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Asymmetric polymerase chain assay combined with propidium monoazide treatment and unmodified gold nanoparticles for colorimetric detection of viable emetic *Bacillus cereus* in milk

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ABSTRACT

Bacillus cereus is a causative agent of an emetic food-borne disease. In this study, visual detection of viable emetic *B. cereus* was developed using a propidium monoazide (PMA)-asymmetric polymerase chain reaction (asPCR) and unmodified gold nanoparticles (AuNPs). To detect only the viable emetic *B. cereus*, PMA treatment was selected before DNA extraction to eliminate the false-positive results from dead bacteria. In the presence of viable target bacteria, the long genomic DNA fragments from the cereulide synthetase gene (*cesB*) were produced by asPCR, which could be effectively absorbed onto naked AuNPs via coordination between Au and the nitrogen atoms of the exposed bases. After adding NaCl solution, visual detection with the naked-eye or with UV–vis spectrophotometer was possible within a few minutes. Under the optimum conditions, the limit of detection (LOD) for viable emetic *B. cereus* reached as low as 9.2×10^1 CFU/mL in 0.01 M phosphate-buffered saline and 3.4×10^2 CFU/mL in milk, which was adequate to meet the maximum limit imposed by the Commission Regulation (EC) No 2073/2005 (500 CFU/mL). The proposed method also exhibited excellent discrimination against 10 common pathogenic bacteria in milk. The PMA-asPCR-AuNPs colorimetric assay offers a promising application in the detection of low concentrations of food-borne pathogens.

1. Introduction

Bacillus cereus is a gram-positive, rod-shaped, and spore-forming bacilli that can be found in diverse environmental conditions, including soil and food such as rice, noodles and milk [1]. It is a causative agent for two types of gastrointestinal disease, namely, emetic and diarrheal syndrome. The emesis type is caused by a heat-stable depsipeptide toxin which is called cereulide, while the diarrhea type is attributed to heat-labile enterotoxins. In reduced oxygen environment, *B. cereus* is able to survive for many years in the form of spores. The spores formed by *B. cereus* may resist pasteurization of milk and can grow if conditions are favorable, leading to food spoilage and production of toxins in food [2]. Besides implementing strict hygienic regulations, the development of accurate methods for *B. cereus* detection in food, especially warmed-up foods or milk, is critical to protect consumers and reduce potential damage.

Culture-based method for *B. cereus* detection is time-consuming (~7d) and lacks specificity [3,4]. Also, injured and non-culturable cells cannot be detected in culture media [1]. Polymerase chain reaction (PCR) assay has been considered as potential alternative approach to the conventional culture-based method for rapid screening of *B. cereus* because of its versatility, low cost, and universality. However, conventional PCR requires laborious and time-consuming gel electrophoresis-based detection and needs skilled personnel. On the other hand, qualitative PCR offers the possibility to quantify the target, but requires complicated instruments and expensive reagents [5]. Therefore, to make up for these shortcomings, it is critical to consider alternative methods to replace gel electrophoresis post-processing of standard PCR reactions that may lead to rapid, simple and sensitive detection of *B. cereus* in foods.

Gold nanoparticles (AuNPs) as biosensors for nucleic acid detection have attracted tremendous interest due to their unique optical,

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physical and chemical properties [6]. For example, AuNPs exhibit distinct colors and strong absorbance peaks in the UV–vis spectrum. This fascinating optical phenomenon of AuNPs is the result of localized surface plasmon resonance (LSPR) or the collective electrons oscillation, which give intense ruby-red color and turn to blue-purple color upon aggregation [7,8]. The dramatic change in color offers a suitable platform for colorimetric detection of DNA, metal ions and biomolecules [9,10]. There are two major types of AuNP-based colorimetric assays, namely, cross-linking assays and non-cross-linking assays [11]. Compared with the cross-linking methods which require expensive covalent modification and long preparation time, non-cross-linking methods achieve more rapid, simple and cost-effective detection [12]. This assay mainly relies on the different affinities of single- and double-stranded DNA (ssDNA and dsDNA) toward negatively charged AuNPs. More importantly, ssDNA is sufficiently flexible and uncoils its bases, which could be adsorbed onto AuNPs to stabilize them against salt-induced aggregation by enhancing the electrostatic repulsion between ssDNA-adsorbed AuNPs. In contrast, dsDNA with double-helix structure is stiffer, thereby, failing to the exposure of DNA bases. The strong electrostatic repulsion between negatively charged surfaces of AuNPs and the high negatively charged phosphate backbones of dsDNA prevents the interaction. Recently, many studies about unmodified AuNP-based colorimetric assays were reported [13–15]. However, most of them used short fragments of ssDNA (5–50 nt) causing the limited sensitivity for nucleic acid detection. To meet these challenges, some efforts have turned to asymmetric PCR (asPCR) amplification, which is performed using a set of specific primers in unequal ratios to produce a large amount of ssDNA amplicons. To guarantee specificity of asPCR, longer genomic DNA amplicons (typically >40 nt) were used [5].

In this work, unmodified AuNP-based colorimetric assay combined with asPCR amplification and propidium monoazide (PMA) treatment for the detection of viable emetic *B.cereus* in milk is reported. The emetic *B.cereus* was first subjected to PMA treatment to eliminate DNA amplification from dead bacteria during asPCR. After that, longer ssDNA amplicons were detected using unmodified AuNP-based colorimetric assay. When the emetic *B.cereus* was absent in the reaction mixture, a distinct ruby-red to blue-purple color change occurred after a few minutes, with no need for any instrumentation.

2. Experimental section

2.1. Reagents and materials

Hydrogen tetrachloroaurate trihydrate ($HAuCl_4{\cdot}3H_2O$), trisodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were obtained from Sigma (St. Louis, MO, USA). PMA was obtained from US Everbright Inc. (Suzhou, China) and dissolved in 20% dimethyl sulfoxide to obtain a stock solution at 1 mg/mL and stored at −20 °C in the dark. TaKaRa MiniBest DNA Fragment Purification Kit were obtained from TaKaRa Biotech Co., Ltd. (Dalian, China). DNeasy Blood and Tissue Kit was obtained from Qiagen Co., Ltd. (Shanghai, China), Lysozyme was purchased from ComWin Biotech Co. Ltd. (Beijing, China).

PCR primers were obtained from Genscript (Nanjing, Jiangsu, China) and the primer set designed for the cereulide synthetase gene (*cesB*) of emetic *B.cereus* are listed in Table 1. The ssDNA (88 nt) was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences (5′ → 3′) was as follows: TAACGGACCATTTGCGAGATTGTATACTCTTTTAGACTCTTTTAACATAAGTTCATCTACTTCTTCTATCCGTAATCCTTCTGGCATC.

2.2. Bacterial culture preparation

Emetic *B. cereus* (JX-CDC PZ0063L), emetic *B. cereus* (JX-CDC JDZ0102Y), and 8 non-target strains, namely, non-emetic *B. cereus* (JX-CDC DA0050L), non-emetic *B. cereus* (JX-CDC PX0093LY), *B. subtilis* (CMCC 65301), *Staphyloccocus aureus* (CMCC 26001), *Listeria monocytogenes* (ATCC 13932), *Salmonella* Enteritidis (ATCC 13076), *Cronobacter sakazakii* (ATCC 45401), and *E. coli* O157:H7 (CMCC 44828) were used in this study. All bacterial strains were grown in Luria Bertani (LB) medium overnight at 37 °C in a rotary shaker at 180 rpm. The viable cell count for each bacterium was determined by surface plating 0.1 mL of the appropriate dilutions onto LB agar at 37 °C for 12–24 h. To obtain dead emetic *B. cereus* cells, the cell suspensions were heated at 100 °C for 20 min. Cell viability was verified by plating onto LB agar for 48 h.

2.3. PMA treatment

Samples were treated with PMA as described previously with slight modifications [16]. Briefly, a 10 μL stock solution of PMA was added to 1 mL of sample solution to create a final concentration of 20 μM. Following 5 min incubation in the dark, samples placed on ice were light exposed for 5 min using a 500-W halogen lamp with occasional shaking. After light-mediated PMA cross-linking, the pellets were collected by centrifugation at 12,000 × *g* for 3 min and washed three times with equal volumes of PBS to remove the free PMA.

2.4. DNA extraction and asymmetric PCR

Genomic DNA from bacterial cultures was harvested as follows: the bacterial pellets were resuspended in 180 μL of enzymatic lysis buffer containing Tris (20 mM), Na_2-EDTA (2 mM), TritonX-100 (1.2%), and lysozyme (20 mg/mL) and incubated at 37 °C for 30 min. Subsequently, bacterial DNA was extracted using DNeasy Blood and Tissue Kit according to the manufacturer's protocol. The DNA was dissolved in 50 μL of sterile water and stored at −20 °C until use.

Asymmetric PCR was performed in a total reaction volume of 50 μL containing 5 μL of target DNA, 3 μL of 10 μM reverse primer, 1 μL of 1 μM forward primer, 25 μL of 2 × Taq mix and 16 μL of sterilized water. The optimized asymmetric PCR conditions were 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 60.6 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min.

2.5. Preparation of AuNPs

The 13 nm AuNPs were synthesized using citrate reduction method with slight modifications as previously described [17,18]. Briefly, 250 mL of 1 mM $HAuCl_4$ solution was heated to boiling followed by the rapid addition of 25 mL of 38.8 mM trisodium citrate solution. The color change in solution from pale yellow to red indicated the formation of AuNPs. After the color change, the solution was further boiled under reflux for 15 min to ensure complete reduction. The solution was cooled to room temperature and stored at 4 °C for further use.

2.6. Colorimetric detection

After brief purification using TaKaRa MiniBest DNA Fragment Purification Kit, the amplified product was mixed with AuNPs to reach a final concentration of 10 nM AuNPs. Then, the mixture was stored at room temperature for 10 min followed by addition of NaCl. The mixture color change was recorded by cameras and an Amersham Pharmacia Ultrospec 4300 pro UV/visible spectrophotometer (England, UK) within 5 min. The detection limit of this colorimetric

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