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# Evaluation and Optimization of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Salmonella enterica* serovar Typhi Isolated from Food Samples

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**ABSTRACT**

**Background:** Foodborne diseases caused by *Salmonella* are considered as a global health concern, especially in low-income countries. Rapid and specific detection of this infective agent is highly important in the outbreak control. The current study aimed to design and optimize a LAMP method and to compare its sensitivity and efficiency with the PCR method in the detection of *S. typhi* in food.

**Materials & Methods:** Food samples including mayonnaise and vegetable salad were inoculated with *S. enterica* serovar Typhi. Sensitivity and detection limit of LAMP test were investigated at different concentrations of contaminated mayonnaise and vegetable salad. *invA* gene was chosen as the target gene for bacterial detection by PCR and LAMP tests.

**Findings:** The detection limit of *Samonella* was estimated to be 16 CFU/mL using LAMP and PCR. LAMP reaction revealed a visible turbidity, indicating the accurate amplification of the selected target gene and proper identification of *Salmonella* at different dilutions of the studied food samples.

**Conclusion:** The present study indicated that LAMP is a rapid, cost-effective, and specific technique for the identification of *Salmonella*. This method could be used in laboratories with minimal equipment without the need for costly molecular detection methods.

**Keywords:** *Salmonella typhi*, *invA*, Mayonnaise, LAMP.

**CITATION LINKS**

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## Introduction

*Salmonella* is a facultative anaerobic, motile, spore-free bacillus, which is considered as the etiological agent of typhoid fever. *Salmonella* is often found in water, soil, as well as in the intestine and feces of vertebrates, including mammals, birds, reptiles, and fish, leading to diseases with a variety of symptoms and complications depending on the host conditions and bacterial serotype [1]. This bacterium is usually excreted in human or animal feces, thereby contaminating water, food, and the environment. Consumption of contaminated foods, such as meat, poultry, eggs, sauces, and vegetables are commonly considered as the transmission route of *Salmonella* infection to humans. In addition, the hands of carriers working in the preparation and distribution sections of the fruit and food industries could also affect water and food contamination [2]. Symptoms including headache, diarrhea, lethargy, abdominal cramps, and myalgia appear within 12 to 72 hours after the consumption of contaminated food. Salmonellosis is a self-limiting disease; however, in children and the elderly, or in people with chronic diseases, infection is worsened, leading to severe and fatal complications such as intestinal perforation and severe hemorrhage, abscess formation, pneumonia, cystitis, and thrombophlebitis. After an acute course of the disease, a group of patients go through the chronic phase and retain the bacteria in their gallbladders for more than 5 weeks up to 3 years, and 3% of the patients become *Salmonella* carriers [1]. Today, more than 2,450 *Salmonella* serotypes have been identified, most of which are potential pathogens in humans and most animals, leading to diseases such as human typhoid fever and enterocolitis. Nontyphoidal *Salmonella* is considered as the main cause of foodborne diseases globally. There are an estimated 200 million to 1.3 billion cases of nontyphoidal salmonellosis worldwide with an annual mortality rate of 3 million [3]. Although current standard methods such as biochemical, molecular, and serological tests as well as culture method are commonly used for the identification of *Salmonella*, they are often time-consuming and have limitations such as high cost or occasional low specificity [4-5].

Molecular methods such as PCR and real-time PCR with high efficiency, sensitivity, and specificity are widely used for *Salmonella* detection. However, these methods also require thermal cycling, expensive devices, and trained personnel [6-7]. Therefore, setting up an accurate, sensitive, simple, and economically viable method for *Salmonella* detection is essential. The loop-mediated isothermal amplification (LAMP) method, developed by a Japanese group in 2000, is an efficient method for detecting food pathogens, which, in addition of being sensitive and specific, rapidly detects pathogens. This method does not require a particular apparatus and could be carried out in different laboratories with minimal facilities [8].

The loop-mediated isothermal amplification method is used for specific amplification and detection of 6 regions of target gene using three pairs of specific primers including two internal primers (FIP, BIP), two external primers (F3, B3), and two loop primers (LF, LB). The reaction process progresses via the strand displacement reaction at a constant temperature. External primers only operate in the non-cyclical stage, and internal primers with both sense and antisense sequences facilitate loop formation. The loop-specific primers bind to the opposite sequence, the sequence that binds to the inner primer in the loop structure, thereby accelerating and facilitating the reaction. The amplification of nucleic acids in LAMP reaction is done through DNA polymerase

with the ability to displace synthesized strands [8-9]. Foodborne diseases caused by *Salmonella* are considered as the major global health problems, especially in developing countries. Therefore, it is important to detect the causative agent rapidly, accurately, and specifically in order to prevent epidemics and subsequent complications [10].

**Objectives:** This study aimed to design and optimize a LAMP method by targeting *invA* gene and to compare its efficacy and sensitivity with the PCR method in the specific detection of *S. typhi* in food samples (vegetable salad and mayonnaise).

## Materials and methods

**Bacterial strains:** *S. enterica* serovar Typhimurium (PTCC1709), *S. paratyphi* A (PTCC 1761), *S. paratyphi* B (PTCC1231), and *E. coli* (PTCC No: 1399) were prepared from Culture Collection of Microorganisms at Industrial and Scientific Research Institute, Iran. *S. paratyphi* C was kindly gifted by Dr. Sajjad Yazdansetad, Golestan University of Medical Sciences, IR Iran. Lyophilized bacterial strains were inoculated in Luria-Bertani (LB) broth and incubated at 37°C for 24 hrs. Then bacterial strains were transferred to *Salmonella-Shigella* (SS) agar culture medium. Macroscopic and microscopic evaluations as well as biochemical tests such as TSI, SIM, methyl-red, Voges-Proskauer, Simmon citrate, urease, glucose, lactose, sucrose, and mannose fermentation were carried out for the confirmation of bacterial strains [11-12].

**Bacterial DNA extraction:** Bacterial DNA was extracted using phenol-chloroform extraction method as previously defined by Kochl et al. (2005) [13] with few modifications. Briefly, a colony of fresh bacterial culture was inoculated in 5 mL of LB broth and incubated overnight at 37ºC in a shaking incubator. Then the medium showing bacterial growth was centrifuged at 13000 rpm for 3 min. The supernatant was discarded, and 200 µL of initial lysis reagent (2 mM sodium EDTA, 40 mM Tris acetate, pH=7.9) was added to the pellet and vortexed well. Then 400 µL of the second lysis reagent (0.8% NaOH, 1% SDS, pH=12.6) was added and incubated at 65°C for 10 min. Next, 600 µL of equilibrated phenol was added to Tris, followed by the addition of 600 µL of chloroform to this solution; after mixing, centrifugation was carried out at 13000 rpm for 5 min. Aqueous phase was collected carefully and transferred to a new microtube. For phenol removal, chloroform was added (2:1 v/v) to the aqueous phase and centrifuged at 13000 rpm for 5 min. Aqueous phase was then re-collected, and to which 90% ethanol was added (0.6:1 v/v). After mixing, the solution was stored at -20ºC for 30 min and then centrifuged at 13000 rpm for 10 min. The supernatant was removed, and 50 µL of washing buffer (ethanol 70%) was added to the pellet and centrifuged at 13000 rpm for 3 min. Washing buffer was removed, and the pellet was left to dry at the ambient temperature. The pellet was solved in 20 µL of distilled water. To assess the quality of the extracted DNA, in addition to calculating the optical density (OD) at 260/280 and 260/230 using a spectrophotometer (Biowave, UK), 5 µL of the extracted DNA was run on 1% agarose gel and electrophoresed at 80 V for 40 min.

**PCR for *invA* gene in *Salmonella*:** Specific primers used for *invA* gene *of S. enterica* serovar Typhi are shown in Table 1 [14]. The PCR reaction was performed in a final volume of 25 µL consisting of 1U SmarTaq DNA polymerase, 2.5 µL of 10X buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.5 mM of each primer (forward and reverse), 2 µL template DNA at a concentration of 500 ng/µL, and 15 µL of distilled water. The thermal program in Thermal Cycler (Labnet MultiGene OptiMax,

USA) was as follows: denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, 51ºC for 1 min, 72ºC for 1 min, and 72ºC for 5 min.

**Table 1)** Specific primers used for *invA* gene of *S. enterica* serovar typhimurium

| Primer Name | Primer Sequence (5'-3') | Amplicon Size (bp) |
|---|---|---|
| F3 | GGCGATATTGGTGTTTATGGGG | 243 |
| B3 | AACGATAAACTGGACCACGG | |

**Loop-mediated isothermal amplification (LAMP):** Designation of LAMP primers used for *invA* gene of *S. enterica* serovar Typhi was carried out using NCBI (GenBank: M90846.1), FASTA sequence of the target gene, and AlleleID software (Premier Biosoft AlleleID v6.01). Figure 1 illustrates the schematic location of external, internal, and loop primers on the target gene. The characteristics of the primers used are shown in Table 2 [14-15]. The LAMP reaction mixture (25 µL) contained 2.5 µL of 10X buffer, 2 µL of 8 mM $MgSO_4$, 1 µL of 0.5 mM dNTP, 2 µL of 0.5 M Betaine, 2 µL of each 0.8 µM BIP and FIP primers, 0.5 µL of 0.2 µM LB primer, 1 µL of 10 U *Bsm* DNA polymerase, 2.5 µL template DNA (500 ng/ µL), and 5 µL of sterile water. The microtube containing the reaction mixture was incubated in a hot water bath at 60ºC for 60 min, then the enzyme was inactivated at 80ºC for 10 min to stop the reaction. The turbidity of the reaction product was visually detected. Then 1 µL of SYBR green fluorescent dye was added, and after exposure to UV, the fluorescent green color in the reaction product was clearly observed. Then 5 µL of amplification product was run on 1.5% agarose gel with loading buffer and electrophoresed at 80 V for 60 min [15].

**Detection limit of LAMP in food samples:** To determine the detection limit of LAMP in food, foods including mayonnaise and vegetable salad were inoculated with *S. typhi*. *E. coli* PTCC1399 and *S. paratyphi* A, B, and C were used as negative controls. For bacterial contamination of the mayonnaise, a colony of fresh bacterial culture was


**Figure 1)** Schematic location of external (F3, B3), internal (FIP: F2, F1; BIP: B1, B2), and loop (LF, LB) primers on the target gene *invA* in *S. enterica* serovar Typhi.

**Table 2)** Sequences of internal, external, and loop primers used in LAMP reaction

| LAMP Primers | Primer Sequence (5'-3') |
|---|---|
| FIP | GACGACTGGTACTGATCGATAGTTTTTCAACGTTTCCTGCGG |
| BIP | CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG |
| F3 | GGCGATATTGGTGTTTATGGGG |
| B3 | AACGATAAACTGGACCACGG |
| LF | GACGAAAGAGCGTGGTAATTAAC |
| LB | GGGCAATTCGTTATTGGCGATAG |

added to 5 mL of LB broth and incubated in a shaking incubator at 37ºC for 24 hrs. After obtaining an OD of 1 at 595 nm, buffered peptone water was used for the preparation of bacterial serial dilution. Then 50, 100, 200, and 300 µL of bacterial suspension were added to 1 mL of mayonnaise as well as 20 g of vegetable salad (ingredients of which included cabbage, tomato, and cucumber). Dilutions of 1:10, 1:50, and 1:100 were prepared from the contaminated mayonnaise and vegetables using buffered peptone water and incubated at 37ºC for 6 hrs. Then sensitivity and detection limit of LAMP reaction were determined on 1 mL of mayonnaise and vegetable salad at dilutions of 1:10, 1:20, 1:50, 1:100, and 1:200. Moreover, the pour-plate technique was performed for each dilution of the inoculated mayonnaise and vegetable salad to determine the bacterial colony forming unit (CFU) [16].

absence of gas and indole production, absence of urease production, and absence of citrate utilization as carbon source by the bacterium.

**PCR assay:** PCR reaction performed on different dilutions (1:10. 1:20, 1:50, 1:100, and 1:200) of food samples (mayonnaise and vegetable salad), by targeting *invA* gene specific for *S. enterica,* resulted in the generation of 243 bp PCR products.

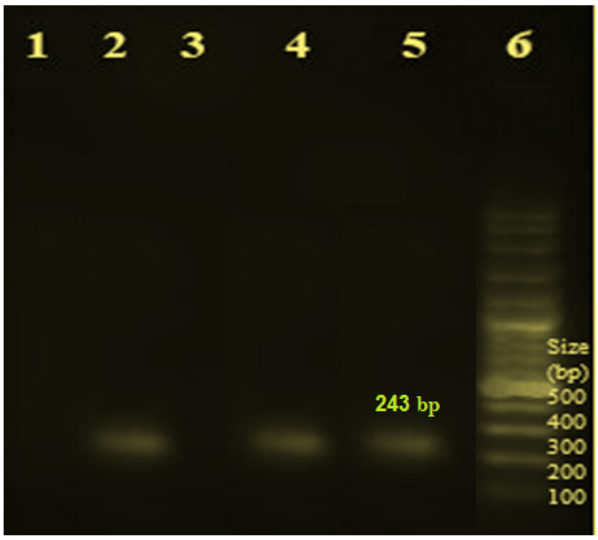


**Figure 2)** Amplification of *invA* gene in *S. enterica* using direct PCR on different dilutions of the studied food samples. Lane 1: negative control; Lane 2: positive control (*S. enterica* serovar Typhi; Lane 3: inoculated bacteria on mayonnaise with dilution of 1:100; Lane 4: inoculated bacteria on mayonnaise with dilution of 1:50; Lane 5: inoculated bacteria on vegetable salad with dilution of 1:50; and Lane 6: DNA marker (100 bp Plus O' Gene ruler, Fermentas, Lithuania).

**Pour plate and bacterial colony count:** Ten test tubes containing 9 mL of sterile physiological serum were chosen. Then 1 mL of bacterial suspension was added to the first tube, and a homogenized suspension was prepared. Serial dilutions of $10^{-1}$ to $10^{-10}$ were prepared by transferring 1 mL of the content of tube 1 to tube 2 and continuing this process until tube 10 in the series. Then 1 mL of each prepared dilution was added

to the plates consisting of 20 mL of molten SS agar and mixed well. After incubating the plates at 37ºC for 24 hrs, colonies of each dilution were counted, and CFU/mL or CFU/g was determined as previously described [17].

## Findings

Gram staining and observation of Gram-negative bacillus confirmed the presence of *Salmonella*. *Salmonella* growth on selective medium of SS agar showed smooth, translucent, black-centered colonies. Biochemical test results showed glucose fermentation, absence of lactose fermentation, hydrogen sulfide production, **LAMP assay:** Bacterial detection limit of LAMP was calculated as 16 CFU/mL, and DNA detection limit of LAMP reaction was measured at 500 pg. The visible turbidity caused by the LAMP reaction on different dilutions of the studied food samples was considered as an indicative of accurate amplification of the target gene and proper detection of *S. enterica*. Furthermore, 1 µL of SYBR Green dye was added to LAMP product, showing fluorescence under UV light (Figure 3). Electrophoresis of LAMP products on 1.5% agarose gel generated ladder-like bands (Figure 4).

In the present study, 9 parameters affecting the LAMP reaction were assessed to optimize *Salmonella* detection, including different concentrations of betaine (at 0-1 M), $MgSO_4$ (at 1-10 mM), *Bsm* polymerase (at 2-10 U), dNTP (at 0.4-2 mM), external primers (at 0.05-0.4 µM), internal primers (at 1.2-2 µM), and loop primers (at 0.2-1 µM), as well as temperature (at 60-65ºC), and time (at 45-65 min). The optimal concentrations for LAMP reaction were as follows: 8 mM $MgSO_4$, 0.4 mM dNTP, 0.4 M betaine, 8 U *Bsm* polymerase, 0.8 µM internal primers, 0.2 µM external primers, 0.8 µM loop primers, and 2.5 µL DNA. Moreover, the optimal temperature and time conditions for LAMP test were 60ºC for 45 min and 80ºC for 10 min.

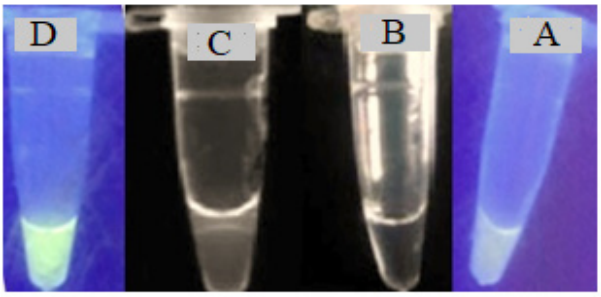


**Figure 3)** Tubes containing LAMP products following the PCR of *invA* gene of *S. enterica* serovar Typhi. A) Lack of amplification after the addition of SYBR Green fluorescent dye under UV light; B) lack of amplification in the absence of SYBR Green dye; C) amplification of the target gene in the absence of SYBR Green dye; D) amplification of the target gene in the presence of SYBR Green fluorescent dye under UV light.

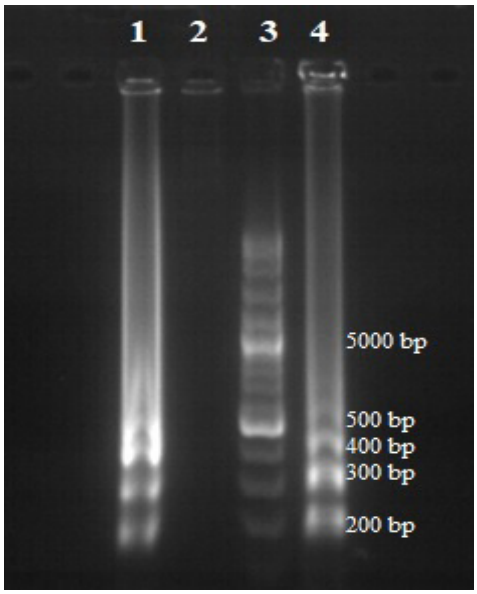


**Figure 4)** Electrophoresis of LAMP product. Lane 1: ladder-like bands generated by isothermal amplification of the target gene of *S. enterica* serovar Typhi in 1:50 dilution of mayonnaise; Lane 2: negative control; Lane 3: DNA marker (100bp Plus O' Gene ruler, Fermentas, Lithuania), Lane 4: positive control.

## Discussion

In the current study, specific detection of *Salmonella* was carried out in a short period of time using LAMP assay and a very simple and cheap thermal block at 60ºC and visual detection of turbidity caused by amplification process. Ladder-like pattern

generated by LAMP reaction following gel electrophoresis indicated that the designed primers specifically detected the target gene in *S. enterica* serovar Typhi, while other strains did not show turbidity or ladder-like band patterns.

Up to now, specific genes *invA*, *invB*, *fiC-a*, *fiC-dT*, *tyv*, *prt*, *int-flom*, *spvC*, *fliC*, *fliB*, *rfbJ*, *STM* 2755, *STM* 2235, and *hilA* have been used for the molecular detection of *S. typhi* in various studies [18] *invA* is one of the major genes involved in *Salmonella* invasion, providing bacterial entry into the deep intestinal layers. This gene is carried by all invasive *Salmonella* strains and is a suitable candidate for rapid and precise detection of bacteria by LAMP test. Recent studies have indicated 100% sensitivity and 96% specificity for *invA*-based detection of *Salmonella* [12, 15]. LAMP assay is highly efficient for the amplification of low copy numbers of bacterial DNA in the absence of thermal cycling. Notomi et al. (2000) indicated that LAMP reaction is applicable on a single copy of DNA [8, 16]. Another advantage of this method is the visualization of amplification products based on the turbidity induced by the release of pyrophosphate from dNTPs in the reaction mixture and its binding to magnesium ions and the subsequent formation of magnesium pyrophosphate [8].

Moganedi et al. (2007) used the PCR method to detect *invA* gene of *Salmonella* in drinking water. The detection limit of PCR pre and post enrichment was $2.6 \times 10^4$ CFU/mL and 26 CFU/mL, respectively [19].

Techathuvanan et al. (2010) used *invA* gene in LAMP assay to detect *S. typhimurium* in pork products. The detection limit of LAMP was reported to be 10 CFU/mL [20]. In accordance with the present study, *Salmonella* detection at 10 CFU/mL and 16 CFU/mL detection limits in contaminated foods was reported by Zhao et al. (1010) [21] and Zhang et al. (2012) [22], respectively.

Hara-Kudo et al. (2005) employed LAMP test for *Salmonella* detection and reported higher sensitivity of this method compared to PCR [9]. Similarly, Wang et al. (2008) reported that the sensitivty of LAMP test for *Salmonella* detection in food leftovers was 10 times more than PCR [23]. In the present study, similar to the PCR method, the detection limit of LAMP was 16 CFU/mL.

dNTPs are required for DNA synthesis; however, their excessive amounts could lead to the reduced specificity of the reaction. In the present study, the least amount of dNTP was used and resulted in better results compared to the use of higher amounts.

Another important factor is betaine which impedes secondary structure formation in GC-rich regions and leads to the utmost DNA amplification. Therefore, the lower amounts of betaine could accelerate the reaction and increase its senstivity as well as turbidity signals [24].

*Bsm* polymerase simultaneously amplifies DNA and detects 6 specific regions of the target sequence by 4 primers in the absence of thermal denaturation and by strand displacement. External primers are involved in the primary step of DNA strand displacement from the internal primers and the formation of dumbbell-like DNA structure. After dumbbell-like structure formation, external primers are no longer required; thus, lowering the required level of external primers. Internal primers are invloved in auto-cycling during DNA amplification. Loop primers increase the sensitivity of LAMP reaction and accelerate DNA amplification by elevating the number of start points for DNA amplification [25]. The optimum reaction temperature was 60ºC, and the reaction time of 45 min improved the quality of the amplified products. After the completion of the reaction and the electrophoresis of the amplified products on agarose gel, ≥241 bp ladder-like bands were

observed. Larger bands were not visible due to the high concentration of agarose gel. The presence of these bands were considered as an indicative of the accuracy of the LAMP reaction.

In the present study, for *Salmonella* detection in food samples (vegetable salad and mayonnaise), the detection limit of LAMP was the same (16 CFU/ml) as the detection level of PCR. According to the obtained results in this study, LAMP is a rapid, cost-effective, and specific method for *Salmonella* detection. This method with acceptable sensitivity and specificity could have field applications and could be used in labs with mnimal equipment without requiring costly molecular detection methods such as PCR and real-time PCR.

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**Ethical Permision:** Not applicable.

**Conflict of Interests:** None.

**Authors' Contribution**: Conceptualization: FF; Data curation and formal analysis: FF, HZ and SY; Investigation: FF, HZ and SY; Methodology and project administration: FF; Supervision: FF; Validation: FF; Writing of original draft: FF, HZ and SY; Writing, reviewing, and editing: FF, HZ and SY.

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