytogenes* in foods (1,6, 25-28). However, all PCR methods have several same intrinsic disadvantages, including requiring either a high-precision instrument for amplication or an elaborate, complicated method for the detection of amplied products (13). These rapid molecular tests might not be the method of choice in basic detection station in developing countries or in eld situations because of the requirement for sophisticated instrumentation and expensive reagents. Now the real-time LAMP assay has many advantages over conventional PCR methods and traditional culture methods, including rapidity, quantitative measurement, a lower contamination rate, higher sensitivity, higher specicity, and easy standardization. In addition, open-tube procedure after amplification potentially acts as a signicant source of cross-contamination. Therefore, it is better to avoid false positive caused by aerosol pollution by using a real-time turbidimeter (29). In general, LAMP was found to be similar or superior to PCR (19).

Since the increase in turbidity of the reaction mixture according to the production of the precipitate correlates with the amount of DNA synthesized, real-time monitoring of the LAMP reaction can be achieved by real-time measurements of turbidity. The quantification of the bacterium load in food samples can also be determined by observing the *L. monocytogenes* LAMP standard curve. An excellent correlation was also observed with 10-fold serial dilution of *L. monocytogenes* cells counted, in parallel, by the LAMP method. The food samples analyzed in this study were naturally contaminated with *L. monocytogenes* when tested with LAMP. Only the positive control food was include inoculation of *L. monocytogenes* cells to simulate naturally contaminated food samples. The State Food and Drug Administration (China) recommend that all ready to eat food (per 25 g) shall not be detectable in *L. monocytogenes*. Without enrichment, DNA amplication assays such as LAMP, lack the needed sensitivity when applied in low concentration contaminated food samples. However, combining with overnight enrichment, the LAMP amplication assays is a desirable approach to achieve the needed sensitivity. In this study, the positive control food illustrates the capability of the real-time LAMP assays when detecting *L. monocytogenes* in spiked food with only 3 CFU. In analysis of the natural food samples, the LAMP and the ISO 11290-1 method demonstrated identical results, suggesting LAMP could possess the potential to be benecial and sensitive tools for the detection of *L. monocytogenes* in food. Moreover, the real-time quantitative LAMP method can be used to support the growth of the pathogen and risk assessment models.

Each test of LAMP method for *hlyA* gene was accomplished within 1 h with high specificity and sensitivity. In addition, through the real-time turbidimeter, the LAMP obtained great advantages in simplicity and quantitative analysis. Future testing with more commercial food samples and shorter enrichment time is desired to further evaluate the efcacy of the assay. In conclusion, this study not only

provides an important diagnostic tool for the detection of *L. monocytogenes* in the eld, but also supplies effective technology of enumeration for quantitative risk assessment of foods.

Acknowledgments This work was supported by the National Natural Science Foundation of China (20877028), the Major State Basic Research Development Program of China (973 Program) (No. 2010CB735700), the Guangdong Nature Science Foundation of China (10451064101005159), and the Fundamental Research Funds for the Central Universities, SCUT (2009ZM0224).

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