Preliminary screening of *Salmonella* in oral drugs by visual LAMP technology

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**Abstract.** Objective According to the Chinese Pharmacopoeia, *Salmonella* should not be detected in oral drugs. In this experiment, LAMP technology was used to detect *Salmonella* quickly, and the detection results were visible to the naked eye with calcein-Mn$^{2+}$ dye, so as to provide pharmaceutical companies with the initial screening of drugs containing *Salmonella*. Methods Four primers (FIP, BiP, F3, B3) were designed to detect *Salmonella* by loop mediated isothermal amplification using inv A gene of *Salmonella*, and then the loop mediated isothermal amplification detection method was established. To verify the specificity of the method, LAMP detection was carried out on 10 kinds of laboratory preserved strains. The sensitivity of the method was obtained by detecting 10 times gradient dilution of *Salmonella* bacteria solution. Finally, the LAMP system of *Salmonella* was used to detect the oral drugs sold on the market and not tested by the Drug Administration, and the sensitivity of the system in the drug was determined. Results After optimization, the specificity of the system was good. For 10 different strains of LAMP detection, only *Salmonella* appeared trapezoidal band, and other strains did not detect the band; Through the experimental verification, the sensitivity of the system was 3.2×10¹ CFU/mL; The system was used for the oral drugs sold in the market and not passed the inspection of the drug administration. The results of LAMP detection showed that no *Salmonella* was detected. However, the results of LAMP detection showed that there were trapezoidal bands in the test results. The sensitivity of the system in the detection of *Salmonella* in oral drugs could reach 3.8×10¹ CFU/mL. Conclusion The visual LAMP detection system for *Salmonella* can be used for the preliminary screening of oral drugs containing *Salmonella*.

**Key words:** LAMP, Loop mediated isothermal amplification, Salmonella inv A, Oral drug, Calcein.
1. Introduction

Salmonella is a common foodborne pathogen, which is a kind of gram-negative bacilli. The main symptoms of Salmonella poisoning are acute gastroenteritis. The symptoms include vomiting, diarrhea, abdominal pain, fever to 38-40 °C and so on. Severe patients will have chills, convulsions, convulsions and coma. As an indispensable part of the life of many patients, the safety and reliability of oral drugs are widely concerned by the people. According to the provisions of the Chinese Pharmacopoeia, Salmonella shall not be detected in oral drugs [1-3]. Therefore, Chinese herbal medicine granules for oral use must be tested for Salmonella before they can be used. At present, the more commonly used microbial detection mainly includes the traditional culture method and Immunology detection method. The traditional isolation and culture detection relies more on manual operation and subjective judgment, which requires a higher level of technical personnel. At the same time, it takes 4-7 to complete the detection. The detection cycle is long; although the immunological detection methods are specific, rapid and sensitive, these effects need to be established on the basis of highly specific antibodies and suitable and stable reaction system; in order to avoid complexity, save time and cost, pharmaceutical companies urgently need a rapid detection method for Salmonella.

Loop mediated isothermal amplification technology is a sensitive chain substitution nucleic acid amplification technique first proposed by Japanese scholar Notomi et al. [4]. This method can amplify the target DNA from several copies to $10^9$ copies in one hour at 65 °C. In addition to the advantages of high specificity and high sensitivity, the operation is very simple, and the requirements for instruments and equipment are low. The reaction can be realized by using water bath pot. The results can be judged by naked eye observation of white turbidity, which is simple and fast. Nowadays, there have been endless examples of detection of Phytophthora cowpea by loop mediated isothermal amplification, such as Xia Xuefeng et al. [5] used the loop mediated isothermal amplification technology to detect Escherichia coli; Xu Jinhe et al. [6] used this technology to detect SARS-COV-2; and Li Jinjin et al. [7] established a ring mediated isothermal amplification detection method for Phytophthora cowpea.

Using the advantages of LAMP technology different from other detection methods, the invA (invasion protein a gene) gene of Salmonella was selected, which is the unique DNA sequence of Salmonella [8]. A group of LAMP citations that can specifically detect Salmonella were designed by using the gene sequence. Then, by adding calcein-Mn²⁺ dye to make the experimental results visible to the naked eye, a visual loop mediated isothermal amplification technique was established for the preliminary screening of Salmonella in oral drugs.

2. Materials and methods

2.1. Basic reagents

2× LAMP PCR Master Mix (univeral) kit (batch number: b532455), calcein and anhydrous manganese chloride were purchased from Shanghai Bioengineering Co., Ltd. 50× Tae electrophoresis buffer, agarose, 2000 bp marker, GelGreen DNA nucleic acid dye, etc. were purchased from Nanjing kingsray Technology Co., Ltd.

2.2. Drugs

The drugs not tested by the Bureau of Drug Administration: Chaihuang granule (batch number: Z20003383), clarithromycin capsule (batch number: H44024313), Gamnua Qingre granule (batch number: Z36021225), Xiaofauling granule (batch number: Z2000384) were given by the pharmaceutical company. The drugs sold on the market: Shanwax Mei Ye granule (batch number: Z2000277113), Angan granule (batch number: Z20003030), Xiaoachaigui antipyretic granule (batch number: Z20050716), Rechin Qing tablet.
2.3. Strain
Salmonella, Digestive bacilli, Candida albicans, Pseudomonas aeruginosa, Pathogenic Bacillus cereus, Proteus, Staphylococcus epidermidis, Streptococcus faecalis, Streptococcus enteritis, Escherichia coli, etc., from the laboratory cryopreserved strains.

2.4. Laboratory equipment
Electrophoretic apparatus (China), thermostatic water bath pot (China), gel imaging analysis system UVI (USA), gas bath thermostatic oscillator (China), incubator (USA).

2.5. Primer Design
The sequence inv A Salmonella gene was obtained from NCBI website, and then the specific primer [9] of inv A gene was obtained by Primer Explore V5 (http://primerexplorer.jp/LAMPv5e/index.html) online design software, as shown in figure 1, the primer sequence is shown in Table 1. Primers were synthesized by Nanjing Kingsley Technology Co., Ltd.

![Figure 1. design of LAMP primers for Salmonella inv a gene on line](image)

| Name of primer | Sequence (5’-3’) | Length of primer (bp) |
|----------------|------------------|----------------------|
| FIP            | ATGATGCCGGCAATAGCGTCAC- | 42                  |
|                | AAAGCAGCTT TACCGTTC |                     |
| BIP            | GATGACCCGCAATGGTATGGAT- | 41                  |
|                | ACCATACCAATGGTACGC |                     |
| F3             | GCGAAGCGTACTGGAAAGG  | 19                   |
| B3             | TCAACAATGCGGGGATCTG  | 19                   |

2.6. Rough extraction of bacterial genomes using boiling water
Put the EP tube containing 1-2 mL bacterial liquid into 100 °C boiling water, after 20 min of water bath, put it into the refrigerator at -20 °C for preservation [10].

2.7. Establishment and Optimization of Visual LAMP System
By referring to the instructions of 2 × LAMP PCR Master Mix (Univeral) kit, the content of primers and the ratio of internal primers (FIP, BIP) to external primers (F3, B3) were tested and adjusted for many times to obtain the optimal LAMP. After the reaction system, a proper amount of Mn$^{2+}$ was added into the system to make it completely react with pyrophosphate ion without affecting the amplification.

LAMP reaction system was heated in water bath at 65 °C for 1 h, then heated in 80 °C water bath for 10 min. after cooling down slightly, appropriate amount of calcein was added into the final reaction solution to make the color difference between the positive and negative control obvious, so as to achieve the visualization effect.
2.8. Specific detection of visual LAMP system

The optimized visual LAMP system was used to detect the genomes of Salmonella, Digestive bacteria, Candida albicans, Pseudomonas aeruginosa, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, Streptococcus enteritidis, Escherichia coli and other strains. The double distilled water was used as the negative control, so as to verify the specificity of the visualized LAMP system [11-12].

2.9. Sensitivity determination of the visual LAMP system

According to 450 μL sterile broth and 50 μL bacterial solution, 9 gradients were diluted successively by 10 times gradient dilution method, and 100 μL of bacterial liquid of 6, 7 and 8 were coated on the plate, and two plates were coated on each gradient to calculate the CFU value of each gradient. Then, the 3-8 gradient bacterial liquid was boiled in boiling water for 20 min, and the visual LAMP system of this experiment was used to evaluate the CFU value of each gradient. The gradient crude extract of the bacterial liquid was detected. The sensitivity of the system was identified by observing the trend of the color and gradient of agarose gel electrophoresis. The sensitivity of the system was [13-14].

2.9.1. Detection of oral drugs using a visual LAMP system. Shanhuameiye granules, Kanggan granules, Xiaoer chaigui Tuire granules, Relinqing tablets, Lianhua Qingwen capsule, Sanqi powder, Xiaopuling granules, Chaihuang granules, clarithromycin capsules, Ganmao Qingre granules and other drugs were dissolved in nutrient broth at the concentration of 0.1 g/mL, and their genomes were roughly extracted by water boiling method, and then visualized LAMP was used. The system detected the crude extract of each oral drug, and observed the difference of color by naked eye and 2% agarose gel electrophoresis. The final result was [15-16].

2.9.2. Determination of Sensitivity of Visual LAMP System in Artificially Contaminated Salmonella Oral Medicines. In this experiment, 200 μL Salmonella solution was artificially added into 5 mL of Chaihuang granule broth which was tested to be sterile, and was shaken overnight in an air bath thermostatic oscillator. According to the method of experiment 2.9, the sensitivity of visual LAMP system in oral drugs was determined.

3. Experimental results

3.1. Establishment of visual LAMP system

After several pre experiments, the optimal visual LAMP total system of 25 μL was determined, including 2 × LAMP Master Mix 12.5 μL, Bst DNA polymerase 0.5 μL, internal primer FIP and BiP (10 μM) 2 μL, external primer F3 and B3 (10 μM) 0.5 μL, Mn²⁺ (15mM) 1 μL, template 1 μL, ddH₂O 6 μL. The system was heated in water bath at 65 ℃ for 1 h, and then passed through Heat in water bath at 80 ℃ for 10 min. When 3 μL of 0.5 mM calcein was added into the final reaction system, the positive result was green and the negative result was brown yellow. By 2% agarose gel electrophoresis, the positive results appeared trapezoidal strip, and the layers were clear and the brightness was clear. No amplification bands were found in the negative control. (see Figure 2)
Figure 2. Visualization of LAMP system color contrast (A) and agarose gel electrophoresis strip (B) (M: 2000 bp DNA marker; 1: Salmonella genome; 2: double distilled water (negative control))

3.2. Specificity detection of visual LAMP system
Using LAMP The genome of Salmonella, enterobacter, Candida albicans, Pseudomonas aeruginosa, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, Streptococcus enteritidis and Escherichia coli were detected. The results showed that except for Salmonella genome, no trapezoidal band was found in other strains, and only the color of Salmonella reaction tube was green. The other reaction tubes were brown yellow, which indicated that the reaction system had good specificity and could be used to detect Salmonella. (see Figure. 3)

Figure 3. Visual lamp system specificity test (A and B) (M: 2000bp DNA Marker; 1: Salmonella genome (Positive control); 2: Candida albicans genome; 3: Pseudomonas aeruginosa genome; 4: Streptococcus enteritidis genome; 5: Pathogenic Bacillus cereus genome; 6: Proteus genome; 7: Staphylococcus epidermidis genome; 8: Streptococcus faecalis genome; 9: Digestive bacteria genome; 10: Escherichia coli genome; 11: Distilled water (Negative control))

3.3. Visual LAMP System Sensitivity Determination
Using 10 times dilution method, the Salmonella original solution was diluted to 9 gradients, and the plate coating was carried out with the bacterial liquid of 6, 7 and 8 gradients. The coated plate was cultured in a 37 °C constant temperature incubator for 24 h, and finally the eighth gradient was about 10 CFU/mL. According to the results, the CFU values of each gradient could be calculated in turn. The LAMP system was used to detect the genomics extracted from 3-8 gradient bacterial solution. The
results showed that the color and band brightness of the final system decreased gradually with the addition of calcein, and the color of the final system could not be distinguished after the 7th gradient, and there were still trapezoidal bands in the 8th gradient, which proved that the sensitivity of the system was $3.2 \times 10^1$ CFU/mL, and the color detection limit was $3.2 \times 10^2$ CFU/mL. (see Figure. 4)

![Figure 4](image-url)

**Figure 4.** Detection limit determination of visual LAMP system (A and B)(M: 2000 bp DNA Marker; 1: $3.2 \times 10^7$ CFU/mL; 2: $3.2 \times 10^6$ CFU/mL; 3: $3.2 \times 10^5$ CFU/mL; 4: $3.2 \times 10^4$ CFU/mL; 5: $3.2 \times 10^3$ CFU/mL; 6: $3.2 \times 10^2$ CFU/mL; 7: $3.2 \times 10^1$ CFU/mL; 8: Distilled water (Negative control))

### 3.4. Detection of oral drugs by visual LAMP system

Using LAMP The nutrient broth solutions of Chimonanthus praecox leaf granules, Kanggan granules, Xiaoer chaigui Tuire granules, Relinqing tablets, Lianhua Qingwen capsules, Sanqi powder, xiaopuling granules, Chaïhuang granules, clarithromycin capsules, Ganmaoqingre granules and other drugs were detected by the system. The results showed that there was no trapezoidal strip in the experimental results, and the color of reaction tubes was brown yellow, which proved that there was no *Salmonella* in the tested drugs. (See Figure 5)

![Figure 5](image-url)

**Figure 5.** Detection of oral drugs by visual LAMP system(M: 2000 bp DNA marker; 1: *Salmonella* genome (positive control); 2: Clarithromycin Capsules; 3: Chaïhuang granules; 4: xiaopuling granules; 5: Ganmao Qingre granules; 6: shanhuaye granules; 7: Kanggan granules; 8: Xiaoer chaigui Tuire granules; 9: Relinqing tablets; 10: Lianhua Qingwen capsules; 11: Sanqi powder; 12: shuangzhishui (negative control))
3.5. Determination of Sensitivity of Visual LAMP System in Artificially Contaminated Salmonella Oral Medicines

To add 200 μL of Salmonella solution to 5 mL of sterile Chaihuang granule broth, in an air bath thermostat, using experiment 1.7. The 8th gradient is about 3.8×10^7 CFU/mL. With this result, CFU values of each gradient can be calculated in turn. Using the LAMP system of this experiment, the crude genome of 3–8 gradient bacteria solution was detected. The sensitivity of the system to oral drugs can reach 3.8×10^1 CFU/mL. The color detection limit is 3.8×10^2 CFU/mL, and the results showed that oral drugs had little effect on visual LAMP detection. (see Figure 6)

![Figure 6](image-url)

**Figure 6.** Visual LAMP system sensitivity determination in artificially contaminated Salmonella oral drugs (A and B) (M: 2000 bp DNA Marker; 1. 3.8×10^7 CFU/mL; 2. 3.8×10^6 CFU/mL; 3. 3.8×10^5 CFU/mL; 4. 3.8×10^4 CFU/mL; 5. 3.8×10^3 CFU/mL; 6. 3.8×10^2 CFU/mL; 7. 3.8×10^1 CFU/mL; 8. Double steam (negative control))

4. Conclusion

Food is the nature of the people, and food is the foundation of the people. Food safety has always been the focus of the masses. Salmonella is a kind of important pathogenic bacteria which endangers human and animal health. Food poisoning can be caused by the feces of people or carriers infected with Salmonella. According to the statistics of various kinds of bacterial food poisoning in the world, the cases of food poisoning caused by Salmonella are the first in the world. It can be seen that the detection of Salmonella in food is particularly important. Oral drugs are widely accepted because they are easy to take and do not need torment. However, due to the complex production process, Salmonella is easy to be infected in the production process. However, drug safety is related to people's health, life safety and social stability. The state has stipulated that all drugs shall not contain Salmonella [1–3]. Traditional culture method for detection of Salmonella is an important detection technology recognized by today's society. However, due to the long detection cycle and cost of money, this method not only reduces the company's profits, but also hinders the rapid circulation of drugs. On the basis of saving the cost of pharmaceutical companies, it is necessary to establish a rapid, specific, convenient and low-cost detection method for qualified drugs to reach patients quickly.

Loop mediated isothermal amplification technology [17–18] as a detection technology rising in recent years, is widely favored in the field of microbial detection due to its advantages of high specificity, high sensitivity, simple operation and low cost. In recent years, the detection methods using loop mediated isothermal amplification technology are also emerging in an endless stream, but there are also some shortcomings in the detection process, such as (1) by the The LAMP primer design is generally aimed at 4–6 regions of target gene, and the design of primer is complex and limited. At the same time, LAMP primer design software has requirements on the length of target gene. Although the use of multiple primers improves the specificity, it also increases the probability of hybridization between primers, which is easy to produce non-specific amplification and lead to false positive results. (2) The concentration of Mg^{2+} in the system should be moderate, less would reduce the activity of enzyme, too much would lead to nonspecific amplification, so it needs a long time of trial. (3) LAMP
reaction products are very stable, not easy to degrade, easy to form aerosol pollution, and the sensitivity of LAMP reaction is very high, even a small amount of positive products will affect the results, which puts forward higher requirements for operators and operation process. As a common microbial detection technology, fluorescent quantitative PCR has the characteristics of fast, high-throughput, strong specificity, sensitivity, high automation, accurate quantification, good repeatability, etc., but it also has some disadvantages, such as the need for professional instruments, high requirements for primers, and the possibility of false positive [19]. However, in terms of economy and simple operation, loop mediated isothermal amplification technology is still slightly superior.

In this study, we designed a set of LAMP which can be used to detect Salmonella. After many experiments, it was found that the color of calcein would be changed by long time heating, so that the color change was not obvious. This experiment analyzed the color development principle of calcein, and finally decided to add calcein at the end of the reaction. After comparing the experimental results, the final concentration of internal primers (FIP, BIP) was 0.8 μM, the final concentration of external primers (F3, B3) was 0.2 μM, the final concentration of Mn²⁺ was 0.6 mM, 2 × LAMP master mix was 12.5 μL, Bst DNA polymerase was 0.5 μL, template was 1 μL, and ddH₂O was 6 μL. The mixed system was heated in a 65 °C water bath for 1 h to make the system fully react. Then the mixture was heated in 80 °C water bath for 10 min. After the Bst DNA polymerase was inactivated, 5 mM was added Calcein makes the system visible. The genome of Salmonella, Enterobacter, Candida albicans, Pseudomonas aeruginosa, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, Streptococcus enteritidis, Escherichia coli were detected by using the optimized system. The results showed that only Salmonella genome showed trapezoidal bands, other strains did not appear trapezoidal bands, and the terminal body. After adding calcein, only Salmonella genome turned green, which proved that the system had good specificity and could be used for the detection of Salmonella. Nine gradients of Escherichia coli were diluted by 10 fold dilution method. CFU values of all gradients were calculated by coating 6, 7 and 8 gradients. Visual LAMP system was used to detect 3-8 gradients. The results showed that the sensitivity of the system was 3.2 × 10⁴ CFU/mL, and the color detection limit was 3.2 × 10² CFU/mL. In addition, LAMP was used in this experiment. The results showed that no Salmonella was detected, but the aseptic Chaihuang granules were active to infect Salmonella. After overnight cultivation in shaking bed, the effect of anti infective granules on Salmonella was not obvious. The sensitivity of LAMP was 3.8 × 10⁴ CFU/mL, and the color detection limit was 3.8 × 10² CFU/mL, indicating that oral drugs had little effect on LAMP detection.

Since the introduction of loop mediated isothermal amplification technology in 2000, scientists have continuously improved and supplemented the technology. For example, Nagamine et al. [20] supplemented the primer design of LAMP technology in 2002, and successfully designed loop primers, which can reduce the reaction time by 1/2. In addition, the research shows that SYBR Green I, hydroxynaphthol blue (HNB), calcein and other dyes can be added into LAMP system, and the reaction results can be directly determined by observing the color difference with naked eyes [21]. In addition, Chen Yin et al. [22] applied gold nano biosensor technology to LAMP detection.

To sum up, based on the needs of the development of the times and the design concept of pursuing economic and environmental protection, this experiment established a visual LAMP detection method for oral drugs containing Salmonella. The method has the advantages of strong specificity, high sensitivity, simple operation and no need of precision instruments, which can bring convenience to pharmaceutical companies.

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