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Original Research

Development of a Visual and Rapid Method Using Loop Mediated Isothermal Amplification in Conjunction With a Specific Molecular Beacon Probe for *Salmonella* Detection

Tianlong Wang[1], Hongwei Song[1], Yan Guo[1], Mengxuan Liu[1], Xiong Xiong[1,*]

[1]College of Food Science and Light Industry, Nanjing Tech University, 211800 Nanjing, Jiangsu, China
*Correspondence: xiongx0624@njtech.edu.cn (Xiong Xiong)
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Abstract

Background: *Salmonella* is a major global threat due to the associated widespread prevalence as a foodborne pathogen; thus, timely diagnostic methods to enable effective disease management are required. Loop-mediated isothermal amplification (LAMP), although renowned for its high sensitivity, specificity, and rapid analysis in foodborne pathogen detection, is frequently hampered by false-positive results due to primer dimer formation. **Methods:** This study introduced a specific molecular beacon (MB) probe to ensure high sequence specificity, and developed a novel MB-LAMP assay for rapid, visual detection of *Salmonella*. The performance of the assay was evaluated using *Salmonella* genomic DNA and artificially contaminated milk samples to assess the assess the analytical sensitivity and practical applicability of the assay in food matrices. **Results:** The MB-LAMP assay demonstrated high efficiency, completing the detection process in less than 50 min, the analytical sensitivity reached 1.11 pg/μL for *Salmonella* genomic DNA. In artificially contaminated milk samples, the method achieved a limit of detection (LOD) of 1000 Colony-Forming Units (CFU)/mL. Notably, the results were readily interpretable by visual inspection without the need for sophisticated instrumentation. **Conclusion:** This MB-LAMP assay provides a highly sensitive, robust, and decentralized molecular diagnostic tool. Indeed, by bypassing traditional cultivation steps and high-end equipment requirements, this assay holds significant potential for real-time food safety monitoring and rapid screening in resource-limited settings.

Keywords: loop-mediated isothermal amplification; *Salmonella*; oligonucleotide probes; milk; food microbiology

1. Introduction

Salmonella is a major zoonotic enteropathogen, primarily colonizing the intestinal tracts of humans, poultry, and livestock. Its contamination stands as one of the formidable challenges in the realm of global food safety, and the transmission to humans occurs predominantly via the consumption of contaminated animal-derived food products, such as undercooked meats, eggs, and dairy items [1,2]. Particularly, during the summer and autumn seasons, *Salmonella* becomes more active, and its resilience allows it to survive in feces, soil, food, and water for months or even years [3]. Infected individuals may present with clinical manifestations including febrile illness, diarrhea, and vomiting, with severe cases potentially resulting in host mortality [4]. According to previous study [5], *Salmonella* accounts for a staggering 20% of bacterial foodborne illness outbreaks between 2006 and 2016, which demonstrated a trend of growth over the last five years [6]. Given the widespread and severe nature of *Salmonella* contamination, rapid and accurate detection methods are of paramount importance for preventing and controlling *Salmonella* infections.

Traditional culture-based methods, although highly specific and accurate, are laborious and time-consuming, since the standard culturing technique for *Salmonella* can take up to 4–7 days for definitive results. An improvement on traditional methods is the use of selective media, such as Rambach agar, which can shorten the detection time while maintaining accuracy [7]. In recent years, enzyme-linked immunosorbent assays (ELISAs) have emerged as a rapid and sensitive alternative, which could specifically quantify antibodies against *Salmonella Typhimurium* and *Salmonella Enteritidis* O-antigens [8]. Additionally, Polymerase Chain Reaction (PCR) technologies have revolutionized *Salmonella* detection due to their speed and sensitivity, and several PCR-based methods, including real-time PCR and multiplex PCR, have been deployed for detection of various *Salmonella* serotypes in food-borne samples [9,10,11].

Although these methods have improved detection speed and sensitivity, they often involve complex operations and are quite expensive. In contrast, the loop-mediated isothermal amplification (LAMP) technique, firstly described by Notomi et al. [12], has demonstrated considerable potential in *Salmonella* detection. LAMP enables DNA amplification under isothermal conditions, typically requiring reaction times of 30 min to 45 min. Its user-friendly nature obviates the necessity for costly equipment, thereby rendering it an exceptionally well-suited method for on-site rapid testing [13,14]. To date, numerous meth-

ods utilizing LAMP technique have been developed for the detection of *Salmonella* [15,16,17]. However, LAMP technology also encounters challenges in practical applications, particularly concerning non-specific amplification and contamination risks associated with tube opening. Non-specific amplification can give rise to false-positive outcomes [18], thereby compromising the detection accuracy. Meanwhile, contamination during tube opening may result in false positives or false negatives due to aerosol exposure [19]. Therefore, the development of a highly specific LAMP detection system is of great necessity.

Molecular beacon (MB) probes are stem-loop oligonucleotide probes labeled with a fluorophore and a quencher at opposite ends. In the absence of the target sequence, the stem-loop conformation brings the fluorophore and quencher into close proximity, resulting in fluorescence quenching. Upon hybridization with the complementary target sequence, the stem-loop structure opens, separating the fluorophore from the quencher and generating a detectable fluorescence signal (Fig. 1) [20]. Therefore, MB probes can serve as sequence-specific fluorescence reporters for LAMP products rather than merely indicating total nucleic acid amplification [21,22]. Previous studies have applied MB-assisted LAMP assays to disease diagnosis and rapid food safety testing [23,24]. In the present study, the MB probe was further integrated into a closed-tube visual LAMP format, enabling target-specific endpoint interpretation while reducing background interference from non-specific amplification and minimizing aerosol contamination associated with post-amplification tube opening.

Although MB-LAMP assays have shown promise for sequence-specific nucleic acid detection, their sequence specificity and endpoint interpretability still require careful optimization [25]. For food safety monitoring, an ideal MB-LAMP assay should not only recognize target-specific amplification products but also provide a visually interpretable readout in a closed-tube format. In the present study, a fluorescence-gap-guided strategy was used to screen the optimal MB stem-loop structure for *Salmonella* detection. The uniqueness of this strategy lies in the integration of target-specific molecular beacon recognition with closed-tube visual endpoint detection. The MB probe functions not only as a fluorescence reporter but also as a sequence-recognition element, because fluorescence is generated only when the probe hybridizes with the target invasion protein A gene (*invA)* amplicon. This design improves the specificity of endpoint interpretation, reduces background interference from non-specific amplification, avoids post-amplification tube opening, and enables result interpretation using only simple heating equipment and Ultraviolet Rays (UV) visualization. Therefore, the optimized MB-LAMP assay provides a specific, closed-tube, and low-equipment-requirement approach for rapid screening of *Salmonella* in food safety monitoring.

The *invA* of *Salmonella enterica* serovar Typhimurium encodes a protein that facilitates the adhesion to and invasion of epithelial cells by interacting with surface proteins [17]. The *invA* gene cluster is implicated in the invasive capability of *Salmonella* towards intestinal epithelial cells, and primers derived from this cluster exhibit species-specificity, making them suitable for the identification of *Salmonella*.

2. Materials and Methods

2.1 Bacterial Strains and Media

The *Salmonella* (ATCC 14028) used in this study was maintained in 10% glycerol and stored at −80 ℃. Frozen cultures were partially thawed at room temperature (22–23 ℃) for 15 min, streaked on Tryptic soy agar slants (HuanKai Microbial, Guangzhou, China), and incubated at 37 ℃ for 24 h. Following overnight incubation, the colonies were transferred to 100 mL Luria-Bertani (LB) (HuanKai Microbial, Guangzhou, China) broth containing 100 mM morpholinethanesulfonic acid (Solarbio, Beijing, China), at pH 7.0 and incubated at 37 ℃ with shaking at 200 rpm for 20−22 h to obtain liquid cultures. The bacterial suspension was diluted and then spread onto plates using the spread plate method. After counting, the concentration of the bacterial suspension was determined to be 1.53×10^9 Colony-Forming Units (CFU)/mL. To determine the analytical sensitivity of the assay at the molecular level, genomic DNA of *Salmonella* was extracted from the pure bacterial culture using a bacterial genomic DNA extraction kit (Tiangen Biotech, Beijing, China). The concentration and purity of the extracted genomic DNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The initial concentration of the purified DNA was determined, and 10-fold serial dilutions were subsequently prepared using sterile nuclease-free water to obtain varying DNA concentrations ranging from 1.11×10^6 down to 1.11×10^{-2} pg/μL. These diluted DNA samples were then used as templates to evaluate the limit of detection (LOD) of the established LAMP assay.

Additionally, ten non-target bacterial reference strains were cultured for specificity validation: *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 13813), *Streptococcus suis* (ATCC 43765), *Streptococcus pyogenes* (ATCC 19615), *Vibrio parahaemolyticus* (ATCC 17802), *Bacillus cereus* (ATCC 14579), *Shigella flexneri* (ATCC 12022).

2.2 Primers Designing and MB Optimization

The *invA* gene was selected as the target and submitted to Primer Explorer V5 (https://primerexplorer.jp/lampv5e/index.html) for the design of primers with parameters: Tm = 60−65 °C, amplicon size <200 bp. *Salmonella enterica* serovar Typhimurium ATCC 14028 served as the reference strain.

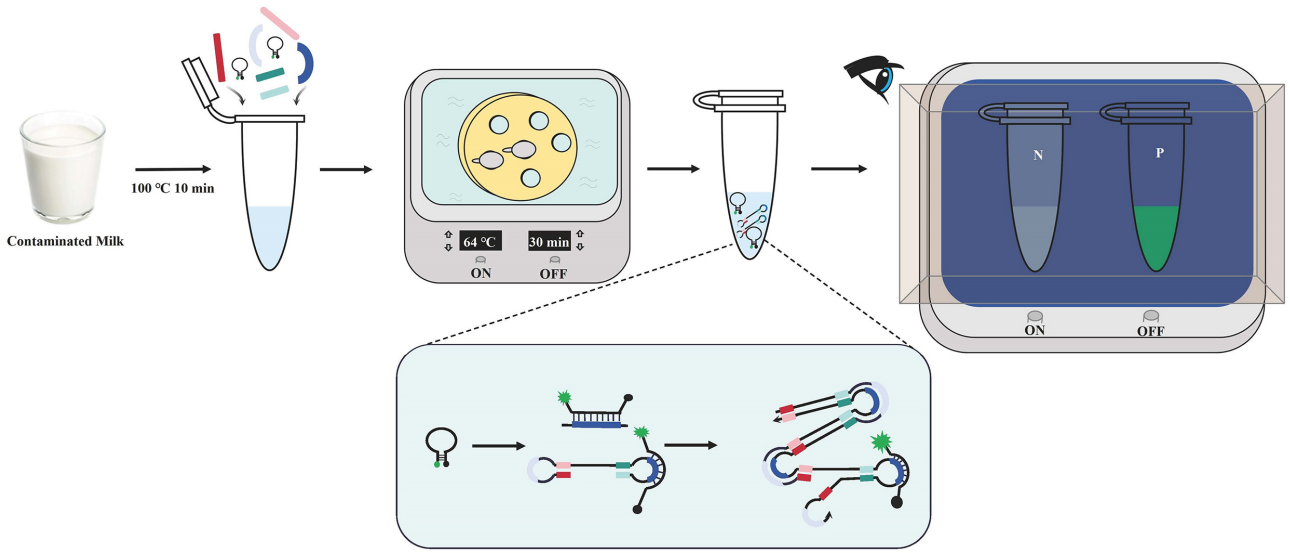

Fig. 1. Schematic workflow and molecular mechanism of the visual MB-LAMP assay for *Salmonella* detection in milk. The diagnostic procedure begins with the rapid thermal lysis of artificially contaminated milk at 100 °C for 10 min to release the target genomic DNA, followed by the assembly of a single-tube reaction mixture containing Bst DNA polymerase, specific primers, and the molecular beacon (MB) probe. Isothermal amplification is subsequently conducted at a constant temperature of 64 °C for 30 min in a water bath, during which the MB probe specifically hybridizes with the amplified *invA* sequences. This hybridization triggers a conformational change in the MB probe, separating the fluorophore from the quencher to emit a bright green fluorescence signal. Finally, the results are visually interpreted under UV light, where positive samples (P) exhibit distinct green fluorescence and negative controls (N) remain colorless, providing a rapid, equipment-free, and aerosol-contamination-proof diagnostic tool for food safety monitoring. MB, molecular beacon; LAMP, loop-mediated isothermal amplification; *invA*, invasion protein A gene; UV, Ultraviolet Rays; P, positive sample; N, negative control.

The design of the MB was grounded in the sequence of the loop primer S-*invA*-loop primer backward (LB)/S-*invA*-loop primer forward (LF) (Table 1). To assess its secondary structure, we utilized the NUPACK web-based tool (accessible at https://www.nupack.org/partition/new) (**Supplementary Fig. 1**). The fluorescence emission of the oligonucleotide was meticulously monitored across a temperature gradient spanning from 35 °C to 97 °C, with measurements taken 10 times at every 1 °C increment. This analysis was conducted using the Gentier 96E real-time PCR system (Tianlong, Xi'an, China). In this study, the suffix "ac" denotes an artificially synthesized complementary oligonucleotide used to hybridize with the corresponding MB candidate for melting curve analysis and fluorescence-gap evaluation.

2.3 Real-Time Fluorescence LAMP Assay

The real-time fluorescence LAMP was performed in a total reaction volume of 20 µL, including 0.2 µM of each outer primer, 0.8 µM of each inner primer and 0.4 µM of each loop primer (Table 1), 2.5 µL 10 × ThermoPol buffer (NEB ENGLAND Biolabs, Nanjing, China), 0.45 mM dNTP mix, 0.5 µM SYTO 9, 2 mM $MgSO_4$, 8 units of Bst DNA polymerase (NEB ENGLAND Biolabs, Nanjing, China) and 1 µL of DNA template solution. The reaction was performed using Gentier 96E real-time PCR system under the following conditions: 64 ℃ for 60 min (one cycle per min). Fluorescence signals were measured at the end of each cycle and a sigmoid shaped fluorescence curve could be observed for successful amplification. The real-time fluorescence LAMP products were interpreted by amplification curve with respect to time (Ct value). One Ct is equal to one min.

2.4 End-Point Visual Detection of the MB-LAMP Products

The reaction mixture (20 µL) was similar to that in the real-time fluorescence LAMP assay described above, including 0.8 µM of each inner primer, 0.2 µM of each outer primer, 0.4 µM of each loop primer, and 0.4 µM of S-*invA*-MB-loop primer backward probe (LBP) (Table 1), 4.5 mM Mg^{2+}, 0.45 mM dNTP mix, 2.5 µL 10 × ThermoPol buffer (NEB ENGLAND Biolabs, Nanjing, China), 8 units of Bst DNA polymerase (NEB ENGLAND Biolabs, Nanjing, China) and 1 µL of DNA template solution. The reaction was performed in a water bath (AoSheng, Hangzhou, China) at 62–65 ℃ for 20–60 min. Positive results can be determined by the green fluorescence under ultraviolet (reaction tubes were laid directly on the UV transilluminator).

Table 1. Sequence of oligonucleotides used in this work.

Code		Sequence (5′ to 3′)
S-*invA*-F3		CATCAGCAAGGTAGCAGT
S-*invA*-B3		AGCATATGTTTTGTTTCCTGA
FIP	S-*invA*-F2	CAGTATTTCTGGGTAACGCA
	S-*invA*-F1c	GGGAGCTTGGCTATGTGTTGC
BIP	S-*invA*-B2	ACGTCAATGAATATTTCGGTAT
	S-*invA*-B1c	GAACGCGCTTGATGAGCTTT
S-*invA*-LF		GGAGTTTCTCCCCCTCTTCA
S-*invA*-LB		ACCACTGTCTGGCGGTGAC
LBP-1		TCCGCGACCACTGTCTGGCGGTGACCCGCGGA
LBP-2		TCGGCCACCACTGTCTGGCGGTGACCGGCCGA
LBP-3		CGCCACACCACTGTCTGGCGGTGACCGTGGCG
LFP-1		TCGCGGAGTTTCTCCCCCTCTTCACCGCGA
LFP-2		CACGCGGAGTTTCTCCCCCTCTTCACGCGTG
LFP-3		TCCCGGAGTTTCTCCCCCTCTTCACCGGGA
LBP-ac		GGTCACCGCCAGACAGTGGT
LBP-3-ac		CGCCACGGTCACCGCCAGACAGTGGT
LFP-ac		TGAAGAGGGGGAGAAACTCC
S-*invA*-LBP		FAM-CGCCACACCACTGTCTGGCGGTGACCGTGGCG-Dabcyl

The underline indicates the loop region of molecular beacon S-*invA*-LBP. FIP, forward inner primer; BIP, backward inner primer; LF, loop primer forward; LB, loop primer backward; LBP, loop primer backward probe; LFP, loop primer forward probe; FAM, 5-Carboxyfluorescein.

2.5 Specificity and Sensitivity Test

The specificity of the newly-established visual MB-LAMP assay was tested using bacterial suspension from the 10 non-target species listed in Section 2.1. The sensitivity of the MB-LAMP assay was assessed using 10-fold serial dilutions of the *Salmonella* genomic DNA templates, ranging from 1.11×10^{6} down to 1.11×10^{-2} pg/μL.

2.6 Interference Evaluation of the Milk and Chicken Matrices

To evaluate the potential inhibitory effects of different food matrices on the amplification system, commercial ultra-high temperature (UHT) pure milk (Guangming, Nanjing, China) and fresh chicken breast were used as representative liquid and solid animal-derived food matrices, respectively. Prior to use, the milk was confirmed to be negative for *Salmonella* and other common foodborne pathogens, and the chicken breast was heat-sterilized after purchase. For the milk matrix, serial dilutions of extracted *Salmonella* genomic DNA were spiked directly into the pretreated sterile milk samples. For the chicken matrix, the heat-sterilized chicken breast was homogenized with sterile phosphate-buffered saline, and the prepared chicken homogenates were spiked with the same serial dilutions of *Salmonella* genomic DNA at concentrations of 11.1, 1.11, 0.11, and 0 pg/μL. After thorough vortexing, the mixtures were immediately subjected to the MB-LAMP assay to assess matrix interference.

2.7 Simulated Detection in Artificially Contaminated Milk Samples

To validate the practical applicability of the method, artificially contaminated milk samples were prepared. Briefly, 1 mL aliquots of the confirmed negative milk were spiked with *Salmonella* bacterial suspensions to final concentrations of 1000, 100, 10, and 0 CFU/mL. Following DNA extraction, the samples were analyzed using the established MB-LAMP assay.

2.8 Statistical Analysis

Statistical analyses were conducted using SPSS Statistics software version 27 (SPSS Inc., Chicago, IL, USA), with results reported as mean ± standard error (n = 3). The comparison of mean values was made, to determine significant difference, using one-way analysis of variance (ANOVA) by Duncan test at the 5% level ($p < 0.05$), with high significance set at the 1% level ($p < 0.01$).

3. Results

3.1 MB Designing and Visual MB-LAMP Optimization

We selected a fragment of the *Salmonella invA* gene for primer design. The target sequence was successfully amplified by both real-time fluorescent LAMP and endpoint visual detection, confirming the feasibility of the designed primer set for *Salmonella* detection (Fig. 2A).

Initially, five stem-loop primers, namely LBP-1, LBP-2, loop primer forward probe (LFP)-1, LFP-2, and LFP-3, were designed using the loop primers S-*invA*-LB and S-*invA*-LF. LBP-2 and LFP-2 were selected through prelim-

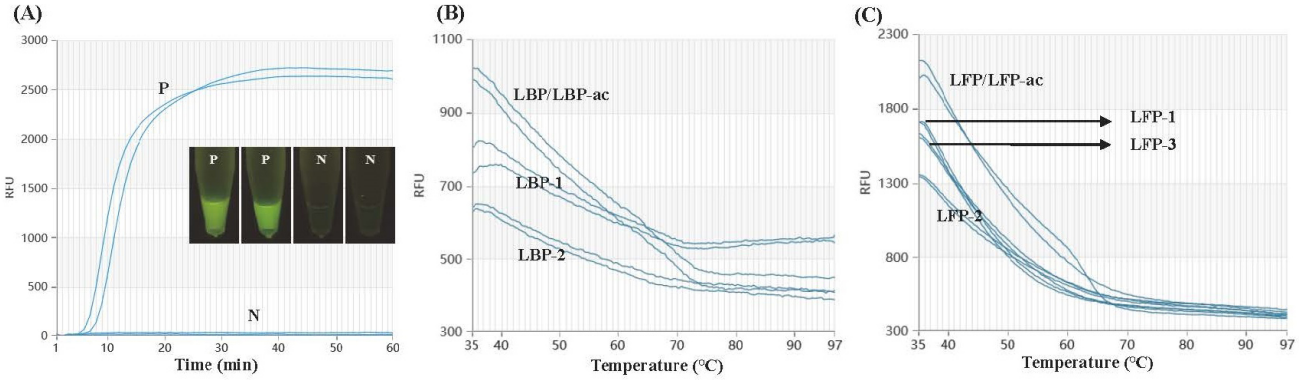

Fig. 2. Real-time LAMP amplification and preliminary screening of stem-loop primers. (A) Real-time fluorescence amplification curve of the *Salmonella invA* target and endpoint visual detection of LAMP products under UV light. P indicates positive reactions, and N indicates negative controls. (B) Melting curve analysis of LBP-derived stem-loop candidates designed based on the S-*invA*-LB loop primer. The fluorescence difference between the LBP/LBP-ac duplex and stem-loop structures at 64 °C was used for preliminary screening. (C) Melting curve analysis of LFP-derived stem-loop candidates designed based on the S-*invA*-LF loop primer. A larger fluorescence difference at 64 °C indicates a more stable MB-target duplex.

inary screening based on the fluorescence difference between the duplex and stem-loop structures at 64 °C (Fig. 2B,C). However, the fluorescence difference between the positive and negative reactions was not sufficiently distinct for endpoint visual discrimination.

Subsequently, LBP-3 was designed with stem-region complementarity to the target sequence and experimentally evaluated. LBP-3, LBP-2, LFP-2, and their corresponding target-complementary duplexes were exposed to a gradual temperature increase from 35 to 97 °C. The fluorescence difference between the duplex and stem-loop structures at 64 °C was compared using three fluorescent dyes, namely SYTO 9, SYBR Green I, and EvaGreen. Among the tested candidates, LBP-3 showed the largest fluorescence difference at the reaction temperature and was therefore selected as the optimal stem-loop structure for the visual MB-LAMP assay (Fig. 3A–O).

After selection of the molecular beacon structure, the concentration of loop primer S-*invA*-LB was optimized. The highest visual fluorescence intensity was observed when 0.2 µM S-*invA*-LB was added to the reaction system (Fig. 4A). When the concentration of S-*invA*-LB was further increased, the endpoint fluorescence intensity decreased (Fig. 4B,C). Therefore, 0.2 µM S-*invA*-LB was selected for the final protocol.

The reaction conditions of the visual MB-LAMP assay were further optimized by evaluating Mg^{2+} concentration, reaction temperature, and reaction time. The optimal reaction conditions were determined to be 4.5 mM Mg^{2+}, 64 °C, and 30 min (Fig. 5A–C). These conditions were used in the subsequent specificity, sensitivity, and simulated sample detection experiments.

3.2 Analytical Sensitivity, Matrix Tolerance, and Specificity

The analytical sensitivity of the visual MB-LAMP assay was evaluated using 10-fold serial dilutions of purified *Salmonella* genomic DNA, ranging from 1.11×10^{6} to 1.11×10^{-2} pg/µL. The lowest concentration that produced a clearly distinguishable positive fluorescence signal was 1.11 pg/µL (Fig. 6A). This result was further cross-validated by agarose gel electrophoresis (Fig. 6B).

To evaluate the matrix compatibility of the proposed MB-LAMP assay, *Salmonella* genomic DNA was spiked into milk samples and chicken matrices at concentrations of 11.1, 1.11, 0.11, and 0 pg/µL. The assay produced clearly observable positive fluorescence signals at concentrations down to 1.11 pg/µL, whereas no visible fluorescence was observed in the negative controls (Fig. 6D,E). These results indicate that the visual MB-LAMP assay had good tolerance to both milk and chicken matrices.

The specificity of the visual MB-LAMP assay was evaluated using *Salmonella* and ten non-target bacterial species, including *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus suis*, *Streptococcus pyogenes*, *Vibrio parahaemolyticus*, *Bacillus cereus*, and *Shigella flexneri*. Green fluorescence was observed only in the *Salmonella* reaction, whereas no visible fluorescence was detected in any of the non-target bacterial reactions or in the negative control (Fig. 6C).

3.3 Detection of Salmonella in Artificially Contaminated Milk Samples

The practical applicability of the visual MB-LAMP assay was evaluated using artificially contaminated milk samples spiked with live *Salmonella* at concentrations of 1000, 100, 10, and 0 CFU/mL. The MB-LAMP assay gen-

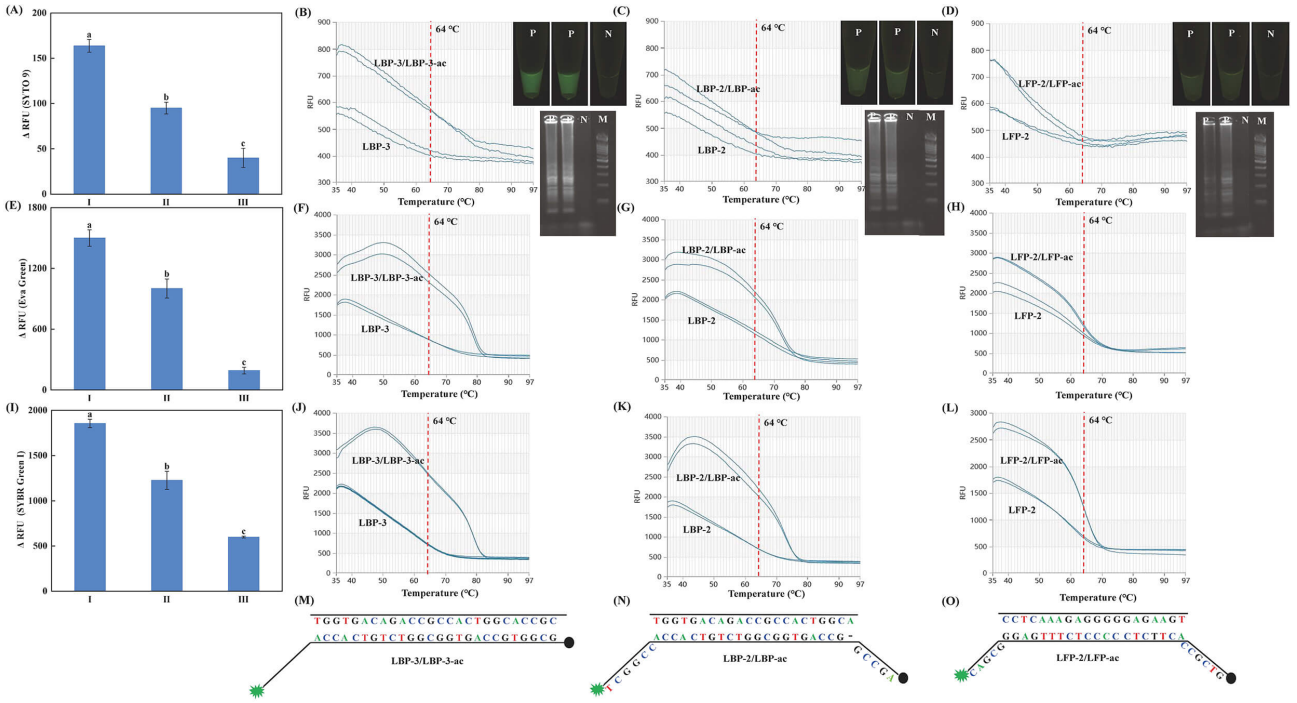

Fig. 3. Screening and structural evaluation of stem-loop primers. (A,E,I) RFU differences between the DNA duplex and stem-loop primer at 64 °C. I, II, and III represent the RFU differences for LBP-3/LBP-3-ac versus LBP-3, LBP-2/LBP-2-ac versus LBP-2, and LFP-2/LFP-ac versus LFP-2, respectively. (B–D) Melting curve analysis using SYTO 9 for LBP-3, LBP-2, and LFP-2, respectively. (F–H) Melting curve analysis using EvaGreen for LBP-3, LBP-2, and LFP-2, respectively. (J–L) Melting curve analysis using SYBR Green I for LBP-3, LBP-2, and LFP-2, respectively. (M–O) Schematic diagrams showing the predicted hybridization patterns between different molecular beacon candidates and their corresponding target sequences. M denotes DL-100 marker, P denotes positive control, and N denotes negative control in the gel images. Different letters (a, b, c) indicate significant difference at $p < 0.05$ (mean ± standard error, n = 3). RFU, relative fluorescence units.

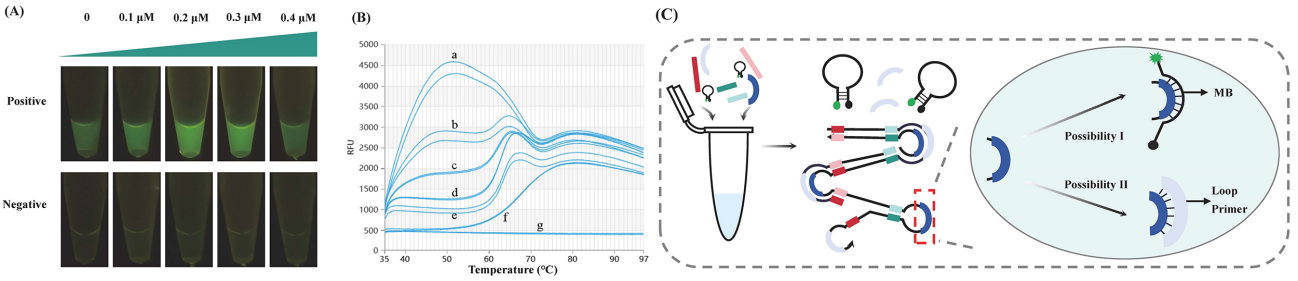

Fig. 4. Optimization of loop primer S-*invA*-LB concentration in the visual MB-LAMP assay. (A) Endpoint visual detection of MB-LAMP products under UV light after addition of different concentrations of loop primer S-*invA*-LB: 0 μM, 0.1 μM, 0.2 μM, 0.3 μM, and 0.4 μM. Positive and negative rows indicate reactions with and without *Salmonella* DNA, respectively. (B) Melting curve analysis of S-*invA*-LBP under different competitive hybridization conditions. Curves a–e represent S-*invA*-LBP with LBP-3-ac and increasing concentrations of S-*invA*-LB from 0 to 0.4 μM; curve f represents S-*invA*-LBP alone; curve g represents the negative control. (C) Schematic illustration of the competitive binding between the loop primer S-*invA*-LB and the molecular beacon probe. Excessive loop primer may compete with the molecular beacon for the same target region, thereby reducing fluorescence intensity.

erated a clearly distinguishable positive fluorescence signal at 1000 CFU/mL, while no positive signal was observed at 100, 10, or 0 CFU/mL (Fig. 6F). Therefore, the LOD of the visual MB-LAMP assay in artificially contaminated milk samples was determined to be 1000 CFU/mL. Although the turbidity and complex composition of milk slightly attenuated the visible fluorescence intensity, the assay still enabled clear discrimination between positive and negative samples in less than 50 min.

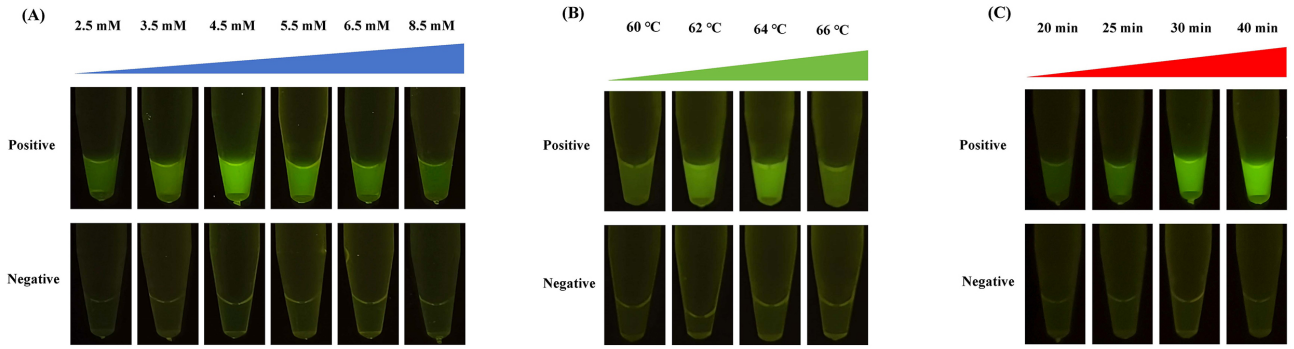

Fig. 5. Optimization of reaction conditions for the visual MB-LAMP assay. (A) Effect of Mg^{2+} concentration on the visual MB-LAMP assay. Mg^{2+} concentrations of 2.5, 3.5, 4.5, 5.5, 6.5, and 8.5 mM were tested. (B) Effect of reaction temperature on the visual MB-LAMP assay. Reactions were performed at 60, 62, 64, and 66 °C. (C) Effect of reaction time on the visual MB-LAMP assay. Reactions were performed for 20, 25, 30, and 40 min. Positive and negative rows indicate reactions with and without *Salmonella* DNA, respectively.

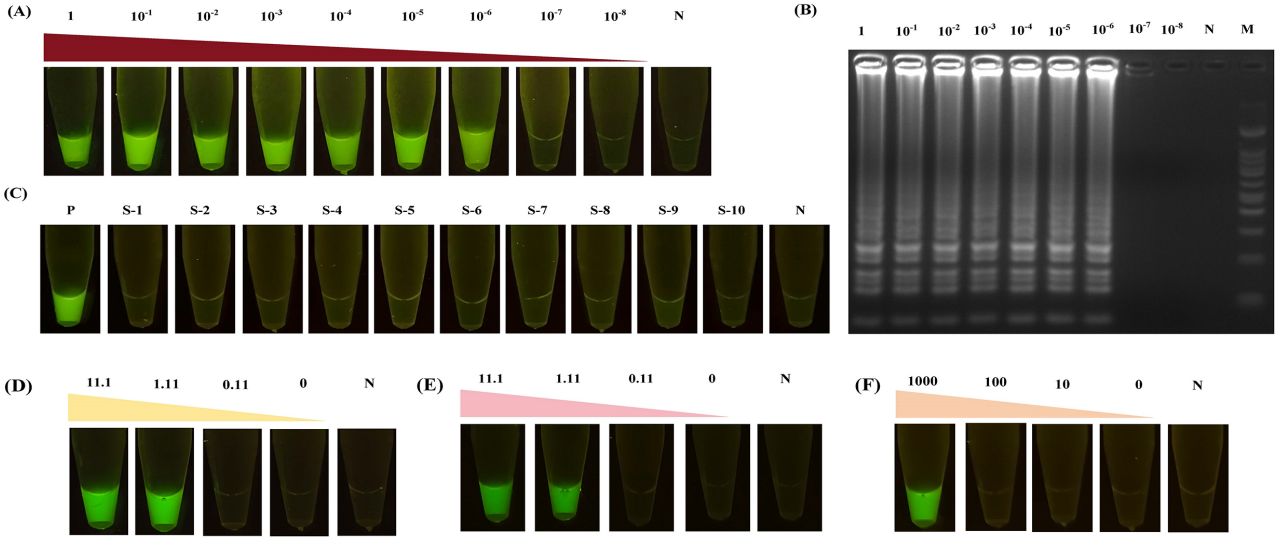

Fig. 6. Analytical sensitivity, specificity, matrix tolerance, and simulated sample detection of the visual MB-LAMP assay. (A) Analytical sensitivity of the visual MB-LAMP assay using 10-fold serial dilutions of purified *Salmonella* genomic DNA, ranging from 1.11×10^{6} to 1.11×10^{-2} pg/μL. The visual LOD was determined to be 1.11 pg/μL. (B) Agarose gel electrophoresis validation of MB-LAMP products generated from the same serially diluted *Salmonella* genomic DNA templates. M denotes the DL-100 DNA marker, and N denotes the no-template control. (C) Specificity evaluation of the visual MB-LAMP assay using *Salmonella* and ten non-target bacterial species. P denotes the positive reaction containing *Salmonella* DNA, S-1 to S-10 denote non-target bacterial species, and N denotes the no-template control. (D) Matrix tolerance evaluation using milk samples spiked with purified *Salmonella* genomic DNA at concentrations of 11.1, 1.11, 0.11, and 0 pg/μL. (E) Matrix tolerance evaluation using chicken homogenates spiked with purified *Salmonella* genomic DNA at concentrations of 11.1, 1.11, 0.11, and 0 pg/μL. (F) Detection of artificially contaminated milk samples containing live *Salmonella* at concentrations of 1000, 100, 10, and 0 CFU/mL. Green fluorescence indicates a positive result. For panels D–F, "0" denotes the matrix sample without spiked *Salmonella* genomic DNA or bacterial cells, and N denotes the no-template control. LOD, limit of detection; CFU, Colony-Forming Units.

4. Discussion

The selection of an appropriate molecular beacon structure is critical for the performance of MB-LAMP assays. Molecular beacons that are either too short or too long may be unsuitable for use as validation probes in LAMP reactions. Although loop primers play an important role in accelerating LAMP amplification, they are not indispensable components of the reaction and can therefore be modified for molecular beacon design [26]. To achieve high specificity, high sensitivity, and low background fluorescence, the molecular beacon should hybridize efficiently with the target sequence at the reaction temperature. This

can be achieved by selecting suitable lengths for the probe and stem-arm regions [27].

In this study, a fluorescence-gap strategy was used to screen the stem-loop structure of the molecular beacon. This strategy relies on the fluorescence intensity difference between the probe-target duplex and the stem-loop structure [28]. Compared with synthesizing multiple dual-labeled molecular beacons, this approach provides a more cost-effective and experimentally feasible strategy for preliminary molecular beacon optimization. The NUPACK prediction was also used to assist the structural evaluation of the molecular beacon.

The effect of MB probe design on assay performance is mainly determined by the thermodynamic balance between the closed stem-loop conformation and the target-bound duplex state. The loop region of the MB probe provides sequence-specific recognition of the *invA* amplicon; therefore, fluorescence is generated only when the probe hybridizes with the correct target sequence, which improves the specificity of endpoint interpretation. The stem region controls the stability of the closed conformation in the absence of target DNA. If the stem is too unstable, the probe may open spontaneously and produce background fluorescence in negative reactions; conversely, if the stem is too stable, target-induced opening may be inefficient and the positive fluorescence signal may be weakened. Therefore, an optimized MB probe should remain closed under non-target conditions but open efficiently after hybridization with the target amplicon at the reaction temperature. In this study, the fluorescence-gap-guided screening strategy enabled the selection of LBP-3, which showed a larger fluorescence difference between the stem-loop structure and the target-complementary duplex at 64 °C. This design contributed to low background fluorescence in negative reactions and strong signal generation in positive reactions, thereby improving visual discrimination between positive and negative samples.

The comparison among SYTO 9, SYBR Green I, and EvaGreen provided useful information for evaluating the fluorescence behavior of different stem-loop candidates. When the temperature of DNA duplexes increases, the minor-groove structure is disrupted, and SYBR Green I and EvaGreen are rapidly released from double-stranded DNA, resulting in a sharp decrease in fluorescence near the denaturation temperature [29]. In contrast, SYTO 9 can bind to a portion of single-stranded regions formed during melting and emit fluorescence, leading to a slower and smoother fluorescence decline [30]. These fluorescence characteristics supported the selection of LBP-3 as the optimal molecular beacon candidate.

After the optimal stem-loop structure was selected, optimization of the visual MB-LAMP system was necessary to balance reaction efficiency and endpoint fluorescence intensity. Unlike singly labeled probes, dual-labeled molecular beacon probes may not efficiently trigger LAMP elongation after target hybridization, which can delay the amplification reaction [25]. This limitation can be addressed by incorporating a cleavable site into the molecular beacon stem region or by adding a loop primer to accelerate amplification [31]. In the present assay, S-*invA*-LB was added to improve amplification efficiency. However, because S-*invA*-LB and the molecular beacon recognize overlapping target regions, excessive loop primer may competitively occupy the target-binding site and reduce molecular beacon hybridization efficiency. This explains why 0.2 µM S-*invA*-LB produced the strongest visual fluorescence signal, whereas higher concentrations attenuated the endpoint fluorescence intensity.

The analytical sensitivity of the visual MB-LAMP assay reached 1.11 pg/µL for purified *Salmonella* genomic DNA. This sensitivity is comparable to previously reported nucleic acid amplification-based methods for *Salmonella* detection, including real-time PCR, duplex PCR, and LAMP-based assays [10,32]. In artificially contaminated milk samples, the assay achieved an LOD of 1000 CFU/mL. The difference between the analytical sensitivity obtained with purified DNA and the LOD obtained in milk samples may be associated with the complexity of the food matrix, DNA extraction efficiency, and potential amplification inhibitors. Nevertheless, the assay retained clear endpoint visual discrimination between positive and negative milk samples.

The specificity evaluation showed that the visual MB-LAMP assay generated a positive signal only for *Salmonella*, while no amplification was observed for the ten non-target bacterial species tested. These results support the specificity of the *invA*-targeted MB-LAMP system under the tested bacterial panel. Compared with traditional culture-based detection, the developed assay shortens the detection time and reduces the need for sophisticated instrumentation. Therefore, this MB-LAMP assay may serve as a rapid preliminary screening tool for *Salmonella* detection in food safety monitoring.

5. Limitations

This study has several limitations. First, although the assay showed good specificity against ten non-target bacterial species, additional *Salmonella* serovars and closely related foodborne bacteria should be tested in future studies to further confirm the inclusivity and exclusivity of the method. Second, the present assay provides qualitative endpoint visual detection, and its quantitative performance remains to be further evaluated. Future work will focus on expanding the bacterial validation panel, increasing the validation scale, and further improving the applicability of the assay for on-site food safety screening.

Although chicken homogenate was included as an additional matrix to evaluate matrix tolerance, further validation using naturally contaminated or live-bacteria-inoculated food samples is still needed to more comprehensively assess practical applicability.

6. Conclusions

In this study, a visual MB-LAMP assay was developed for rapid detection of *Salmonella*. By integrating a target-specific molecular beacon probe targeting the *invA* gene with LAMP amplification, the assay achieved sensitive and specific detection of *Salmonella* genomic DNA. The analytical sensitivity reached 1.11 pg/μL, and the assay successfully detected *Salmonella* in artificially contaminated milk samples with an LOD of 1000 CFU/mL in less than 50 min.

The main advantage of the developed assay is the combination of high sequence specificity, closed-tube visual readout, and low equipment requirements. By integrating target-specific MB recognition with LAMP amplification, the assay reduces false-positive interpretation caused by non-specific amplification, minimizes aerosol contamination associated with post-amplification tube opening, and supports rapid preliminary screening of *Salmonella* in food safety monitoring settings with limited laboratory infrastructure. However, further validation using naturally contaminated samples, a broader range of Salmonella serovars, and additional food matrices is still required before routine application.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article and its supplementary information files. The oligonucleotide sequences used in this study are provided in Table 1. Additional raw data, including original fluorescence images and gel electrophoresis images, are available from the corresponding author upon reasonable request.

Author Contributions

TW: Investigation, methodology, writing (original draft); HS: resources, investigation; YG, ML: Investigation; XX: methodology, data curation, writing (review and editing), project administration. All authors have contributed to the editorial changes made to the manuscript. All authors read and approved of the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JFSFQ53033.

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