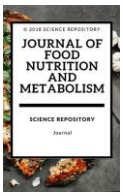

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Research Article

Evaluation of a Loop-Mediated Isothermal Amplification (LAMP) Method for the Detection of *Salmonella* spp. in Terms of Sensitivity and Applicability

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ABSTRACT

Salmonella spp. are important food-borne pathogens that can cause diseases in humans. Many detection methods have been established in *Salmonella* spp. using loop-mediated isothermal amplification (LAMP) or reverse transcription loop-mediated isothermal amplification (RT-LAMP). The detection limits of these assays varied from 1 CFU/reaction to 104 CFU/reaction, from 100 fg genomic DNA/reaction to 10 pg genomic DNA/reaction, or from 2.0×101 CFU/mL to 107 CFU/mL for food samples. In this study, LAMP assays were developed using genomic DNA for the detection of *Salmonella* spp. Two sets of LAMP primers were designed using the *inv*A gene and the 16S-23S rRNA intergenic spacer region (ITS) of *S. enterica* as the target sequences for two LAMP assays. The detection limits of the two methods were respectively 20 pg *S. enterica* DNA/reaction and 10 pg *S. enterica* DNA/reaction at the optimized temperature, and the LAMP methods were of high repeatability and specificity for *S. enterica* detection. This study provides a baseline for the application of LAMP for the detection of food-borne pathogenic bacteria.

Introduction

Food-borne diseases caused by pathogenic bacteria have become a major global public health issue, among which salmonellosis is the illness most often attributed to the consumption of poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce contaminated by *Salmonella* spp. [1, 2]. *Salmonella* spp. have been identified as the most frequent cause of food-borne infection outbreaks in many countries [3]. Therefore, rapid and sensitive methods for the detection of *Salmonella* spp. are needed to control and prevent salmonellosis outbreaks. Many methods, including culture-based, immunology-based, and nucleic acid-based, have been developed, among which more than twenty loop-mediated isothermal amplification (LAMP) or reverse transcription loop-mediated isothermal amplification (RT-LAMP) methods have been established targeting different specific genes of *Salmonella* spp. [3].

Most of these LAMP methods used the invasion gene *inv*A as the target sequence [4-13]. For example, Chen *et al.* developed specific LAMP methods utilizing the *inv*E gene and 3 serotype-specific genes, *fli*C, *lyg*D and STM4495 for the detection of *Salmonella* and 3 common *Salmonella* serotypes [14]. In the study of Tang *et al.*, the LAMP assay with the *fim*Y gene as the target sequence was established to detect *Salmonella* species in possibly infected ducks [15]. Okamura *et al.* developed the LAMP assays that amplified the *rfb*J gene for detection of the O4 group of *S. enterica* and the insertion element of SE for detection of the O9 group of *Salmonella* in chickens [16, 17]. A *sef*A-based LAMP method was developed by Gong *et al.* for detection of *S. enteritidis* and *S. gallinarum* in chickens [18]. The *pho*P, *his*J, and *rfb*S genes of *Salmonella* spp. have also been used as target sequences for LAMP assays [2]. In spite of different genes as target sequences, all of these LAMP or RT-LAMP assays were of high specificity for *Salmonella* spp., but they varied greatly in sensitivity (detection limit)

When CFU/reaction, cfu/test, copies/reaction, and cells/test were used as the measurement units, the detection limits of 1 CFU per reaction, >2.2 cfu/test, 4 CFU/reaction, 6.0 cfu/test and 4.8 cfu/test, 15 copies/reaction, and 10^4 CFU/reaction, have been reported [4, 9, 12, 15, 18, 19]. With

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CFU/mL, CFU/25 ml, CFU/g and CFU/25g as the measurement units for detection of *Salmonella* spp. in food produces, the detection limits were determined to be 2.0×10^1 CFU/mL, 10^2 CFU/25g, 200 CFU/g, 10^3 CFU/ml, 10^4 CFU/25 ml, 10^4-10^6 CFU per 25 g, and 10^7 CFU/mL [8, 11-13, 17, 19].

When pg DNA/tube, fg DNA/tube, and pg/μL were used as the measurement units, the sensitivity of the LAMP assays was found to be 100 fg DNA/tube, 1 pg DNA/tube, and 10 pg/μL [3, 5, 7]. In conclusion, since the results of these LAMP assays in detection limits have been confusing to interpret, the LAMP technology has not been practically applied for the detection of *Salmonella* spp. In this study, genomic DNA from pure bacterial cultures of *Salmonella* spp. was used as template, LAMP assays with the *inv*A gene and 16S-23S rRNA intergenic spacer region of *S. enterica* as the target sequences were developed, the specificity and sensitivity of LAMP assays were also evaluated.

Materials and Methods

I Primer Design for LAMP Assay

Two sets of LAMP primers targeting the *inv*A gene (GenBank accession No. CP041208.1) and the 16S-23S ribosomal RNA intergenic spacer (ITS) (GenBank accession No. CP050716.1) of *Salmonella enterica* serotype Newport were designed using PrimerExplorer 5 (Link), and one set of primers was selected with Oligo 7 (Molecular Biology Insights, Inc. Colorado Springs, USA). The primer sequences are listed in (Table 1).

Table 1: LAMP primers targeting *inv*A and ITS of *Salmonella enterica*.

Target (GenBank accession no.)	Primer	Sequence (5'–3')
*inv*A (CP041208.1)	F3	GGAAAAAGAAGGGTCGTCGT
	B3	ATGCTGTTATCGTCCAGGC
	FIP	CCGGCTCTTCGGCACAAGTAATTTTTGGACTGATTGGCGATCTCG
	BIP	AAGCTCAACTTGCGGAGCGTTTTTAACAATACTTCCGGCAGGC
	LF	GGTACGGTCTCTGTAGAGACTTTAT
16S-23S ribosomal RNA intergenic spacer CP050716.1	F3	TCACACAGATTGTCTGATGA
	B3	CGTGGAATAACGAAGCATAC
	FIP	TTATCAGGGGTGCGCTCTAATTTTAACGAGCAGTAAAACCTCTA
	BIP	GGTGAGGTCGGTGGTTCAAGTTTTTATGTGAGTTATTTCACAACGC
	LF	CCACCTGAGCTACAAGCCTG
	LB	AGGCCTACCAAATTTTCCCTGAAT

II Bacteria Strains and Genomic DNA Extraction

The *Salmonella enterica* and non-*Salmonella enterica* strains used in this study are listed in (Table 2). All of the strains except the *Listeria monocytogenes* strains were cultured overnight at 37°C in Luria-Bertani (LB) broth, and the *L. monocytogenes* strains were cultured in DifcoTM Buffered *Listeria* Enrichment Broth Base (Becton, Dickinson and Company). Genomic DNA from the pure cultures was extracted using the DNeasy® Blood & Tissue Kit (QIAGEN N.V. Corporate), and the DNA templates were used to determine the sensitivity and specificity of the LAMP assays.

III Sensitivity Determination

The LAMP assay using the *inv*A gene primers was performed in a 25 μL reaction mixture containing 0.8 μM each of forward inner primer (FIP) and backward inner primer (BIP), 0.2 μM each of forward outer primer (F3) and backward outer primer (B3), 0.4 μM of forward loop primer (LF), 1.4 mM dNTPs, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, 0.8 M betaine, 1×EvaGreen, 1×Rox, 8 U Bst 2.0 WarmStart DNA polymerase (New England Biolabs, Beverly, MA, USA), and serial dilutions ranging from 200 pg to 0.2 pg of *Salmonella enterica* genomic DNA [15]. The reaction mixtures were heated at the optimized temperature of 65°C for 60 min (30 s per cycle) using a StepOne™ System (Applied Biosystems, Foster City, CA, USA).

The LAMP assay with the 16S-23S ribosomal RNA intergenic spacer as the target sequence was modified according to the method of Wang *et al*. [20]. The reaction was performed in a 10 μL reaction mixture containing 0.8 μM each of forward inner primer (FIP) and backward inner primer (BIP), 0.2 μM each of forward outer primer (F3) and backward outer primer (B3), 0.4 μM of forward loop primer (LF) and backward loop primer (LB), 1.0 mM dNTPs, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, 7.5% DMSO, 1×EvaGreen, 1× Rox, 3.2 U Bst 2.0 WarmStart DNA polymerase (New England Biolabs, Beverly, Mass., USA.) and serial dilutions ranging from 10 pg to 0.01 pg of *Salmonella enterica* genomic DNA. The reaction mixture was heated at the optimized temperature 57°C for 60 min (30 s per cycle) using a StepOne™ System (Applied Biosystems, Foster City, CA, USA) [21].

IV Specificity Determination

The specificity of two LAMP assays with invA primers and ITS primers were determined using the bacterial strains listed in (Table 2), and the amount of DNA template used was 100 pg per reaction.

Results

I Detection Limit of the LAMP Assays

As shown in (Figure 1), the detection limit of the LAMP assay with *inv*A primers was 20 pg per reaction, while the sensitivity of the LAMP assay with 16S-23S ribosomal RNA intergenic spacer as target sequence was 10 pg per reaction, and there was little significant difference in terms of detection limits for these two LAMP assays.

Table 2: Bacterial strains used in the study.

Bacterial Strain (Serotype)	Bacterial Strain (Serotype)
Escherichia coli O121:H19	*Listeria monocytogenes* J1-094 (1/2c)
Escherichia coli O26:H11	*Listeria monocytogenes* C1-115 (3a)
Escherichia coli O111:H8	*Listeria monocytogenes* J1-031 (4a)
Escherichia coli O145:H2	*Listeria monocytogenes* W1-110 (4c)
Escherichia coli O103:H2	*Listeria monocytogenes* ATCC19115 (4b)
Escherichia coli O45:H12	*Listeria innocua* ATCC51742
Listeria monocytogenes J1-225 (4b)	*Listeria invanovii* ATCC49954
Listeria monocytogenes J2-020 (1/2a)	*Salmonella* Typhimuriam
Listeria monocytogenes J2-064 (1/2b)	*Salmonella enterica* serotype Newport
Listeria monocytogenes J1-169 (3b)	*Escherichia coli* O157:H7 933
Listeria monocytogenes J1-049 (3c)	*Escherichia coli* O157:H7 B1409
Listeria monocytogenes M1-004 (N/A)	
Listeria monocytogenes M1-004 (N/A)	

All of the strains were from the culture collection of Eastern Regional Research Center (ERRC), Agricultural Research Service (ARS), United States Department of Agriculture (USDA).

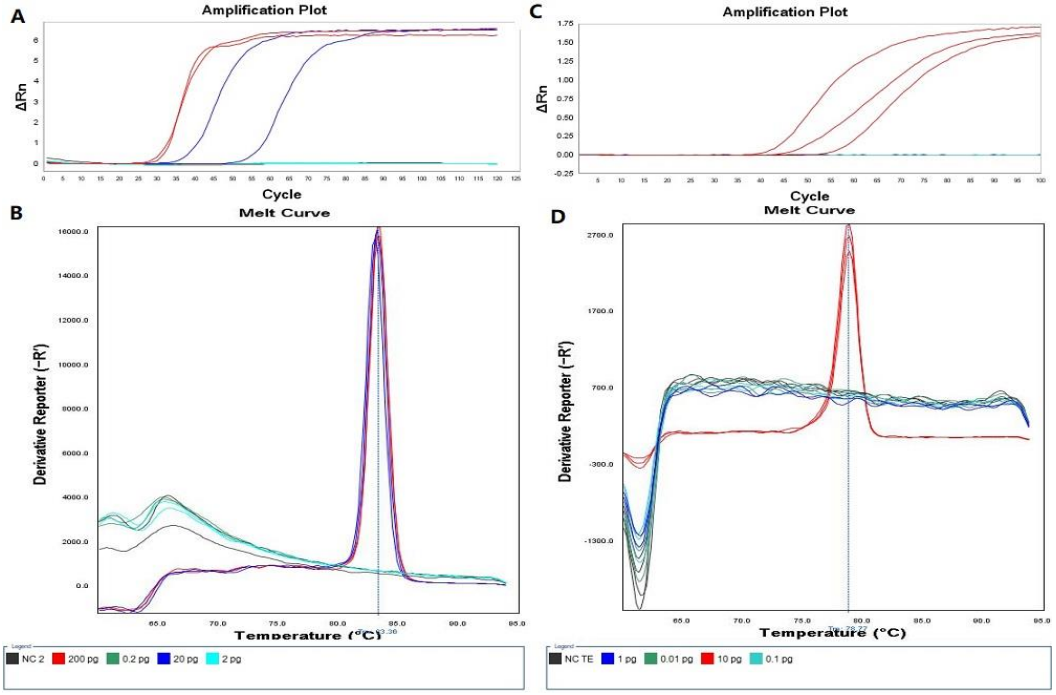

Figure 1: Detection limits of two LAMP assays. **A)** Amplification plot of LAMP assay with *inv*A primers. **B)** Melt curve of LAMP assay with *inv*A primers. **C)** Amplification plot of LAMP assay with ITS primers. **D)** Melt curve of LAMP assay with ITS primers.

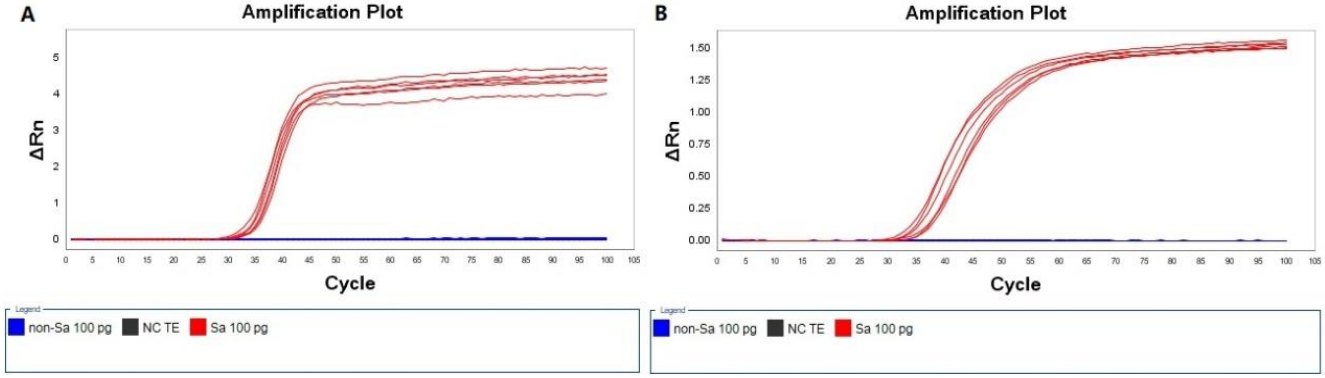

Figure 2: Specificities of the LAMP assay with *inv*A primers and ITS primers. **A)** Amplification plot of the specificity determination for *inv*A LAMP assay. **B)** Amplification plot of the specificity determination for ITS LAMP assay; non-Sa: 22 non-*Salmonella enterica* strains in (Table 2).

II Specificity of the LAMP Assay with ITS Primers

The specificities of the LAMP assay with ITS primers and the LAMP assays with *inv*A primers were determined. As shown in (Figure 2), as for both LAMP assays, the six repeats of *Salmonella enterica* serotype Newport were positive, while the two repeats of 22 non-*Salmonella enterica* strains and two negative controls were negative; therefore, the LAMP assays were highly specific for *Salmonella enterica*.

Discussion

Two LAMP assays with the *inv*A gene and 16S-23S rRNA intergenic spacer region of *S. enterica* as the target sequences were established in this study. The sensitivity of the assay with ITS primers (10 pg per reaction) was slightly higher than that of the assay with *inv*A primers (20 pg per reaction), which is equivalent to 3×10^4 copies/reaction. The sensitivity was similar to that of the LAMP assays developed by Liu *et al.* and Zhang *et al*., but it was much lower than that of other reported LAMP assays in which there was up to four orders of magnitude difference [3-5, 9, 12, 14, 15, 18, 19].

One important factor for differences in sensitivity is that the DNA preparation methods can have a great effect on the LAMP reaction. When the DNA template was prepared by a boiling method, the detection limit was as low as 1 CFU per reaction; it was proposed that the boiling and cooling process provided the single-strand template for the LAMP reaction [4, 5, 10, 12-15]. A commercial DNA preparation kit was used by Liu *et al.*, and in our study, the prepared DNA template was double stranded, and the sensitivity was as low as 10 pg per reaction [3]. The mechanisms of Polymerase Chain Reaction (PCR), Helicase-dependent Amplification (HDA) and Recombinase Polymerase Amplification (RPA) assays for amplification of double stranded DNA are well-established; however, there is so far no report on the mechanism of the LAMP reaction with double stranded DNA as template, which warrants further study.

Other factors related to sensitivity include the method used to determine results, and use of enrichment, reverse transcription, etc. It was reported by Zhang *et al.* that the reduction of the detection limit, from 10^4 copies/reaction to 10^2 copies/reaction, could be the result of using visual determination by fluorescence instead of turbidity [9]. The LAMP assays could detect 10^4 CFU/25 ml without enrichment and 100 CFU/25 ml with an 8 h enrichment for *Salmonella* spp., Techathuvanan *et al.* improved the *Salmonella* detection limit from 10^6 CFU/25 g to 10^2 CFU/25 g for both pork chop and sausage samples using a 10 h enrichment [8, 12]. With detection of *Salmonella* spp. with RT-LAMP, the 6 h enrichment made little difference and only improved the detection limits from 10^7 CFU/25 mL to 10^6 CFU/25 mL [11]. It could be that competitive or non-competitive inhibitors existed between reactions catalyzed by reverse transcriptase and Bst DNA polymerase (Large Fragment), which also needs further study.

The LAMP assays with the *inv*A gene and the 16S-23S ribosomal RNA intergenic spacer as the target sequence were established in this study. Like most previous reports, they were highly specific to *Salmonella enterica* [2]. It was also proven by Wang *et al.* that the presence of high concentrations of non-target genomic DNA neither adversely affected the amplification efficiency nor generated significant background [5]. In conclusion, the LAMP assay can be used to detect *Salmonella* spp. in different food matrices sensitively and selectively, given that a proper DNA preparation method was used after enrichment.

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Conflicts of Interest

None.

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