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Establishment and Application of Rapid Quantitative Detection Technology for *Bacillus cereus*

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Abstract: Infections caused by *Bacillus cereus* often occur, causing a certain degree of health hazards and economic losses to people around the world. Rapid quantitative detection is of great significance for prevention. This study aims to develop a simple and rapid quantitative detection method for *B. cereus*, which can be used to monitor the contamination of *B. cereus* in food samples. A fluorescence real-time quantitative PCR assay was employed to solve this problem. According to the conservative sequence of the gyrB gene in *B. cereus*, primers were designed online using primer-blast of the NCBI website. The recombinant plasmid standard was constructed by genetic engineering technology. Reaction parameters of real-time PCR were also optimized in this study. After the establishment of this method, 16 rice samples collected from a canteen were tested, one of which was positive and the content of *B. cereus* was 7.13×10^2 copies/g. The analysis of the melting curve indicated that the system was specific and sensitive, therefore which made it an effective approach for the rapid quantitative detection of *B. cereus* in food samples. This result is of particular significance for application of predicting the levels of *B. cereus* in food and other samples.

Keywords: *Bacillus cereus*; Detection and Quantification, Real-Time PCR; Melting Curve Analysis

Introduction

Bacillus cereus is a common foodborne pathogenic bacterium, which is widely distributed in nature. It usually exists in soil, water, air, animal intestines, and many raw and processed foods (Ehling-Schulz *et al*., 2019; Sarrıas *et al*., 2002). It is a gram-positive bacterium. The suitable growth temperature range is 20~45°C and it grows slowly or does not grow below 10°C (Toril and Granum, 2013). Foodborne diseases caused by *B. cereus* mostly occur in warm seasons and often contaminate foods containing more carbohydrates, such as rice (Albaridi, 2022; Yu *et al*., 2020; Sidiqui *et al*., 2019; Forero *et al*., 2018; Glasset *et al*., 2016; Bilung *et al*., 2016; Jiang, 2013). Rice and other foods are easy to be polluted by *B. cereus* and multiply in large quantities after being placed under high temperatures for a long time. Most of the contaminated foods are completely normal in appearance and properties, without obvious deterioration. Collective poisoning caused by *B. cereus* contamination of rice often occurs (Huang *et al*., 2019; Zhang *et al*., 2017; Dong *et al*., 2014; Wang and Wei, 2012; Fei, 2012). Infection with *B. cereus* mainly produces food poisoning symptoms such as diarrhea and vomiting. In addition, it can also cause other non-gastrointestinal infections (Jiang *et al*., 2018). Different microbial pathogens have different infectious doses (i.e., the number of infectious units required to cause an infection) (Ishida *et al*., 2018). Studies have shown that the number of *B. cereus* causing diarrhea and other foodborne diseases in food generally ranges from 10^5 to 10^8 cfu/g (Lin and Xu, 2018). Therefore, the establishment of a rapid quantitative detection technology for *B. cereus* is conducive to monitoring the contamination of this bacterium in food (Zhang *et al*., 2019).

At present, the detection and identification of *B. cereus* are still mainly at the level of isolation, culture, staining observation, and biochemical identification. These traditional methods have a long detection cycle, cumbersome operation, and many experiments involved, requiring a large number of personnel to participate (Yang *et al*., 2019). However, some modern methods, such as molecular biology and immunology methods, either need to isolate pathogens in advance, lack sufficient sensitivity or are difficult to quantify, or the subsequent steps are cumbersome and prone to pollution, resulting in false positive results, etc., (Yang *et al*., 2019; Mandal *et al*., 2011). To avoid the occurrence of the above, the fluorescent quantitative PCR technology came into being. In this study, primers were designed according to the commonly used target molecule gyrB gene in bacterial classification and identification based on nucleotide sequence and the fluorescent real-time quantitative PCR detection technology based on SYBR Green was employed to establish a fast and

sensitive quantitative detection technology for *B. cereus* in food, especially in rice samples. This study will provide a reference for rapid quantitative detection of pathogenic bacteria in food samples.

Materials and Methods

Bacterial Strains, Samples, and DNA Extraction

The standard bacterial strains used in this study were obtained from the Centers for Medical Culture Collection (CMCC), Beijing, People's Republic of China. The target bacterial cultures were serially diluted to the appropriate inoculation level and confirmed by plate counting in triplicate. Rice samples were taken from a college canteen, 4 samples were taken every day and a total of 16 samples were collected for four consecutive days. The samples were placed in a sterilized jar by aseptic operation, weighed, soaked in sterile physiological salt water, fully shaken and mixed, and stored at 4°C for subsequent use as food sample materials.

To extract bacterial DNA, the above 50 mL sample solution was taken, the food residues were removed by low-speed centrifugation and the bacterial cells were collected by high-speed centrifugation. The bacterial pellets were subjected to DNA extraction using lysis by boiling (Li *et al*., 2013; Li and Mustapha, 2002). The specific operation steps were as follows: High-speed centrifugation, cell collection, cell suspension in pure water, boiling in water, low-speed centrifugation to remove impurities and the supernatant directly as a template (Barbosa *et al*., 2016; Zhang *et al*., 2013). The bacterial DNA samples (in sterile redistilled water) were stored at 4°C for use and 1 μL of supernatant was used in the PCR manipulation described below.

Design and Specificity of Primer

According to the commonly used target molecule gyrB gene in the research of bacterial classification and identification based on the nucleotide sequence, primers were designed online by using Primer Blast of NCBI website (https://www.ncbi.nlm.nih.gov/tools/ primer-blast/).

The nucleotide sequences finally determined by multiple sequence comparisons were pasted into the Primer Blast interface. After setting various parameters reasonably, several pairs of primers were automatically generated by the system. The BLAST analysis (https://www.ncbi.nlm.nih.gov/blast/) was conducted to check whether there were complementary pairings within the primer and between multiple primers. Then the homology of DNA outside the target sequence on the primer and template was compared. Finally, the primer sequences selected for subsequent detection were determined. The primer sequences of *B. cereus* were shown in Table 1 and the amplified fragment length of the primer was 123 bp. The primer sequences were synthesized by Kinst Technology Co., Ltd. (Nanjing). The specificity of primers was also evaluated by cross-reaction and melting curve analysis of fluorescence quantitative PCR.

Construction of Standards and Standard Curves

B. cereus was cultured, the colonies were collected, the cells were lysed by boiling and the template was prepared. Using the specific primers, ordinary PCR was performed, electrophoresis separation was adopted and the DNA in the gel was recovered. Then, it was connected with pMD 19-T Vector and transformed into the competent *Escherichia coli*. The white colonies were screened with ampicillin and their specificity was identified by colony PCR and sequencing. The plasmid was extracted with MiniBEST Plasmid Purification Kit. The plasmid was quantitated by the nucleic acid quantitative analyzer and diluted in multiple proportions to prepare gradient concentration reference standards, which were amplified by ABI PRISM 7500 fluorescent PCR and used to construct standard curves. A recombinant plasmid reference standard for real-time quantitative PCR detection of *B. cereus* was constructed and its practicality was also evaluated through experiments.

Real-Time PCR Amplification and Melting Curve Analysis

The template DNA of 16 rice samples and the reference standards were simultaneously detected by fluorescent quantitative PCR. The reaction system was SYBR Green fluorescent real-time quantitative PCR system, which was operated according to SYBR Premix Ex Taq™ (TaKaRa) instructions. The reaction system was 12.5 μL SYBR® Premix Ex Taq™ (TaKaRa) (2 ×), 1.0 μL primer (5 μm), 0.5 μL ROX Reference Dye II (50 ×), 1.0 μL DNA template, 9.0 μL ddH_2O, total volume 25 μL. The cycle parameters were set as follows: Predenaturation at 95°C for 10 sec; denatured at 95°C for 5 sec, annealed at 55°C for 34 sec, and cycled for 45 times after optimization. Fluorescence values were collected after annealing at 55°C and the 60~95°C melting curve analysis step was added after the above amplification. At the same time, the national standard method of food safety, food microbiological examination (GB 4789.14, 2014) (National Food Safety Standards, 2014), was also used to detect *B. cereus* for comparison and validation.

Table 1: Specific primers used for the detection of *B. cereus* in this study

Primers	Sequence 5'-3'	Target gene	Locus	Product size (bp)
Bc-gyrB-1	GCTTCACCATCTTGTTTGG	gyrB	NC_004722	123
Bc-gyrB-2	CGCCCATTATCCGTTACA			

Results

Specificity of Primers

The primer sequence was compared by BLAST and no nontarget bacteria were found to match it completely. The mixed bacteria of common intestinal pathogens were used to make templates for PCR amplification and no non-specific bands were found in the agarose gel electrophoresis profile in Fig. 1. According to the analysis of the melting curve, only a single melting peak appeared and no other non-specific peak was present in Fig. 3, which indicated that the primers designed and used in this study were specific to *B. cereus*.

Results of Standards and Standard Curves

Ten times gradient serial diluted plasmid standard of *B. cereus* (whose concentration was 1×10^4 to 1×10^9 copies/mL) was used as a template for fluorescence quantitative PCR amplification. The results were shown in Fig. 4. It could be seen that the Threshold Cycle (CT) values of the intervals between the gradients were equal. The logarithm of the diluted copy number of the standard was taken as the abscissa and the Ct values were taken as the ordinate to establish the standard curve of fluorescence real-time quantitative PCR. The results were shown in Fig. 2. The linear regression equation was $y = -1.94368x+32.29984$, $R^2 = 0.99564$. There was a good linear relationship within the concentration range of 1×10^4 to 1×10^9 copies/mL, which indicated that it can be used as a standard curve to detect the contamination amount of *B. cereus* in the food samples.

Detection of the Food Samples

The sterile physiological salt water immersion solution of 16 rice samples, after discarding the rice grain precipitation, was used to prepare template DNA by boiling method, and fluorescent quantitative PCR amplification was carried out together with the plasmid standard with 10 times gradient serial dilution. Each sample was repeated three times. The test results showed that one rice sample was positive (Fig. 5) and the content of *B. cereus* was 1.91×10^2 copies per gram of rice. This was very similar to the result of the national standard method 1.88×10^2 cfu/g, but the national standard method took 3 days, while the method established in this study only took less than 5 h.

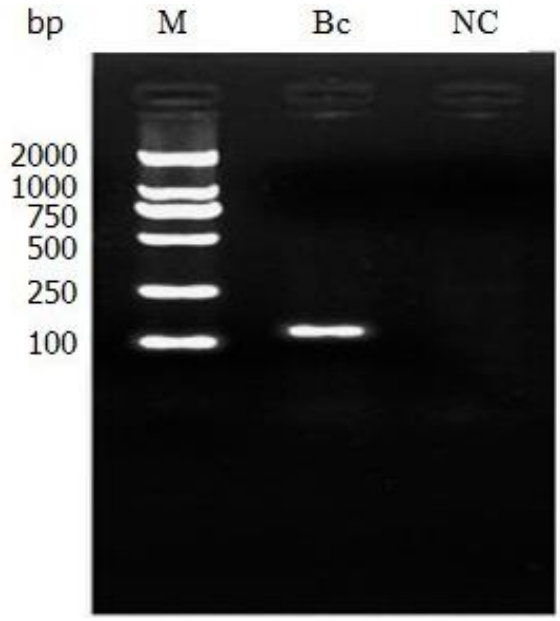

Fig. 1: Agarose gel electrophoresis profile of PCR product. Lane M: DL2000 DNA marker, Lane Bc: PCR product of gyrB gene, Lane NC: Negative control

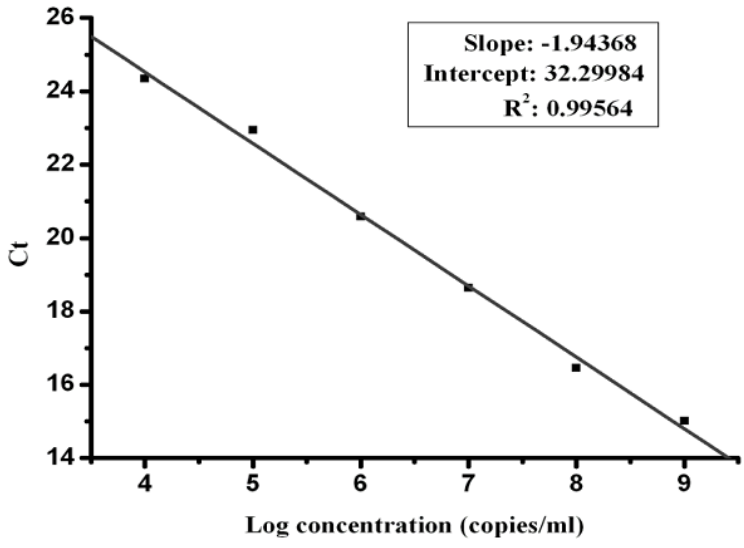

Fig. 2: Standard curves

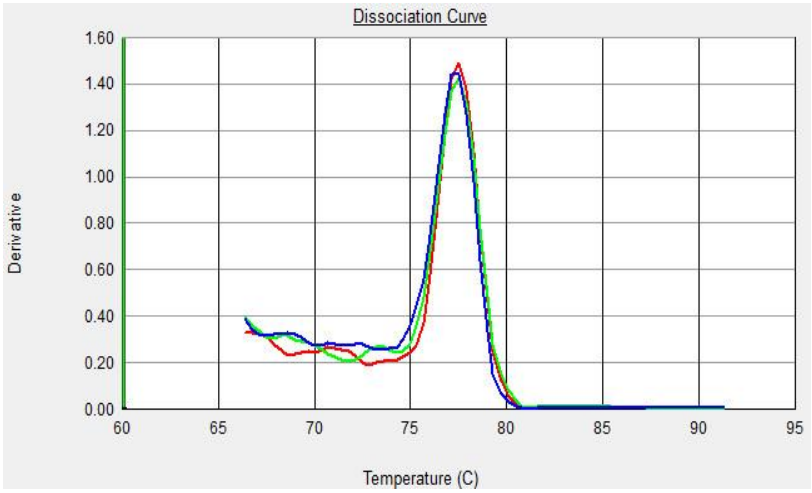

Fig. 3: The melting curve of amplification products of *B. cereus*. The melting temperatures occur at 77.1°C. No nonspecific peaks are present

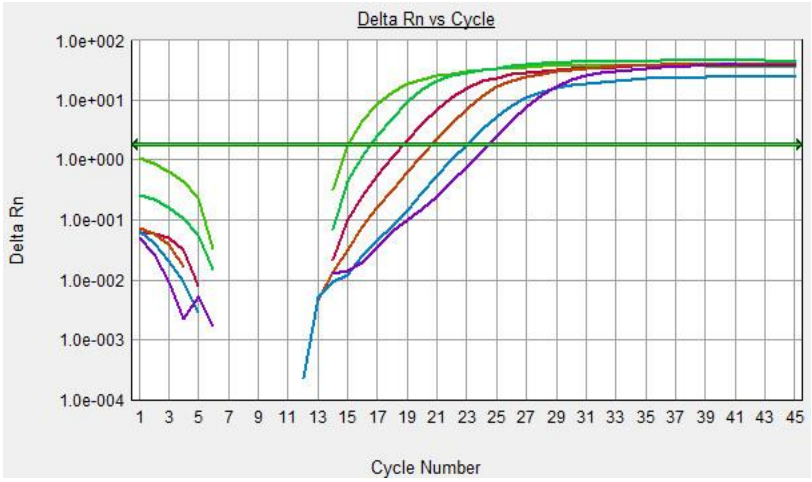

Fig. 4: The amplification profile of 10-fold serial dilution of *B. cereus*. The concentrations of bacteria range from 1×10^4 - 1×10^9 copies per milliliter

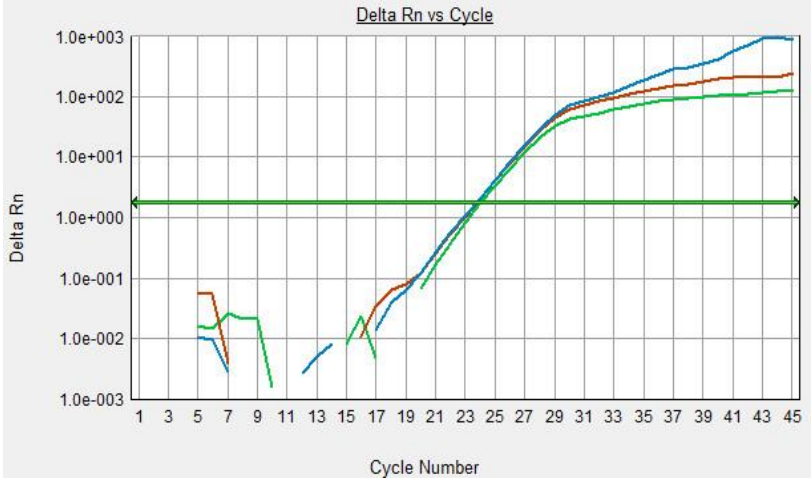

Fig. 5: Test results of food samples

The gene encoding the B subunit protein of DNA gyrase (DNA gyrase subunit B, gyrB) is ubiquitous in various bacteria, which is conservative and variable. It is often selected as a target molecule in the research of bacterial classification and identification based on nucleotide sequence (An *et al*., 2010; Li *et al*., 2008; Hao and Han, 2008). In this study, the gyrB gene of *B. cereus* was selected as the target molecule to design primers. After BLAST comparison, cross reaction, and fusion curve analysis and evaluation of fluorescence quantitative PCR, the primers showed good specificity, which can be used for the specific detection of *B. cereus* in fluorescence real-time quantitative PCR.

Discussion

Fluorescent quantitative PCR detection technology not only retains the specificity and sensitivity of conventional PCR detection but also omits the electrophoresis steps of conventional PCR. The whole process is controlled by a computer. It can simultaneously complete the amplification and quantification of up to 96 samples, which is suitable for the detection of a large number of samples and greatly improves the detection time limit. The method established in this study has a small workload and does not require early enrichment culture. It takes less time than traditional detection methods and the detection results can be obtained in 4-5 h, which provides a technical means for the supervision of *B. cereus* contamination in related foods. The only drawback of this method is that the detection requires expensive fluorescent quantitative PCR instruments and experienced operators.

B. cereus often has potential opportunities to contaminate food during the processing and storage before the food supply (Cattani *et al*., 2016). This bacterium is very easy to reproduce in rice and other foods. Especially in summer, if rice and other foods are not eaten immediately after processing and stored in the refrigerator, they are easily contaminated by *B. cereus* and multiply in large quantities (Sidiqui *et al*., 2019; Wang *et al*., 2016). Food poisoning is likely to occur when the number of *B. cereus* entering the digestive tract exceeds 10^6 through contaminated food, which should be highly paid attention to by catering staff and families.

Conclusion

Using SYBR Green-based fluorescent real-time quantitative PCR, we have developed a rapid quantitative detection technology for *B. cereus* from food samples, which has the advantages of strong specificity, high sensitivity, good accuracy, and short time consumption. This method was employed to detect 16 rice samples collected from a school canteen, among which one sample was found to be contaminated with *B. cereus*. The method established in this study will provide technical support for food safety monitoring of *B. cereus* contamination and rapid response to food poisoning events. Because they can quantify the level of bacterial contamination in samples and are easy to automate, the technology will inevitably become a routine means of bacterial detection.

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Author's Contributions

Feilong Sun: Developed the idea, performed the experiments and prepared the manuscript.

Hanyu Li: Participated in partial experiments, acquired and interpreted the results.

Jingxue Qian: Analyzed the data and participated in manuscript preparation.

Rumeng Luo and Mingyue Zhang: Participated in partial experiments.

Ethics

The authors declare that they have no conflict of interest. All authors have read and approved the manuscript and no ethical issues involved.

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