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RESEARCH NOTE

Development and Application of a Rapid and Simple Loop-mediated Isothermal Amplification Method for Food-borne *Salmonella* Detection

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Abstract A loop-mediated isothermal amplification (LAMP) method for rapid detection of the food-borne *Salmonella* strains had been developed and evaluated in this study. The optimal reaction condition was found to be 65°C for 45 min, with the detection limit as 1 pg DNA/tube and 100 CFU/reaction. Application of LAMP assays was performed on 214 food-borne *Salmonella* strains using a rapid procedure and easy result confirmation, where the specificity of LAMP and polymerase chain reaction (PCR) assays was 97.7% (209/214) and 91.6% (196/214), respectively; with a 100% specificity for both assays.

Keywords: loop-mediated isothermal amplification (LAMP), *Salmonella*, rapid detection, food-borne pathogen, *invA*

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Introduction

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

Salmonella is a major food-borne pathogen worldwide, also regarded as the leading microbe in food contamination and responsible for diverse and various food poisoning cases of humans (2-4). Various serotypes of *Salmonella* are implicated in food-borne infections and reported to contaminate food products such as eggs, milk, poultry, meat, and vegetables, which is the main cause of human gastrointestinal and other related diseases. With increased awareness in public health and as a major concern of food industry and safety, development of a rapid, sensitive, cost-effective, and easy-operating bacteriological *Salmonella* detection is of the utmost importance and urgent necessity. Traditionally, 4 to 6 days is required for the conventional detection method to obtain a confirmed result. Polymerase chain reaction (PCR)-based and real-time PCR-based assays have been employed and developed for the past

decades (5-11). 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.

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, which is different from PCR in that 4 or 6 primers perform the amplification of the target gene. The amplification uses isothermal conditions between 60-65°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 (12). LAMP constituted a potentially valuable tool for rapid diagnosis of food-borne pathogens, which had also been reported and applied in the field of bacteriological *Salmonella* detection (13-17). Nevertheless, post detection by electrophoresis or real-time turbidimeter was required in these assays. In the current study, we develop a simple LAMP assay and apply it to detection on a large scale of *Salmonella* strains from various food samples, in which only visual observation of color changes is employed for the confirmation of results and no more than 60 min is needed for the whole process.

Materials and Methods

Bacterial strains Thirty-nine 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 assay (Table 1). Application of the optimized LAMP and PCR assays was performed on a total of 214 food-borne *Salmonella* strains, which were isolated from various food samples during 2001-2008 and had been preliminarily identified in the Lab of Clinical Microbiology of Zhongshan Supervision Testing Institute of Quality & Metrology.

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

Reference strains	No. of strains	Culture	LAMP	PCR
Gram-negative organisms				
Salmonella typhimurium ATCC 14028, WF 04313	2	+	+	+
Salmonella choleraesuis ATCC 13312	1	+	+	+
Salmonella enteritidis WF 05148, WF 07086	2	+	+	+
Salmonella typhi WF 03201, WF 05026, WF 08138	3	+	+	+
Salmonella paratyphi WF 06426	1	+	+	+
Salmonella aberdeen WF 04542	1	+	+	+
Salmonella gallinarum WF 05938	1	+	+	+
Psuedomonas aeruginosa ATCC 27853	1	-	-	-
Klebsiella pneumoniae ATCC 13883	1	-	-	-
Enterobacter cloacae ATCC 23355	1	-	-	-
Acinetobacter baumannii GH31	1	-	-	-
Vibrio parahaemolyticus O3: K6 WF 01031, WF 04506, WF 06215	3	-	-	-
Vibrio parahaemolyticus O4: K37 WF 01309, WF 04238	2	-	-	-
Vibrio cholerae SK10	1	-	-	-
Vibrio vulnificus ATCC 27562	1	-	-	-
Vibrio mimicus ATCC 33653	1	-	-	-
Escherichia coli O157: H7 WF01201 ATCC43889, NCTC12900	3	-	-	-
Escherichia coli O127: H6 WF073522	1	-	-	-
Escherichia coli C600 ATCC 25922, ATCC8739, C600, DH5α	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	39			

Primer design The protocol was designed to detect the genus specific target *invA* to differentiate *Salmonella* and non-*Salmonella* strains. 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) and F2 (ACRCGCCATGGTATGGATTTGTGAC CATCACCAATGGTCAGC); backward inner primer (BIP) consisted of complementary sequence of B1 (B1c), a T-T-T-T linker and B2 (ATGATGCCGGCAATAGCGTCA AGCCAGCTTTACGGTTCCT). The outer primers F3 (TCAACAATGCGGGGATCTG) and B3 (GAAGCGTA CTGGAAAGGGAA) located outside of the F2 and B2 regions, while loop primers LF (TCCGCTCTGTCTACTT ATACCAT) and LB (TGATAAACTTCATCGCACCGTC AA) 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 (18-23). To ascertain the detection limits of LAMP and PCR assays, template DNA from *Salmonella Typhimurium* ATCC14028 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 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, USA), 1 X thermopol buffer (New England Biolabs, 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 μL 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, Germany) and cloned into the pMD18-T vector (TaKaRa, Japan). The sequencing was done by an ABI PRISM 310 genetic analyzer (PE Biosystems, USA).

Application of LAMP assays on a large scale of *Salmonella* strains Two-hundred-and-forteen food-borne *Salmonella* 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). This experiment was performed twice to ensure reproducibility.

Results and Discussion

Optimization of the conditions of LAMP assays The specific amplification generated many ladder-like pattern bands on agarose gel due to its characteristic structure up to the loading wells, with a 205 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. 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 90 min. While with loop primers, the amplification was initially detected at 30 min, and reached maximal at 45 min. LAMP assays were

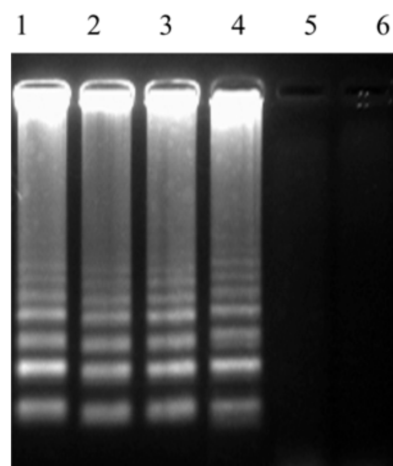

Fig. 1. Sensitivity of LAMP assays for detection of *Salmonella* strain ATCC14028. Lane 1, 1 ng template DNA; lane 2, 100 pg template DNA; lane 3, 10 pg DNA; lane 4, 1 pg DNA; lane 5, 100 fg DNA; lane 6, 10 fg DNA

performed with omission of 1 or 2 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 assays The detection limits of LAMP assay was found to be 1 pg DNA/tube and 100 CFU/reaction (LAMP was positive for sample containing 1×10^4 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 more sensitive than PCR (Fig. 1). Simultaneously, high specificity was acquired when LAMP assay was subjected to 39 reference strains, with no false positive amplification observed (Table 1).

Application of LAMP assays on *Salmonella* isolates The established LAMP assays were applied to detect 214 *Salmonella* strains using a simple DNA preparation process, and results confirmed by observation directly by naked eye and under UV light. Of a total of 214 strains, 209 and 196 were detected to be positive by LAMP and PCR, respectively. The sensitivity of LAMP and PCR assays was 97.7 and 91.6%, with a 100% high specificity obtained by both assays.

In conclusion, this LAMP assay was demonstrated to be a useful and powerful tool for rapid detection of *Salmonella* strains. Comparing with previously reported LAMP assays for the detection of *Salmonella* strain, the improved LAMP method in the present study offers advantages on easiness in operation and time consumption. The total detection time, including DNA preparation, LAMP reaction, and results determination, was approximately 60 min. In addition, instead of PCR machine or real-time turbidity in previous studies (24), simple equipments as water bath and direct observation were used for reaction and determination, with high sensitivity and specificity as 97.7 and 100%, respectively. Undoubtedly, rapidness, easiness, and cost-effectiveness of LAMP assay will aid in the broad application of bacteriological detection of *Salmonella*.

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