Preliminary screening method of Escherichia coli in oral drugs by visual LAMP technology

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Abstract. According to Chinese Pharmacopoeia, Escherichia coli should not be detected in oral drugs. In this study, a visual LAMP method for rapid detection of Escherichia coli was developed to replace the traditional microbial limit test method. Methods Four specific primers (FIP, BiP, F3, B3) for loop mediated isothermal amplification were designed by using uidA gene of Escherichia coli, and calcein-Mn²⁺ dye was added to the system to establish a visual loop mediated isothermal amplification detection method for Escherichia coli. Finally, the visual LAMP system was used to detect the presence of E.coli and the sensitivity of the system in drugs. Results The specificity of the optimized system was good, using this system to detect 10 different strains, the results showed that only Escherichia coli genome appeared, the system turned green, the other systems were brown yellow; the detection limit of gradient dilution Escherichia coli was 10² CFU/mL by LAMP. Visual LAMP was used to detect the oral drugs sold in the market and not passed the test of the drug administration, and no E.coli was detected, but the oral drugs that were artificially contaminated with E.coli were visualized by LAMP. The color detection limit of the system in oral drugs was 1.8×10² CFU/mL. Conclusion The visual LAMP detection system can be used for the preliminary detection of oral drugs containing Escherichia coli.

Keywords: LAMP; Loop mediated isothermal amplification; Escherichia coli uidA; Oral drug; Calcein.

1. Introduction

According to the Chinese Pharmacopoeia, Escherichia coli should not be detected in oral drugs (quoted from Chinese Pharmacopoeia [1-3]). Chinese herbal medicine granules are made by the compatibility of traditional Chinese medicine under the guidance of the basic theory of traditional Chinese medicine, because of its variety of drugs, wide intake channels, thus increasing the risk of E.coli contamination. Chinese herbal medicine granules and other oral drugs must first pass the microbial limit test of the State Food and drug administration; and the content of Escherichia coli is a
must test item, and the microbial limit test takes about 1 month, and the test cost is 3 Therefore, pharmaceutical companies have to carry out preliminary screening before drug inspection. The traditional cultivation method is not only very cumbersome, but also requires high technical level of operators, and the detection cycle is long. Therefore, there is an urgent need for a rapid and effective method for preliminary screening of Escherichia coli in oral drugs and food.

Loop mediated isothermal amplification technology is the basis of the third generation PCR amplification. It is a nucleic acid isothermal amplification technology proposed by Japanese scholar Notomi et al. [4] in 2000. It has the advantages of strong specificity, independent of instrument, isothermal amplification, and direct observation of results. At present, novel coronavirus pneumonia is widely used in the rapid detection of pathogenic bacteria such as viruses and bacteria. For example, Wu Tao and [5] used LAMP technology to get a new method for rapid detection of new crown pneumonia virus. For example, [6] and Wei Zhenyuan used loop mediated isothermal amplification technology to detect Salmonella, and [7] such as Wei Wei Jun used LAMP technology to detect fungi.

In this experiment, we selected the uidA gene of E.coli by using the advantages of LAMP technology. This gene is a gene that catalyzes the hydrolysis of terminal non reducing β-glucose residue and releases β-glucosidase in the genome of E.coli. It is an important marker for identification of E.coli [8]. Using the conserved sequence of the gene, we designed a group of LAMP Primers. Calcein is a kind of fluorescent dye, which can combine with Ca²⁺, Mg²⁺, Mn²⁺ and other metal ions and show different colors. The single calcein is green in the system, but the color changes to brown yellow after adding Mn²⁺. With the amplification of LAMP, pyrophosphate ion produced by the reaction reacts with Mn²⁺, the reaction system turns green when precipitate is formed. In this experiment, calcein Mn²⁺ dye is added to the reaction system to dye the reaction system, thus a visual loop mediated isothermal amplification technique is established for preliminary screening of Escherichia coli in oral drugs.

2. Materials and methods

2.1. Reagents, drugs and strains
2 × lamp PCR Master Mix (universal) kit (batch No.: b532455), calcein and anhydrous manganese chloride were purchased from Shanghai Bioengineering Co., Ltd. Agarose, 50 × Tae electrophoresis buffer, GelGreen DNA nucleic acid dye and 2000 bp marker were purchased from Nanjing Kingsray Technology Co., Ltd. The drugs that failed to pass the inspection by the drug administration: Clarithromycin Capsules (batch No.: H44024313), Chaihuang granules (batch No.: Z20003383), xiaopuling granules (batch No.: Z20003384) and Ganmao Qingre granules (batch No.: Z36021225) were donated by pharmaceutical companies. The drugs on the market: Chimonanthus praecox leaf granules (batch No.: Z20027113), Kanggan granules (batch No.: Z20003030), Xiaoer chaigui Tuire granules (batch No.: Z20050716), Relinqing tablets (batch No.: 200307), Lianhua Qingwen capsule (batch No.: z20040063) and Sanqi powder (batch No.: 190315) were purchased from huangqingren pharmacy of Nanchang City, Jiangxi Province. Escherichia coli, Candida albicans, Pseudomonas aeruginosa, Streptococcus enteritidis, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, digestive bacteria, Salmonella, etc. were isolated from the frozen strains in our laboratory.

2.2. Experimental equipment
Constant temperature water bath pot (China), electrophoresis device (China), gel imaging analysis system UVI (USA), constant temperature incubator (USA), gas bath constant temperature oscillator (China).

2.3. Primer design
The uidA gene sequence of E.coli was obtained from NCBI website, and then the sequence was analyzed by primer explore V5(http://primerexplorer.jp/LAMPv5e/index.html)On line design software
specific LAMP primers for uidA gene [9], which were synthesized by Nanjing kingsray Technology Co., Ltd., and the primer sequences are shown in Table 1.

| Name of primer | Sequence(5′-3′)                                      | Length of primer(bp) |
|----------------|------------------------------------------------------|----------------------|
| FIP            | TGCGATGGATCCCGCGATAATT-TGATTACCGACGAAAACGGC         | 42                   |
| BIP            | CCGAACACCTGGGTTGGACGATA-TCACACAGCGCGTGGTTAC          | 41                   |
| F3             | ACTGGCAGACTATCCCGC                                    | 18                   |
| B3             | GCTGACATCACCATGCGC                                     | 19                   |

2.4. Extraction of bacterial genome by boiling method
Put 1-2 mL bacterial liquid into 100 °C boiling water, after 15 min water bath, put it into -20 °C refrigerator for preservation [10].

2.5. Establishment and optimization of visual LAMP system
According to the instructions of 2 × lamp PCR Master Mix (universal) 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, and appropriate concentration of Mn2+ was added to obtain the optimal visual LAMP reaction system.

The LAMP reaction system was heated in water bath at 65 °C for 1 h, and then heated at 80 °C for 10 min. after that, appropriate amount of calcein was added into the final reaction system to distinguish the negative and positive results.

2.6. Specificity detection of visual LAMP system
In order to verify the specificity of the visualized LAMP reaction system, E.coli genome was used as positive control and double distilled water as negative control The system detected the genome of Candida albicans, Pseudomonas aeruginosa, Streptococcus enteritidis, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, digestive bacteria, Salmonella and other strains [11-12].

2.7. Detection limit determination of visual LAMP system
Using 10 times gradient dilution method, nine gradients were diluted according to 900 μL aseptic broth and 100 μL bacterial liquid. 200 μL of bacterial liquid in 6, 7 and 8 gradients were absorbed and coated on the plate. 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 15 min, and the visual LAMP system of this experiment was used to determine the CFU value of each gradient The detection limit of the system was determined by comparing the differences of color changes [13-14].

2.8. Detection of oral drugs by visual LAMP system
Clarithromycin capsules, Chaihuang granules, xiaopuling granules, Gannmaoqingre granules, Chimonanthus praecox leaves granules, Kanggan granules, Xiaoer chaigui Tuire granules, Relinqing tablets, Lianhua Qingwen capsules, Sanqi powder and other drugs were dissolved in nutrient broth at the concentration of 0.1 g/mL. the genome was extracted by boiling method, and then the visualized LAMP was used The crude extracts of each oral drug were detected by the system, and the results of [15-16] were determined by observing the color changes of the final system and agarose gel electrophoresis.
2.9. Detection limit determination of visual LAMP system in oral drugs contaminated with Escherichia coli

In order to determine the detection limit of visual LAMP system in oral drugs, 100 μL Escherichia coli solution was artificially added into 10 mL of Chaihuang granule broth which was tested to be sterile, and was shaken overnight in an air bath thermostatic oscillator. The experiment was carried out according to the method of experiment 1.7 [17-18].

3. Experimental results

3.1. Establishment of visual LAMP system

After comparing the results of several validation experiments, it was found that the color change of calcein was affected by long-time heating. Considering various factors, this experiment decided to add calcein after the reaction, and finally determined the optimal LAMP total system of 25 μL, including 2 × LAMP Master Mix 12.5 μL, Bst DNA polymerase 0.5 μL, internal primer FIP, BiP (10 μM) 2 μL, external primer F3, B3 (10 μM) 0.5 μL, MnCl₂ solution (15mM) 1 μL, template 1 μL, ddH₂O 6 μL. The system was heated in 65 °C water bath for 1 h and then heated at 80 °C for 10 min. The color of positive test system was green, and the negative test was brown yellow after adding 3 μL calcein (500 μM) into the final system. (see Figure. 1)

![Figure 1](image)

**Figure 1.** Visualization of LAMP system color contrast (A) and agarose gel electrophoresis strip (B)(M: 2000 bp DNA marker; 1: Escherichia coli genome; 2: double distilled water (negative control))

3.2. Specificity detection of visual LAMP system

Using LAMP system [19-20] was used to detect the genome of Candida albicans, Pseudomonas aeruginosa, Streptococcus enteritidis, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, enterobacter, Salmonella and other strains, and the E.coli genome was used as a positive control and distilled water as a negative control Other systems are yellowish brown. The electrophoretic results were in accordance with the color contrast, which proved that the system was practical. (see Figure. 2)
Figure 2. Visual lamp system specificity test (A and B)(M: 2000bp DNA Marker; 1: *Escherichia coli* 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: *Salmonella* genome; 11: Distilled water (Negative control))

### 3.3. Detection limit determination of visual LAMP system

Using 10 times dilution method, the *E.coli* original solution was diluted in 9 gradients, and 200 μL of bacterial liquid in 6, 7 and 8 gradient was coated on the plate. Two plates were coated on each gradient. The coated plate was cultured in 37 °C constant temperature incubator for 24 h. The average value of single colony of these three gradients was calculated by colony counting. Finally, the eighth gradient was determined to be about 10 CFU/mL, through the results, the CFU values of each gradient can be calculated in turn. The detection limit of calcein LAMP system was 10^2 CFU/mL (see Figure. 3)

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

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

Clarithromycin capsules, Chaihuang granules, xiaopuling granules, Ganmao Qingre granules, Chimonanthus praecox leaves granules, Kanggan granules, Xiaoer chaigui Tuire granules, Relinqing tablets, Lianhua Qingwen capsules, Sanqi powder and other drugs were dissolved in nutrient broth at the concentration of 0.1 g/mL. The results showed that there was no trapezoidal band in the test results, which proved that there was no *Escherichia coli* in the tested drugs. (see Figure. 4)
3.5. Detection limit determination of visual LAMP system in oral drugs contaminated with Escherichia coli

100 μL Escherichia coli was added into 10 mL of Chaihuang granule broth, which was tested to be sterile, and was shaken overnight in a gas bath thermostatic oscillator. The eighth gradient was calculated to be about $1.8 \times 10^4$ CFU/mL by using the method of experiment 1.7. The CFU values of each gradient can be calculated in turn. The LAMP system was used to detect the genomics extracted from 3-8 gradient bacterial solution. The detection limit of the system can still reach $1.8 \times 10^2$ CFU/mL, which proves that oral drugs have little effect on LAMP system. (see Figure. 5)

4. Conclusions

As a common food borne pathogen in our life, E.coli is widely concerned by people. People infected by E.coli are easy to suffer from many digestive system diseases, such as acute gastroenteritis and chronic enteritis, which can lead to diarrhea in mild cases and collapse or even death in severe cases. As an oral medicine, Chinese herbal medicine granule is a unique prescription of the people's Republic of China. It can save people from fire and water in many aspects. However, due to its diverse ingredients and wide sources, it increases the risk of E.coli infection, which is extremely unfavorable for patients. The state attaches great importance to drug safety, which stipulates that all drugs and foods shall not contain Escherichia coli [1-3]. Up to now, the State Food and drug administration still uses the traditional culture method for the detection of E.coli. Although this method has a long detection cycle and complicated operation, it is regarded as the gold standard for microbial identification because of its obvious results and free from the restriction of bacterial life and death. Due to the long testing cycle and high cost of the drug administration, the pharmaceutical company
will conduct a pre inspection in the company before sending the drug to the drug administration, so as to prevent the failure of the drug test after the drug is sent to the drug administration, resulting in unnecessary losses. Nowadays, most pharmaceutical companies still use the traditional cultivation method for drug screening, which greatly reduces the company’s profit, but also hinders the circulation of drugs. In order to make high-quality drugs reach the hands of patients quickly and save manpower and material resources, pharmaceutical companies need a fast, effective, specific and highly sensitive oral drug Methods of screening Enterobacteriaceae.

As a new technology which can be used in medicine, animal medicine and food safety, the ring mediated isothermal amplification technology has attracted the attention of the field of life science because of its high sensitivity, short reaction time, no special instruments, simple operation and convenient results detection. But there are some problems in the technique, such as (1) primer design has some difficulties. Because of the variety, it is easy to produce nonspecific amplification (2) with high sensitivity and easy to be polluted. In addition, the experiment should be carried out in different regions. (3) The amplification products are of many kinds, and the target DNA fragment is difficult to separate, which is not conducive to the analysis of gene sequence. (4) The length of the target band should be controlled within 300 bp, and it is difficult to expand if it is more than 500 bp. QPCR is by PCR The technology derived from the technology can monitor the product content in real time, and is also a common detection method in various fields. It has the characteristics of fast, sensitive, high throughput, strong specificity, high automation, good repeatability and accurate quantification. It is widely used in various domains, but there are also some shortcomings such as high primer requirements, need professional instruments, and there are some shortcomings such as false positive Where [22]. Both have their own advantages in detection, and they are hard to distinguish between them. But in recent years, scientists have a deeper knowledge in the research of ring mediated isothermal amplification. The research on end product detection shows that SYBR Green I can be added to LAMP system I, hydroxynaphthol blue (HNB), calcein and other dyes can directly determine the reaction results by observing the color difference of the naked eye. This method can replace agarose gel electrophoresis, greatly saving time and cost. The HNB can be added to the system before reaction, which can eliminate the false positive result of [23] after the reaction. In terms of primer design, Nagamine et al. [24] can reduce the reaction time by about 50% by adding ring primers into LAMP reaction system.

In this experiment, Escherichia coli uidA was used A group of loop mediated isothermal amplification specific primers were designed to detect E.coli. After several experiments on the concentration and proportion of primers, it was found that calcein was not suitable for long-term heating treatment. Therefore, after comprehensive consideration, it was decided to add calcein after reaction, and the final concentration of internal primers (FIP, BIP) was 0.8 μM, the final concentration of external primers (F3, B3) was 0.2 μM, and the final concentration of Mn²⁺ was 600 μM. after the reaction, 3 μL calcein (500 μM) was added into the system. The electrophoretic bands of the final system were clear and bright, and the color contrast of the final system was the most obvious. The optimized system was used to detect the genome of Candida albicans, Pseudomonas aeruginosa, Streptococcus enteritidis, pathogenic Bacillus cereus, proteus, Staphylococcus epidermidis, Streptococcus faecalis, digestive bacteria, Salmonella and other strains. The results showed that the system had good specificity and could be used for the detection of Escherichia coli. In this experiment, the E.coli solution was diluted to 9 gradients by 10 times dilution method. The CFU value was calculated by plate coating on 6, 7 and 8 gradients, and LAMP detection was carried out on 3-8 gradient. The results showed that the detection limit of the system could reach 10² CFU/mL. In addition, LAMP was used in this experiment The system has tested clarithromycin capsules, Chaihuang granules, xiaopuling granules, Ganmaoqingre granules that have not passed the test of the drug administration, and the drugs sold in the market, such as shanhuameiye granules, Kanggan granules, Xiaoer chaigui antipyretic granules, Relinqing tablets, Lianhua Qingwen capsules, Sanqi powder and other drugs. The results showed that no E.coli was detected, but the aseptic Chai The
results showed that the visual LAMP detection technology was a good method to identify the presence or absence of *E. coli*, and it could be used for the detection of oral drug *E. coli*.

To sum up, the visual LAMP detection method established in this experiment has the advantages of strong specificity, high sensitivity, simple operation and no need of precision instrument, but it is difficult to distinguish the false positive caused by dead bacteria and live bacteria. Some scholars used propidium monoazide (PMA) pretreatment of bacteria combined with LAMP reaction can selectively amplify the DNA of living bacteria, reducing the false positive rate [25]. Since the Chinese Pharmacopoeia stipulates that oral drugs should not contain *Escherichia coli*, this method can be applied to screening and detection of *Escherichia coli* in oral drugs and food.

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