Original Article

Real-Time Loop-Mediated Isothermal Amplification (LAMP) Method for Quantitative Salmonella Typhi Detection Based on ViaB Gene

Sohrab M[1], Majidzadeh K[2], Morovvati A[3], Soleimani M[1,4*]

1. Department of Microbiology, Islamic Azad University, Qom Branch, Qom, Iran
2. Academic Center for Education, Culture & Research (ACECR), Breast Cancer Research Center (BCRC), Tehran, Iran
3. Department of Microbiology, Islamic Azad University, Qom Branch, Qom, Iran
4. Department of Microbiology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran

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Abstract

Background & Objective: There is a vast range of diseases caused by Salmonella including gastroenteritis, enteric fever, bacteraemia, and focal infection. Salmonella infection is usually transmitted to humans by consuming foods or water contaminated with animal or human waste. This study aims to design a quantitative-LAMP method for real-time detection of Salmonella enterica serovar Typhi (S. Typhi), the causative agent of Typhoid fever.

Materials & Methods: A new LAMP primer set was designed and used based on the ViaB gene to specifically detect S. Typhi. The genome of some Salmonella-related and non- Salmonella-related bacteria was subjected to the LAMP assay for detection of S. Typhi to evaluate its analytical specificity, its analytical sensitivity and limit of detection (LOD). Further, turbidity in tubes was measured by a turbidimeter, and then a standard curve was depicted by plotting (Tt) time threshold values against the logarithm of ViaB gene copy number and this standard curve helped the researchers a lot to obtain a quantitative method.

Results: The Salmonella Typhi LAMP assay specifically assessed the ViaB gene. It was shown that the analytical sensitivity of the assay was 0.28 fg using agarose gel electrophoresis or amplification plots in Loop amp real-time turbidimeter system, whereas the amplification was 2.8 fg under direct observation of fluorescent color changes. In the proposed methods, the lower limit of detection (LLOQ) of the assays was ~1 and 8 copies of the ViaB gene, respectively. In the LAMP assay for the quantitative detection of Salmonella Typhi, the linear correlation (r2= 0.97) was calculated between the log copy number and Tt values.

Conclusions: The Salmonella Typhi LAMP assay is a simple and accurate tool to detect the causative agent of Typhoid fever that may be designed for clinical laboratories.

Keywords: LAMP, Salmonella Typhi, Typhoid fever, Specificity and Sensitivity

Introduction

Salmonella is characterized by a genus of rod-shaped, Gram-negative, non-spore-forming, and motile bacteria with peritrichous flagella (1). There is a vast range of diseases caused by Salmonella ranging from gastroenteritis, enteric fever (caused by typhoid and paratyphoid serotypes), bacteraemia and focal infection to convalescent lifetime carrier state (2). The salmonella infection is usually caused by consuming foods or water contaminated with animal or human waste (1-3). Salmonella enterica subspecies enterica serovar Typhimurium, which is shortened to Salmonella Typhi, cause a severe systemic infection called typhoid, also known as enteric fever. Salmonella Typhi is the

***Corresponding Author: Soleimani Mohammad,**
Department of Microbiology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran
Email: soleimanidor@yahoo.com
https://orcid.org/0000-0002-8270-9499

human-specific causative agent of typhoid fever that infects approximately 22 million people and causes 216,000 deaths in underdeveloped countries every year (4-6). The death rate of infected people hospitalized in developing countries ranges from 2% in Pakistan and Vietnam to 30%-40% in Indonesia and Papua New Guinea (7). To the best of our knowledge, PCR and Real-time PCR are the most popular diagnostic methods for detection of Salmonella Typhi (8-9); however, they sometimes come across with undesirable obstacles, for example, time and cost are always important elements in clinical diagnosis. The cost has beneficiary roles in methods for detection and diagnosis in clinical and industrial domains. An example of the high cost of real-time PCR and PCR can be observed in the need for thermocycler. With the ease and accessibility of equipment that provide quick diagnosis, it is crucial to run the experiment using non-sophisticated equipment (10).

On the other hand, the absence of sensitive, reliable, and rapid clinical diagnostic methods for the detection and treatment of Salmonella Typhi is so noticeable (11). In addition, some serological tests such as the Widal tests, despite their low sensitivity and specificity and lack of practical values in the endemic area, are still used (12-13). Another reason for the application of simple, cheap, and accessible methods such as the Widal test in the diagnosis of typhoid fever is the low accessibility of new methods in the detection of Salmonella Typhi in some developing countries (14). The low standards of antigens and contribution of antigenic factors to distinguish Salmonella Typhi from other Salmonellae, as well as high risk of false-positive reaction as a result of non-typhoid fever in the analysis of the Widal test in the area that Salmonella Typhi is regarded as endemic agent (14), have highlighted the need to develop faster and more accurate techniques to detect this microorganism. One of the novel nucleic acid amplification methods with high sensitivity, rapidity, and specificity is Loop-Mediated Isothermal Amplification (LAMP) which works under isothermal conditions (15). The LAMP assay is applicable for the molecular diagnosis of various organisms such as bacteria, viruses, fungi, and parasites.

The present study aims to design and promote the LAMP assay to introduce a specific and sensitive method for the rapid and cost-effective detection of Salmonella Typhi within a conserved region of the viaB gene and also develop a sensitive quantitative-LAMP assay (Q-LAMP) to quantify this pathogen in various samples. It is worth mentioning that other researchers have proved the usefulness of this gene in achieving a rapid, susceptible, and specific molecular detection method (16).

Materials & Methods

Sequence Alignment and Primer Design

In the GenBank of NCBI, the viaB sequences of Salmonella Typhi were collected with particular accession numbers of CP003278.1, AE014613.1, AL627283.1, X67785.1, D14156.1, and CP000857.1, and then multiple alignments were carried out with CLC sequence Viewer 6.4 (CLC bio Aarhus, Denmark). An online software program (primer explorer V4) from EIKEN CHEMICAL CO., LTD (http://primerexplorer.jp/e/) (Table 1), were also used to design a set of six LAMP primers, including loop primer. The primer's specificity was confirmed through the in-silico analysis by BLAST and Primer-BLAST tools on the NCBI server (http://www. ncbi.nlm.nih.gov/). The primers were custom synthesized in a commercial source (Bioneer, South Korea).

The LAMP assays

The LAMP assay was conducted with 25µl volume containing 40 pmol of each inner primer (FIP and BIP), 5 pmol of each outer primer (F3 and B3), 20 pmol of the loop primer (LB) (Table 1), 1.4mM each of deoxynucleotide triphosphate, 1M betaine (Sigma-Aldrich, USA), 20 mM Tris-HCl (pH 8.8), 10mM KCl, 10 mM (NH2) SO_4, 8 mM MgSO4, 0.1% Triton X-100, 8 U of Bst DNA polymerase, large fragment (New England Biolabs, Ipswich, MA, USA), and 2µl of genomic DNA of Salmonella Typhi PTCC 1609.

The LAMP products were visualized under daylight or UV light by adding 1 µl of a fluorescent detection reagent consisted of 625µM Calcein (Dojindo Molecular Technologies, Inc, Japan) and 12.5 mM $MnCl_2$ (Sigma-Aldrich, USA) to some of the reaction mixture tubes. The mixtures were incubated at 63°C for 35 or 60 min in a Loop amp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan), and then the turbidity data of the reactions were collected at 650 nm every 6 seconds. In the end, the Bst DNA polymerase was inactivated by heating at 80°C for 5 min. To evaluate the results, the researchers applied electrophoresis on 2% agarose gel observed the turbidity directly and inspected fluorescent emission under the UV light. The specificity of the LAMP assay was confirmed in the process of viaB gene amplification by digesting the amplified products by bglII restriction enzyme (Fermentas Vilnius, Lithuania) at 37 °C for 7 h and then electrophoresing it on 2% agarose gel (17).

Specificity of the LAMP assay

Similar to the above-mentioned procedure, the specificity of LAMP reactions was evaluated using the genomic DNA of Salmonella Typhi and genomic DNA of non-Salmonella organisms. The DNA of the negative controls are as follows: Shigella sonnei ATCC 9290, Klebsiella pneumoniae ATCC 7881, E.coli, Staphylococcus aureus ATCC 25922, Enteropathogenic Escherichia coli (EPEC) ATCC 43887, Escherichia coli O157:H7 ATCC 43895, Yersinia enterocolitica ATCC 23715, Streptococcus pneumonia ATCC 25923, Coxiella burnetii Nine Mile strain ATCC VR-615 and Bacillus subtilis ATCC 6051. The results of the reactions were evaluated and confirmed in the loopamp real-time turbidimeter and then were electrophoresed on 2% agarose gel.

Thymine Adenine Cloning and Preparation of Standard Plasmid

Thymine Adenine cloning (i.e., TA cloning) of the product was performed after the PCR amplification of the viaB gene of Salmonella Typhi with F3 and B3 primers. Hence, the PCR product was purified by a PCR Purification Kit (Bioneer, South Korea). In the next step, linear pTZ57R/T vector was ligated into the purified viaB gene fragment (227 bp) by T4 DNA Ligase (1 U/µL), based on the work instructions of InsTAcloneTM PCR cloning kit (Fermentas Vilnius, Lithuania). Competent cells of E.coli JM107 were transformed by the ligation reaction product. To select and blue/white screening of the desired clones, the researchers incubated transformed cells at 37°C for 24h on Luria-Bertani medium (Merck, Germany) containing 38.4µg/ml IPTG (isopropyl-beta-D-thiogalactopyranoside) (Sigma, St. Louis, MO, USA), 40µl/ml X-gal (5-bromo-4-chloro-3-indolyl beta-D- galactoside) (Sigma, St. Louis, MO, USA), 50µg/ml nalidixic acid and 100µg/ml ampicillin (Merck, Germany). In the next step, some white colonies were extracted by AccuPrep® Plasmid Mini Extraction Kit (Bioneer, South Korea) for target plasmid, and the insertion of viaB gene was confirmed by PCR through the outer primers (i.e., F3 and B3). The final investigation and confirmation of the positive clones were done by bglII restriction enzyme. The pTZ57R/T-viaB, which confirmed plasmid, was quantified by UV absorbance measurement at 260 and 280 nm. It was used as a positive control in sensitivity tests, quantitative evaluation and LAMP assay (18).

Analytical Sensitivity and Quantification

A 10-fold serial dilution of pTZ57R/T-viaB plasmid (from 280 nanograms to 0.28 femtograms = from 8×10^8 copies to ~1 copies) was applied in the LAMP experiment to examine the analytical sensitivity of the assay. The results of the amplified target sequence were analyzed through the following methods: real-time turbidity measurement in Loopmap real-time turbidimeter, visual observation of turbidity by the naked eye, direct and under UV lamp visualization of fluorescent and electrophoresis on 2% agarose gel. Finally, the limit of detection (LOD) of the assay was determined based on each detection method. To improve the assay and

obtain a quantitative LAMP method (Q-LAMP), the researchers used Loopmap real-time turbidimeter to monitor the increase of turbidity resulting from the magnesium pyrophosphate, a byproduct of the LAMP reaction. A standard curve for Q- LAMP was depicted using serial dilutions of pTZ57R/T-viaB plasmid (from 280 ng to 0.28 fg). For each dilution, the copy number was plotted against the time threshold (Tt) value, the time at which the turbidity passed the threshold level. Then linear regression was calculated using the Microsoft Excel program (17-18).

Results

Analysis of the LAMP Products, Specificity, and Cloning

The turbidity was observed by the naked eye due to isothermal amplification of viaB gene or the formation of the white precipitate of magnesium pyrophosphate in the reaction tubes. Owing to the combination of Calcein with magnesium ions in the mixture, the fluorescence emission from intense green fluorescent protein (GFP) was observable under UV light (254~366nm). Amplification of the real-time turbidimeter with an OD of 0.65 confirmed the amplification of the viaB gene (see Figure 1). Detailed ladder-like DNA profiles correspond to the LAMP products were shown by electrophoresis on 2% agarose gel and the predicted theoretical size of LAMP products (87bp and 91bp) expected to be yielded by bglII restriction enzyme digestion was perfectly matched with the actual results (data are not shown here). The reduction of incubation time with the loop primer was also demonstrated. The minimum time for the isothermal amplification was 50 min in the LAMP reaction without the loop prime. However, it was 32 minutes for a detectable signal in the LAMP reactions with the loop primer. The real-time turbidimeter graph analysis has clearly illustrated the exclusivity of amplification for Salmonella Typhi, presenting the specificity of the LAMP assay. However, this result was negative for 11 non-Salmonella bacteria species (data are not shown).

Only tubes containing Salmonella Typhi genomic DNA showed the characteristics of ladder-like multi bands on gel agarose electrophoresis. This result, in agreement with the BLAST analysis, confirmed the specificity of the assay. In cloning the experiment and creating a plasmid positive control, PCR was performed with the outer primers (i.e., F3 and B3) on extracted recombinant plasmids from white color colonies and showed a 227 bp band on 2% agarose gel. Moreover, the process of the pTZ57R/T-viaB was confirmed by bglII digestion and showed a band of about 90 bp as expected (data are not shown).

Sensitivity and quantitative capability of the LAMP assay

The 10-fold serial dilution of pTZ57R/T-viaB (from 280 ng to 0.28 fg equals from 8×10^8 to 80 copies) and the LAMP reaction were the reliable and straightforward methods for identification of limit of detection or analytical sensitivity of the assay. Figure 2A shows a judgment graph of the real-time turbidimeter stating that a decrease in the copy number of the viaB gene can increase the time threshold (Tt). The results of the judgment plot showed that the corresponding agarose gel electrophoresis and visual detection of fluorescent emission due to the creation of Calcein magnesium complex, and the limit of detection of the assay were estimated to be ~1 copy per reaction tube in the presence of the agarose gel electrophoresis and the real-time turbidimeter detection methods and 8 copies in the present visual detection of the fluorescent emission (Figure 2). The average Tt value for template range of ~1-8×10^8 copies of viaB in 25µl volume was between 35 and 63 min (Figure 2). Further, the standard curve generated by the turbidity-based real-time LAMP assay showed a linear relationship between the log copy number of the viaB gene and the corresponding Tt values with a correlation coefficient (r2) of 0.97 (data are not shown).

Table 1. Primer sequences of a LAMP assay for rapid detection of Salmonella Typhi based on ViaB gene

Primer	Sequence (5´-3´)	Reference
F3-ST-viaB	TCATTTCAAGTTCGCGACTA	
B3-ST-viaB	TTCCATACCACTTTCCGAA	
FIP-ST-viaB	TGTTGCCGCATCGAAAACAGCTTGCACGTTTTTGGTTGA	This study
BIP-ST-viaB	CAACACCAAAGTGGTGAAAAGATCTTCATCTTTACTATATCCTTACGGT	
LB-ST-viaB	TTGACGGAGCAGAGAGATTATCG	

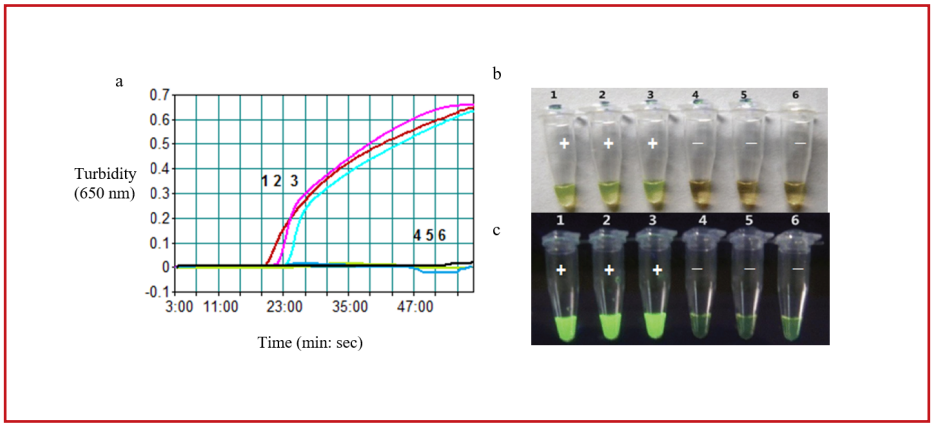


Figure 1. Detection of the LAMP reaction. Tubes 1-3 containing pTZ57R/T- ViaB; tubes 4-6, negative control (without DNA). Analysis of the LAMP reaction products using amplification graph depicted by the real-time turbidimeter. Visual detection of the LAMP reaction products using Calcein under daylight (b) and under UV light (c).

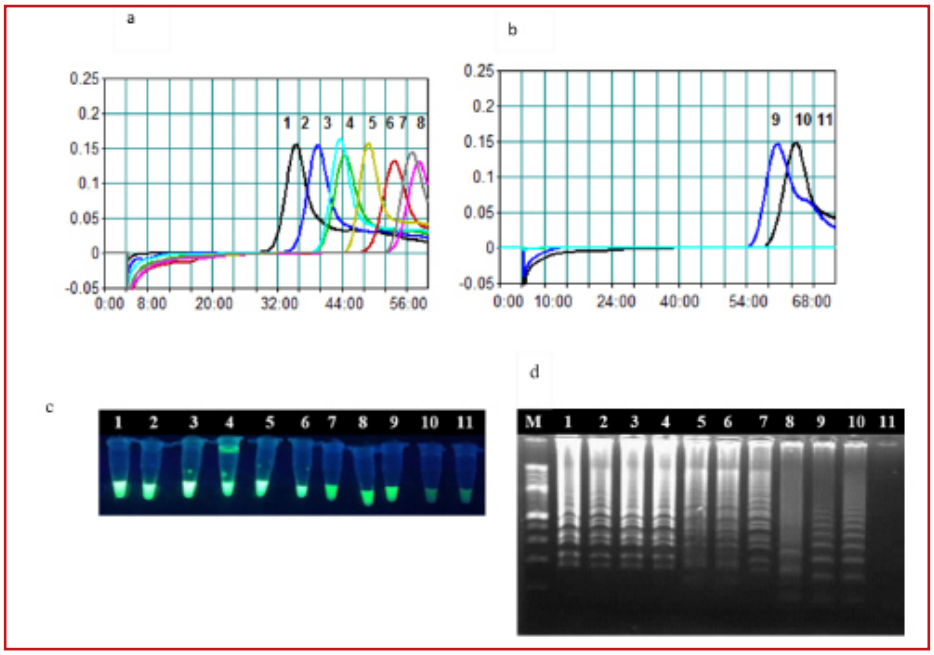


Figure 2. Identification of Sensitivity of the LAMP assay based on a 10-fold serial dilution of pTZ57R/ T-ViaB. (a) A representative judgment graph of the real-time turbidimeter. Curves 1–10, serial dilution from 280 ng to 0.28 fg equals from 8×108 to 80 copies; curve 11, negative control. (b) Visual inspection of Calcein fluorescence. Tube 1-10, serial dilution, same as above; tube 11, negative control. (c) Electrophoresis of the products on 2% agarose gel and inspection of ladder-like pattern. Lane M, 100 bp DNA ladder; lanes 1–10, the serial dilution; lane 11, negative control.

Discussion & Conclusion

Salmonella Typhi causes Typhoid fever, a severe infection in the reticuloendothelial system characterized by systemic illness with high fever, diarrhea, constipation, and abdominal pain (19-21). Hence, the accurate and quick detection of the agent plays a key role in public health to manage infection and disease, especially in epidemic events. Although the isolation of Salmonella Typhi from blood and bone marrow is regarded as the gold standard, there are some main obstacles such as poor sensitivity in endemic areas associated with high rates of antibiotic use and long-time consumption(19). This study represents a specific, sensitive, rapid, and considerably cost-effective method for qualitative and quantitative detection of Salmonella Typhi. This method can compete and can be compared with any other DNA amplification methods that are routinely used in the detection and diagnosis of different microorganisms in the laboratory. The LAMP method offers a lot of advantages compared with other methods in the diagnosis of infectious diseases (19). In addition, the LAMP assay can be examined by naked eyes; in other words, there is no need for the use of complex and expensive equipment. In this study, the analytical specificity of the assay was 100%, with emphasis on the designed primers that were specific for the viaB gene and the amplification process, which was done only by the DNA of Salmonella Typhi. Furthermore, the speed and efficiency of amplification were raised by applying the designed loop primer, which is attached to the stem-loops during the reaction process (22). The sensitivity of the assay was demonstrated by the detection of ~1 copy of the Salmonella Typhi ViaB gene in a reaction tube. This sensitivity is outstanding and comparable with other diagnostic methods such as real-time PCR. Moreover, either false positive and negative results or cross-reactivity with other species was not observed. It can be assumed that the high specificity of the reaction in the LAMP assay would be due to the use of six primers. Indeed, the generated fragments from the viaB LAMP products which were digested by bglII were entirely specific. To detect the LAMP products, the researchers needed to use Calcein as a fluorescent dye. To the best of our knowledge, by adding the fluorescent dye to the reaction, the fragments were observed by naked eyes due to the change of color by fluorescence. Thus, because of this kind of system for observation, first, there will be no need for electrophoresis and secondary which is more important, the reaction will be prevented from contamination during electrophoresis or dying by ethidium bromide (23). Although there is no difference in sensitivity of the electrophoresis and judgment graph of the real-time turbidimeter (~1 copy), the detection methods were nearly 10-fold more sensitive than Calcein fluorescent detection (8 copies). Therefore, Calcein fluorescence is a trusted non-complex assay that eliminates the use of sophisticated patterns and instruments in the diagnosis of typhoid fever.

In the quantitative section of this study, the linear correlation (r2= 0.97), which was between the log copy number and Tt value, illustrates the measurability of the LAMP assay in its reactions. Hara-kudo, et al. (2005) illustrated the limit of detection of LAMP assay in the diagnosis of Salmonella against the PCR and real-time PCR. The limit of detection in LAMP assay was between 1.9 and 2.2 cells per test tube which were comparable with detection of Salmonella by PCR (16 cells/ test tube) (24). Furthermore, the sensitivity of the real-time PCR was higher (5 cell/ tube test) than that of PCR in the same experiment as Hara-kudo (25). This comparison shows the priority of sensitivity level in different assays, and LAMP assay obtains the highest sensitivity approximately eight times. Massi, et al. (2005) can quantify the copy number of the Salmonella Typhi flagellin gene in the range of 100 to 106 per reaction by TaqMan assay. They chose the flagellin gene because it uniquely belonged to Salmonella Typhi (26). Indeed, in the present study, the sensitivity limit was ~1 copy with the lowest concentration of 0.28 fg whereas Calcein fluorescence illustrated 8

copies number in the same concentration, which demonstrates the high sensitivity of the LAMP assay in comparison with other methods such as PCR and real-time PCR. During the last decades, a lot of methods have been carried out to diagnose Salmonella Typhi to achieve the results in a short time with high specificity and accuracy. The first outstanding detection was the nested PCR that approached the high detection rate compared to the blood culture method and Widal test. However, it has some negative points which take a long time to show the results, and more importantly, it has more contamination compared to PCR assay. Furthermore, detection of amplification in conventional PCR assay which can be performed by agarose gel has a limitation in sensitivity and speed (26). To complete the comparison of different assays, real-time PCR, in which a fluorescent signal is a major factor in detection, has more advantages than conventional PCR, such as quantitative detection, which is not an entirely desirable assay in detection (26). Time consumption, high cost, and complexity of required equipment are the disadvantages of these methods. LAMP assay's current new method performs more efficiently in time, cost, specificity, and sensitivity. The Salmonella Typhi LAMP method was designed and presented in this study because of its high specificity and sensitivity compared to other molecular methods. It does not require expensive equipment and reagents and is a good candidate for clinical laboratories, especially in underdeveloped areas. Accurate results obtained using this method for the quantitative determination of Salmonella Typhi also indicate the feasibility of this method for quantifying bacteria in the studied samples.

Authors' contribution statements

M. Soleimani is the corresponding author, performed the laboratory tests, drafted the manuscript, and participated in data analysis. K. Majidzadeh-A designed and supervised the study, analysed and interpreted the data, and reviewed the manuscript. M. Sohrab and A. Morovvati participated in laboratory work and contributed to data analysis. All authors read and approved the final version of the manuscript.

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Conflicts of interest

All authors declare that there is no conflict of interest.

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