A multiplex loop-mediated isothermal amplification assay for rapid detection of *Bacillus cereus* and *Staphylococcus aureus*

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Summary

*Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) are major human foodborne pathogens that may produce a variety of toxins and cause diarrhea, food poisoning, and even death. In order to monitor and prevent the spread of these pathogens, a multiplex loop-mediated isothermal amplification (multi-LAMP) assay was developed to simultaneously and rapidly detect *B. cereus* and *S. aureus*. The sensitivity and specificity of the loop-mediated isothermal amplification (LAMP) reactions were determined via electrophoresis. The multi-LAMP showed 100% inclusivity and exclusivity, the sensitivity was 10 fg/μL and was 10 times more sensitive than that of polymerase chain reaction (PCR), the results were consistent with those of conventional PCR assay, and the entire assay should be finished within 40 min. This multi-LAMP assay was confirmed as a rapid and reliable diagnostic technique upon application for clinical samples and food samples. To our knowledge, this is the first study to report the application of multi-LAMP to detect *B. cereus* and *S. aureus*.

**Keywords:** Multi-LAMP, *Bacillus cereus*, *Staphylococcus aureus*, pathogen, rapid detection

1. Introduction

*Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) are the most common causes of foodborne pathogens in developed and developing countries. In the United States, during 2009-2015, Foodborne Disease Outbreak Surveillance System (FDOSS) received reports of 5,760 outbreaks that resulted in 100,939 illnesses, 5,699 hospitalizations, and 145 deaths (1). 1,229 foodborne outbreaks caused by *B. cereus* and *S. aureus* were reported; 39% were reported with a confirmed etiology. Vomiting was commonly reported in *B. cereus* (median, 75% of cases) and *S. aureus* outbreaks (median, 87%) (2). At the same time, China, Japan, South Korea and other countries had also reported the outbreak of foodborne epidemics (3,4). Rice or soy dishes were commonly implicated in *B. cereus* (50%) outbreaks, and meat or poultry dishes were commonly implicated in *S. aureus* (55%) outbreaks (5). These pathogens cause illness through preformed toxin production in improperly handled foods or in vivo toxin production within the gastrointestinal tract after consumption of a contaminated food (6,7). Therefore, the development of a rapid and ready-to-use method for simultaneous detection of these pathogens is of great importance to improve food safety and protect human health.

Many methods have been developed to detect *B. cereus* and *S. aureus*, including convention culture-based, immunology-based, and molecular methods (8,9). Conventional culture-based methods are safe and simple and Cost efficiency, but time-consuming. Immunology-based assay, including enzyme-linked immunosorbent and immunofluorescence, are fast but not very effective in terms of sensitivity (10). Molecular methods such as PCR and nucleic acid probe technology, Which are rapid, sensitivity and
specificity, however, the instruments used in these methods are expensive and profession (11,12).

The LAMP technique is a novel constant-temperature nucleic acid amplification technique invented by Japanese scholars in 2000 with high sensitivity, specificity, and rapidity for the low-cost detection of pathogens (13-17). Song found that a loop-mediated isothermal amplification (LAMP) method for detecting Shigella and enteroinvasive Escherichia coli, the LAMP method efficiently detected the gene within 2 h at a minimal amount of bacteria (8 CFU) per reaction (18). Wan selected the heat-labile enterotoxin gene to design LAMP primers, optimized the reaction conditions of LAMP, and examined the specificity and sensitivity of the method (19). All the related reports did not study the B. cereus and S. aureus, and the LAMP assay can only detect a gene in a single reaction. In the present study, a multi-LAMP assay was developed to simultaneously detect B. cereus and S. aureus. The aim of this study is to establish a rapid and low-cost method for detecting B. cereus and S. aureus, and getting a better understanding of the distinguishing epidemiologic and clinical characteristics of outbreaks caused by these pathogens, it will help investigators determine which one pathogen was the likely cause.

2. Materials and Methods

2.1. Bacterial strains

11 bacterial strains were used in the present study (Table 1). They were cultured for 24-48 h at 37°C in nutrient broth. B. cereus and S. aureus were used as standard strains to develop the multi-LAMP assay.

2.2. DNA extraction

DNA from 11 compared strains used in this study was extracted according to DNA purification Kit (Shanghai Weijie Biological Engineering Co., Ltd., Shanghai, China). The DNA extracted was used as template in the later assay determining the optimum reaction conditions and analyzing the specificity of multi-LAMP in detecting two goal strains.

Use the bacterial genomic DNA extraction kit, follow the instructions in the instructions: 1.5 mL of the bacterial solution, centrifuge (Tabletop refrigerated centrifuge, Thermo Fisher Scientific, Chengdu, Sichuan Province, China), add buffer GA suspension, 37°C for 30 min, add 20 μL of proteinase K and mix well into 220 μL buffer GB, mix well, Warm bath at 70°C for 30 min. 220 μg of absolute ethanol was added, mix well, transfer to the adsorption column CB3, centrifuge, add 500 μL buffer GD and centrifuged at 10,000g for 2 min. 600 μL of the rinse solution PW was added to the adsorption column, centrifuged, and finally 75 μL of the elution buffer TE was added to the collection tube to extract the genomic DNA of the bacteria. The DNA of the extracted strain was detected by electrophoresis on a 0.7% agarosegel (DYY-8B type steady current electrophoresis instrument, Liuyi Instrument Factory, Beijing, China).

2.3. LAMP primer design

The result of comparison of similar strain sequences in the Gene Bank database showed that the nhe and nuc genes are well conserved. Then the LAMP primers (Shanghai Shenggong Company, Shanghai, China) were designed on the website (http://primerexplorer.jp/e/). According to the characteristics of LAMP primer design, two groups of LAMP primers were selected, including external primers F3 and B3, internal primers FIP and BIP, as shown in Table 2.

2.4. Optimization of LAMP reaction system

The reaction system of LAMP was optimized. First determine the composition of the 25 μL reaction system: 10 × Thermopol Buffer 2.5 μL Betaine (4 mol/L) 5 μL; four primers each (F3:B3:FIP:BIP = 5:5:40:40, μmol/L) 2 μL; Bst DNA polymerase large fragment (8 U) 1 μL; dNTPs (10 mmol/L) 4 μL; MgSO4 (100 mmol/L) 1.5 μL; template 1 μL; sterile deionization 2 μL of water.

The following changes were attempted in the reaction to optimize the clearness of strips. (i) Optimization of amount of Mg2+ addition amounts were increased progressively from 1.3 μL to 1.7 μL i.e., 1.3, 1.5, 1.6, 1.7 μL; (ii) the temperatures were increased from 58°C to 64°C, i.e. 58, 60, 62, 64°C, (iii) the concentrations of primer were increased from1.8 μL to 2.4 μL, i.e., 1.8, 2.0, 2.2, 2.4 μL. According to the principle of the same amplification efficiency of the two LAMP systems, the 25 μL multiple LAMP reaction conditions were finally determined.

2.5. Specificity of multi-LAMP assay

All 9 bacterial strains in Table 1 were used as templates

### Table 1. Bacterial strains used in the present study and their sources

| No. | Bacterial species | Source |
|-----|------------------|--------|
| 1   | Listeria monocytogenes:1.4255 | CDCP   |
| 2   | Escherichia coli:1.2574 | CDCP   |
| 3   | Salmonella enteritidis:50400 | CGMCC  |
| 4   | Listeria ivanovii:1.1527 | CDCP   |
| 5   | Lactobacillus delbrueckii subsp:50400 | CDCP   |
| 6   | Saccharomyces cerevisiae:1.10599 | CDCP   |
| 7   | Enteroinvasive E.coli:ATCC44338 | CGMCC  |
| 8   | Shigella flexneri:1.1059 | CDCP   |
| 9   | Enterotoxigenic E.coli:44274 | CGMCC  |
| 10  | Bacillus cereus:NC7401 | CGMCC  |
| 11  | Staphylococcus aureus:1.6739 | CDCP   |

CDCP, Sichuan Center for Disease Control and Prevention, CGMCC, China General Microbiological Culture Collection Center.
3. Results

3.1. Optimization of LAMP reaction system

The three reaction conditions of Mg^{2+} concentration, temperature and primer concentration were optimized. The results of LAMP reaction of B. cereus were detected by 0.5% agarose gel electrophoresis (Figure 1). It can be known from the specific ladder strips in the group diagram of Figure 1 that the LAMP reaction was ideal, and the designed primer for the nhe gene can specifically and accurately identify the B. cereus. From Figure 1A, 25 μL of LAMP reaction system can be obtained, and the optimum amount of MgSO_4 is 1.5 μL. From Figure 1B, the optimum temperature was 60°C, and the optimal concentration of primers is 2.0 μL from Figure 1C. The LAMP reaction system was optimized based on the LAMP amplification assay of B. cereus, and the determination of amplification conditions laid the foundation for further identification of two pathogenic bacteria by multi-LAMP.

3.2. Specificity of multi-LAMP

The two pathogenic bacteria B. cereus and S. aureus were specifically detected by multiple LAMP, and the results are shown in Figure 2. It can be seen from Figure 2 that the pathogenic bacteria B. cereus and S. aureus bands in the LAMP amplification result was positive. The other 9 strains (1 to 9 in Figure 2) was negative. Comparing the blanks in 0, the positive results were accurate. It was indicated that the B. cereus and S. aureus, which were detected by multi-LAMP, were successful, and the reaction was rapid, simple and specific, which provided a basis for rapid detection of food pathogenic bacteria.

3.3. Sensitivity of multiple LAMP and PCR assays

The template concentration of the pathogens were 10-fold gradient dilution from 10 ng/μL DNA to 10^{-7} times (1 fg/μL), and multiplex LAMP amplification was performed to detect the reaction. The multiple LAMP experiment was repeated 2 times, LAMP products were subjected to electrophoresis on 2.0% agarose gel.

A PCR assay was performed to compare its sensitivity and the clinical detection rates with those of the LAMP assay. Each plasmid sample was amplified in 20 μL reaction mixtures containing 10 μL PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, People’s Republic of China), 400 pM primers (Table 3), and 1 μL DNA template. The cycling conditions were as follows: 3 mins at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; 5mins at 72°C. The PCR products were analyzed electro-phonetically on a 2% agarose gel (13).
Figure 1. Electrophoresis of LAMP products. M indicates DL 2000 DNA Marker, 1~4 in A indicates 1.3, 1.5, 1.6, 1.7 μL of MgSO₄, 1~4 amplification temperature in B is 58°C, 60°C, 62°C, 64°C, 1~4 primer in C Concentrations 1.8, 2.0, 2.2, 2.4 μL.

Figure 2. Detection specificity of LAMP reaction for strain. M in A indicates DL 2000 DNA Marker, 1~9 corresponds to the positive plasmid of 9 strains in sequence; 0 in B is blank control, 10 indicates B. cereus, 11 indicates S. aureus, 1~9 indicates 9 strains, M indicates DL 2000 DNA Marker.

Figure 3. The sensitivity of LAMP and PCR assays. M indicates DNA Marker, 1~8 is the DNA template mass concentration 10, 1 ng/μL; 10, 1 pg/μL; 100, 10, 1 fg/μL.
LAMP sensitivity detection is ideal, and the 10-fold gradient dilution of the two bacterial DNA templates to 10⁶ times (10 fg/μL) can still enlarge the clear ladder-like strips, while the dilution is not detected at a low concentration of 10⁻² times (1 fg/μL). Simultaneously, the bacterial samples were also subjected to traditional PCR. It was found that the sensitivity of the multiple LAMP amplification detection of pathogens in the experiment was 10 times higher than that of PCR.

4. Discussion

The multiple LAMP reaction was used to detect the two pathogenic bacteria. In the experiment, two sets of primers were able to specifically amplify, indicated that the primers were specific. The sensitivity of the multiple LAMP reaction to identify pathogenic bacteria was also high, (reaching 10 fg/μL), which is higher than the sensitivity of 0.2 ng/L in the pistachio DNA detected by Liu and Huang (20). Simultaneously, we compared the sensitivity of LAMP with that of traditional PCR analysis, reporting that the detection limit of the LAMP assay was 10-fold that of conventional PCR analysis, and the results of LAMP being consistent with those of conventional PCR analyses (21). It shows that this method can identify food samples contaminating pathogens quickly, conveniently and accurately. Compared with traditional PCR, the present method exhibited the following advantages: 1) high specificity and sensitivity and ease of operation; 2) multiplex detections of genes using the same detection system, thus reducing manual operation; 3) a total operating time of < 40 min, as opposed to 90 min to detect via conventional PCR analysis; 4) greater user-friendliness than conventional PCR analysis, with no requirement of specialized instruments and complicated operations.

This experiment only uses a simple agarose gel electrophoresis test to obtain an ideal positive test result. However, the LAMP reaction only has negative and positive results. It is prone to false positives during the experiment, and the detection sensitivity is too high and it is easy to cause pollution, which has a great impact on the results. Therefore, it is necessary to pay attention to the addition of multiple LAMP reagents of different species in different rooms and places, personnel exchange to avoid contamination and false positives, and the length of the reaction target sequence is controlled below 300 bp, once non-specific amplification occurs, not easy to identify (22). If these deficiencies of LAMP are further improved and improved, the potential of multiple LAMP in food and sanitation identification of pathogenic bacteria can be made even greater.

Acknowledgements

This work was supported by a grant (Innovation project of science and technology, Sichuan Province, China. No. 12205484) We thank Dr. Yongxin Li of The Sichuan University for her useful suggestions and ideas.

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(Received October 2, 2019; Revised November 23, 2019; Accepted December 5, 2019)