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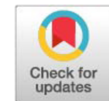
# Establishment of LAMP Assay for Detection of *Bacillus cereus*

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**Authors' Contribution**
**XY-m designed the research. SZ-y and YJ-b adjusted and revised the experimental process. BR-r collected experimental data. CY-h analysed the results. ZS-y, PJ and BH-x took respositility of reserach related to LAMP assay. RH-l donated the experimental strains and related reagents, and provided useful suggestions and ideas for the research.**

**Key words**
**Loop-mediated isothermal amplification (LAMP), *Bacillus cereus*, *entFM* gene, Hydroxynaphthol**

## ABSTRACT

*Bacillus cereus* is a common foodborne and opportunistic pathogen. It is widely distributed in soil, water and animal intestines. Its special spore structure easily causes foodborne infection to humans or animals. In order to monitor and prevent the spread of *B. cereus*, a rapid and sensitive loop-mediated isothermal amplification assay has been developed to detect it in uniformly items. According to the *entFM* gene of *B. cereus*, four primers were designed, and reaction components and conditions optimized. Sensitivity was compared between optimized LAMP assay and the conventional PCR. The optimal reaction conditions of LAMP assay (25uL each reaction) comprised 1.0 mol/L betaine, 6 m mol/L $Mg^{2+}$, 1.4 mmol/L dNTPs, 1.6 μmol/L inner primers (1:1), 0.2 μmol/L outer primers (1:1), 2.5 μL 10×thermpol reaction buffer, 1 μL Bst DNA Polymerase (8U/μL), 1 μL template DNA, at 65 ℃ incubating 60 min. Two strains of *B. cereus* out of 18 strains were positive result by LAMP assay. The detection limit of *B. cereus* genomic DNA(gDNA) by LAMP was 0.755 pg/μL which was 100 times more sensitive than conventional PCR assay. The CFU limit of LAMP assay was $14\times10^3$ CFU/mL. The artificial polluted samples (chicken meat) can be detected by LAMP, when at least 45 min must be needed to enrich bacteria. Visual dye hydroxynaphthol (HNB) successfully used in LAMP assay was established for detection of *B. cereus* in meat.

## INTRODUCTION

*Bacillus cereus* is widely distributed in nature and various foods. It was first isolated from air in a cow shed more than one hundred years ago and reported for the first time in Norway 1950. Since then, similar food poisoning has been reported in many countries (Stenfors *et al*., 2008).

Food poisoning by *B. cereus* has obvious seasonality, especially from June to October. Long storage time or incomplete food heating results in the massive reproduction of the bacteria and the production of toxins in food (Sun *et al*., 2016). Rice or soy dishes were commonly implicated in *B. cereus* outbreaks (50%). *B. cereus* which infects human or animal causes sickness through preformed toxin production in improperly handled foods or in vivo toxin production within the gastrointestinal tract after eating of a contaminated food. The symptoms that human and animal are infected of *B. cereus* are abdominal pain, vomiting, diarrhea and so on (Deng *et al*., 2020).

Therefore, the development of a ready to use and rapid method for detection of *B. cereus* is of great importance to improve food safety and protect human health. The detection assays of *B. cereus* involve routine detection method such as isolation and identification or rapid detection assay including immunology-based and molecular biology. Physiological and biochemical identification test of *B. cereus* is a common a common and accurate diagnostic assay *B. cereus*. Molecular biology for example PCR assay and immunological technology for instant ELSIA are rapid detection assay that can get the results in several hours. Li *et al*. (2021) have established a PCR assay to realize the rapid detection by amplifying the *hblA* gene of pathogenic *B. cereus*. ELISA have been invented as an assay for detection of *B. cereus* by Tallent *et al*. (2015). Isolation and culture method maybe the most accurate, but it is needed that professional researchers

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operate the experiment with high cost and long time in a potential threat environment. Rapid detection assays have advantages of high speed, high sensitivity, reliability and accuracy, but the equipment are high cost and too professional.

In 2000, a novel nucleic acid amplification assay named loop-mediated isothermal amplification (LAMP) have been invented in Japan (Notomi *et al*., 2000). Four primers are used in LAMP assay for amplifying target gene under 60-65°C constant temperature for 30 ~ 60min. Incili *et al*. (2019) have improved that specificity and sensitivity values of the LAMP assay are equal or higher and less time-consuming than ISO and VIDAS UP methods that are international standards. A LAMP assay established by Horiuchi *et al*. (2019) for detection of *Helicobacter pylori* specimens was $10^{-1}$ CFU/tube (37 min reaction time), which was 10-fold more sensitive than polymerase chain reaction. Many other studies have shown that LAMP is more sensitive and efficient than conventional PCR assay (Babu *et al*., 2020; Liu *et al*., 2019; Sheet *et al*., 2016; Mu *et al*., 2016; Xu *et al*., 2014; Porcellato *et al*., 2016). The purpose of this study is to establish a LAMP assay for detection of *B. cereus*.

## MATERIALS AND METHODS

*Main reagents*

dNTPs, betaine and Dnase type I digestive enzymes were purchased from Shanghai Sangon Biotech Co., Ltd; $Mg^{2+}$($MgSO_4$), thermal buffer and Bst DNA polymerase were gotten from New England (Beijing) Co., Ltd; High purity DNA template preparation kit was bought from Bao biology Co., Ltd; LB liquid culture medium was purchased from Haibo biological Co., Ltd; 50×TAE buffer, DL 2000 DNA maker and nucleic acid electrophoresis dye were purchased from Tiangen Biochemical Co., Ltd; hydroxynaphthol blue (HNB) indicator was purchased from Haiji biological Co., Ltd. Eighteen strains of common foodborne pathogenic microorganisms used in this study were donated by the microbiology laboratory, Institute of zoonosis, Jilin University. Single colony of Bacteria cultured on tryptone soybean agar plate was inoculated into LB liquid medium at 37°C for 12-16 h. The harvested bacteria were used for extraction of genomic DNA (gDNA) and stored at -20°C. The DNA extracted were used as templates in the later optimum reaction conditions and analyzing the sensitivity and specificity of LAMP.

*LAMP primers design*

Through pre experiment, *entFM* virulent gene of *B. cereus* was chosen to design the LAMP assay primers. Primers were designed using the special design software primer Explorer v4 software program (http://primerexplorer.jp/elamp4.0.0/index.html). The LAMP primers were synthesized by Shanghai Biotechnology Co., Ltd. The primer sequences are shown in Table I.

*Optimization of reaction conditions*

25 μL LAMP reaction system was as follow: 1 μL Bst DNA Polymerase(8U), 2.5 μL 10× thermpol reaction buffer, outer primers (B3 and F3 0.2 μmol/L each), 1 μL genome template (gDNA) kept constant volume or concentration through the whole research. Concentration of betaine, $Mg^{2+}$, dNTPs, inner primers (BIP and FIP), reaction time and temperature were optimized. The following changes were attempted to optimize the results. The condition of betaine ranged from 0 to 1.4 mol/L *i.e.* 0, 0.2, 0.4, 0.6, 0.8,1.0, 1.2, 1.4 mol/L. The condition range of $Mg^{2+}$ was 1.0 ~ 8.0 mmol/L, *i.e.* 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mmol/L. dNTPs was optimized from 1.0 to 2.4, *i.e.* 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4 mmol/L. The test range of inner primers (each) were operated from 0.2 to 1.6 μmol/L, *i.e.* 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 μmol/L. The reaction temperature ranged from 55 to 80 °C to find the optimum reaction temperature, *i.e.* 55, 60, 65, 70, 75, 80°C. The selection of reaction time ranged from 30 to 80 min, *i.e.* 30, 40, 50, 60, 70, 80 min. Finally, LAMP system was supplemented to 25 μL with sterilized $ddH_20$. The LAMP experiments were repeated 3 times at least.

**Table I. LAMP primers sequence.**

| Primer name | Primer sequence | Primer position $^α$ |
|---|---|---|
| F3 | 5'- GATACATCTTCAATCGCTGG -3' | 3530994-3531013 |
| B3 | 5'- GGAAGTATACTAAATCACCTGG -3' | 3531204-3531183 |
| FIP | 5'- TGAATCCACTGCAATCAAAACCA | 3531090-3531068 |
| | TTTT | Primer linked base |
| | TAAATGGTTCACCATACAGAACA -3' | 3531028-3531050 |
| BIP | 5'- TGGTCATAAAGGCGCTCGTC | 3531113-3531132 |
| | TTTT | Primer linked base |
| | CTAGTTTTTGTTTTAGAGCTCCAG-3' | 3531172-3531149 |

α, according to the primers position marked by *B. cereus entFM* gene (GenBank accession No. CP 015589.1).

*Specificity of LAMP assay*

Genomic DNA of eighteen strains (*Bacillus cereus* (CGMCC 1.195, CMCC(B) 63303), *Yersinia enterocolitica* (CGMCC 52225), *Vibrio prholyticus* (CGMCC 1.1615), *Candida albicans* (ATCC 1.2258), *Salmonella choleraesuls* (CGMCC 1.1859), *Corynebacterium glutamicum* (CMCC(B)46117), *Staphylococcus aureus* (CMCC 1.2328), *micrococcus lysodeikticus* (CGMCC 1.634), *Bacillus subtilis* (CGMCC1.1630), *Escherichia coli* (CMCC 1.2385), *Micrococcus luteus* (CGMCC 1.193), *Edwardsiella tarda* (ATCC 15947), *Salmonella paratyphosa* (CMCC 50093), *Salmonella typhi* (CMCC 50071), *Salmonella Schott* (CMCC 50094), *Salmonella gallinarum* (CMCC 50118), *Salmonella paratyphosa* (CMCC 50093)) were used as templates to determine the specificity of LAMP reaction. Sterilized $ddH_20$ instead of gDNA was taken into the LAMP assay as the negative control. After the reaction, the results were detected by 2% agarose gel electrophoresis to verify the accuracy and specificity of LAMP assay for detecting *B. cereus*.

*Sensitivity of LAMP assay*

*Sensitivity of LAMP assay*

Genomic DNA of *B. cereus* extracted in step 2.1 was determined the initial concentration of genome with BioTek take 3 microplate spectrophotometer under Gene 5 software. The initial gDNA was diluted with 10-fold gradient from $10^{-1}$ to $10^{-8}$ times with sterilized $ddH_20$ and then performed in LAMP assay. The LAMP experiment was repeated 3 times at least, and reaction products were subjected to electrophoresis on 2.0% agarose gel.

*Sensitivity of PCR assay*

Referring to literature (Porcellato et al., 2016), A conventional PCR assay was performed to compare its sensitivity with the above LAMP assay. The primer sequences: Upstream: 5′-gctaaaaggtacttagcttagg-3′; downstream: 5′-tatatacattatgcgtcatcac-3′. The PCR reaction system (25 μL) included 2 × PCR Master Mix 12.5 μL, 0.2 μmol/L upstream primer, 0.2 μmol/L downstream primer, 1 μL gDNA, and finally supplemented to 25 μL with sterilized $ddH_20$. The reaction condition was as follows: 94℃ for 5 min; 94℃ 30 s, 57℃ 30 s, 72℃ 30s, 35 cycles; 72℃ 7 min. At the end of the reaction, 2% gel electrophoresis was performed. The size of the amplified target fragment was 297 bp. The experiment was repeated 3 times at least.

*The CFU limit of LAMP assay*

The pure culture same as *B. cereus* initial gDNA was diluted with 10-fold gradient from $10^{-1}$ to $10^{-8}$. Through calculation, the colony detection limit of LAMP assay was obtained. The experimental procedure was repeated at least three times.

*Artificial polluted samples detection by LAMP assay*

Chilled chicken was bought in local supermarket and sterilized at 121℃ for 15min. 5 g chicken muscle were added into sterilized normal saline and homogenized, and the volume is finally kept at 100 mL. The limit of *B. cereus* CFU for LAMP assay was added into the chicken homogenate and extracted with the high purity gDNA template preparation kit. The optimized LAMP assay was used to detect the extracted gDNA. This step was repeated at least three times.

*Visual dye hydroxynaphthol blue (HNB) used in LAMP assay*

According to the color development principle of hydroxynaphthol blue (HNB) indicator, LAMP assay was established in 25uL including 2.5 μL10 × theromal buffer, 1 μL Bst DNA Polymerase(8U), 0.2 μmol/L external primers (each), 1μL gDNA, 5 μL HNB solution, and the above optimized conditions. Finally, sterilized $ddH_2O$ was supplied to 25 μL in LAMP assay. At the end of the reaction, 2% gel electrophoresis was performed. This part of the experiment was repeated at least three times.

## RESULTS

*Optimization of reaction conditions*

It can be known from the specific ladder strips that the LAMP reaction is ideal. The designed primers for the *entFM* gene can specifically and accurately identify the *B. cereus*. The optimum amount of betaine was 1.0 mol/L. $Mg^{2+}$ was optimized as 6 mmol/L. And the optimal concentration of dNTPs was 1.4 mmol/L. The concentration of inner primer was chosen as1.6 μmol/L, so the rate between external and inner primers is 1:8. The optimized temperature and time was respectively 65°C and 60 min.

*Specific results of LAMP assay*

Only 2 strains of *B. cereus* were positive results (Fig. 1), so it can be inferred that the LAMP assay established in this study for the detection of *B. cereus* has high specificity.

*Sensitivity of LAMP assay*

The initial concentration of gDNA extracted from *B. cereus* was 75.5ng/μL. The results showed that the detection limit of LAMP assay was $7.5\times10^{-4}$ ng/μL (0.755pg/mL, Fig. 2A). The detection limit of conventional PCR assay was $7.5\times10^{-2}$ ng/μL (75.5pg/ μL Fig. 2B), it can be seen that LAMP assay were $10^2$ times higher sensitivity than conventional PCR.

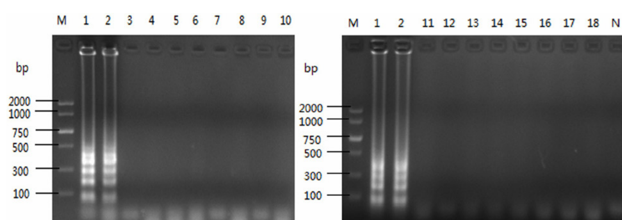


Fig. 1. Detection specificity of LAMP reaction for strains. M, DL2000 DNA marker; N, negative control. 1-2, *Bacillus cereus;* 3, *Yersinia enterocolitica;* 4, *Vibrio prholyticus*; 5, *Candida albicans*; 6, *Salmonella choleraesuls*; 7, *Corynebacterium glutamicum*; 8, *Staphylococcus aureus*; 9, *Micrococcus lysozyme*; 10, *Bacillus subtilis*; 11, *Escherichia coli*; 12, *Micrococcus luteus*; 13, *Edwardsiella tarda*; 14, *Salmonella paratyphosa*; 15, *Salmonella typhi*; 16, *Salmonella Schott*; 17, *Salmonella gallinarum*; 18, *Salmonella paratyphosa.*

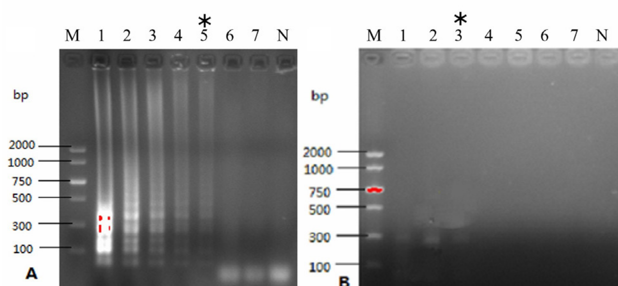


Fig. 2. The sensitivity of LAMP and PCR assays. M, DL2000 DNA marker N: negative control. 1, 7.5 ng/μL; 2, $7.5\times10^{-1}$ ng/μL; 3, $7.5\times10^{-2}$ ng/μL; 4, $7.5\times10^{-3}$ ng/μL; 5, $7.5\times10^{-4}$ ng/μL; 6, $7.5\times10^{-5}$ ng/μL; 7, $7.5\times10^{-6}$ ng/μL; *, gDNA detection limit of LAMP or PCR assays. A, The sensitivity of LAMP assay; B, The sensitivity of PCR assay.

*The CFU limit of LAMP assay*

According to the colony count of $10^{-8}$ times diluted *B. cereus* culture medium plate (Fig. 3), the CFU was 14 CFU/mL. So the limit of LAMP assay was $14\times10^{3}$ CFU/mL.

*LAMP assay detection of manually spiked contaminated samples*

When LAMP assay was operated for detection of manually spiked samples, a short time enrichment process is needed. The results are shown in Figure 4, obvious amplification bands were at 45 min and 60 min. Therefore, the enrichment time is at least 45 min, so that the LAMP assay can detect *B. cereus* in meat.

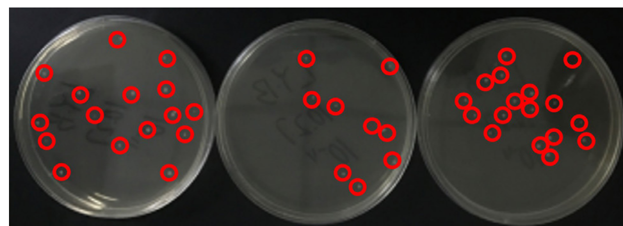

Fig. 3. the colony counts of 10-8 times diluted *B. cereus.* The average colony count of the three plates was 14 CFU/mL.

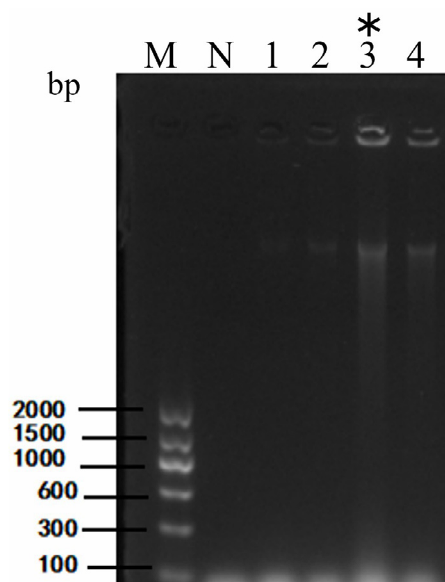


Fig. 4. results of enrichment time for detection of manually spiked contaminated samples. M, DL2000 DNA marker; N, negative control; 1, 15 min; 2, 30 min; 3, 45 min; 4, 60 min; *, indicated the shortest time for enrichment.

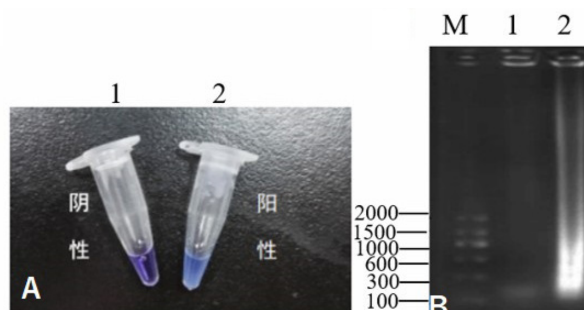


Fig. 5. The results of HNB used in LAMP assay. M, DL2000 DNA marker; 1, negative control; 2, HNB used in LAMP assay. A, indicated results of HNB in LAMP assay with naked eye; B, electrophoresis results of HNB in LAMP assay.

### *Visual dye HNB used in LAMP assay*

As it showed in Figure 5A, the positive result was observed as watched with naked eye and the negative one is navy blue. In Figure 5B, the 2% nucleic acid gel electrophoresis showed that HNB positive result had a specific band on the imager.

## DISCUSSION

The spore produced by *B. cereus* is an important factor leading to foodborne diseases. The spore has strong resistance to high temperature, drying, ultraviolet, ionizing radiation, toxic chemicals and other disinfection methods. It has been reported that spores of *B. cereus* are successfully revived from amber with a history of 25-40 million years and salt water with a history of 250 million years (Sagripanti *et al*., 2007; Henriques and Moran, 2007). The spores of some kind of *B. cereus* are more heat-resistant than those of thermophilic *Bacillus subtilis* and *Bacillus licheniformis*, so *B. cereus* can withstand more procedures in cooking food. Therefore, the characteristic of *B. cereus* that is difficult to sterilize is the main reason why it is easy to cause food pollution and disease (Dun *et al*., 2009).

As an important foodborne pathogenic microorganism, *B. cereus* mainly infects people with immune deficiency, such as the elderly, children, newborns and so on. It may cause bacteremia, endocarditis, meningitis and human eye diseases (Jessberger *et al*., 2020; Huang *et al*., 2020).

Yan *et al*. (2015) from National food safety risk assessment center in China researched that *B. cereus* was detected in 57 of 135 infant formula milk powder, and 24 strains of *B. cereus* were extracted. Among the 24 strains, the proportion of *B. cereus* with *nhe* gene was 92.98% (53/57), followed by *entFM* gene (71.93%). The proportion of the above two (*nhe* and *entFM*) virulent genes is 70.18%. According to the Blast (n) function in NCBI website, it is found that *nhe* gene has high homology with *Bacillus anthracis*, *Salmonella* and other kind of bacteria. At the same time, we found that the specificity of LAMP primers designed from *nhe* gene was poor. The *entFM* virulent gene of *B. cereus* has high specific without homology with other bacteria.

## CONCLUSIONS

A LAMP assay is established for detection of *Bacillus cereus* in manually spiked contaminated samples. The optimized LAMP assay is as follow: 1.0 mol/L betaine, 6 mmol/L $Mg^{2+}$, 1.4 mmol/L dNTPs, 1.6 μmol/L inner primers (BIP and FIP, each), 0.2 μmol/L outer primers (B3 and F3, each), 2.5 μL 10×thermpol reaction buffer, 1 μL Bst DNA Polymerase (8U/μL), 65℃ incubating 60 min, filled up with $ddH_2O$ to 25uL. 2 strains of *Bacillus cereus* in 18 strains common foodborne pathogenic were positive result by LAMP assay. The detection limit of *Bacillus cereus* gDNA was 0.755 pg/μL which was 100 times more sensitive than conventional PCR assay. The CFU limit of LAMP assay is $14\times10^3$ CFU/mL. The artificial pollution samples can be detected by LAMP with 45 min enrichment time. Hydroxynaphthol blue (HNB) is successfully applied in this experiment, and satisfactory results are obtained.

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### *Statement of conflict of interest*

The authors have declared no conflict of interest.

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