Multiplex PCR assay for the detection of enterotoxigenic *Bacillus cereus* group strains and its application in food matrices

T. D. Kalyan Kumar · H. S. Murali · H. V. Batra

Received: 5 June 2008 / Accepted: 4 March 2009
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Abstract *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* are the major concerns for the food safety in terms of frequency and/or seriousness of the disease. Being members of the same group and sharing DNA homology to a larger extent, they do create problems when their specific detection/identification is attempted from different food and environmental sources. Numerous individual polymerase chain reaction (PCR) and few multiplex PCR (mPCR) methods have been employed to detect these organisms by targeting toxin genes but with lack of internal amplification control (IAC). Therefore, we attempted a mPCR with IAC for the detection of enterotoxigenic *B. cereus* group strains by selecting *hbl A*, *nhe A* and *cyt K* genes from *B. cereus*, indicative of the diarrheal potential and *cry I A* and *pag* genes, the plasmid borne phenotypic markers specific to *B. thuringiensis* and *B. anthracis* strains, respectively. Multiplex PCR assay validation was performed by simultaneous comparison with the results of single-target PCR assays and correlated to the classical conventional and biochemical identification of the organisms. The mPCR was able to detect as low as 10^1 – 10^2 organisms per ml following overnight enrichment of spiked food samples (vegetable biriyani and milk) in buffered peptone water (BPW). The presence of these organisms could also be detected by mPCR in naturally contaminated samples of rice based dishes and milk. The high throughput and cost-effective mPCR method described could provide a powerful tool for simultaneous, rapid and reliable detection of enterotoxigenic *B. cereus* group organisms.

Keywords *Bacillus cereus* · *Bacillus thuringiensis* · *Bacillus anthracis* · Toxins · mPCR

Introduction

Bacillus cereus, *Bacillus thuringiensis* and *Bacillus anthracis*, which form a part of *B. cereus* group are ubiquitous in their distribution and have a marked role in human activity [1]. *B. cereus* associated food poisoning occurs round the year without any specific geographic distribution. Some strains of the *B. cereus* are able to grow even at refrigeration temperatures there by posing a serious concern for food safety [2, 3]. *B. cereus* causes diarrheal and emetic syndromes by eliciting a variety of extracellular toxins [4–6] including three main types of enterotoxins namely hemolysin BL (HBL), non-hemolytic enterotoxin (NHE) and cytotoxin K (Cyt K) [7–10]. HBL and NHE are three component (tripartite toxins) toxic proteins. While cytotoxin K is a pore forming toxin causes necrotic enteritis [11].

Bacillus thuringiensis is an insect pathogen that presents many phenotype characteristics that are common to *B. cereus* strains [12]. *B. thuringiensis* produces a parasporal crystal during the sporulation of its growth cycle, which is the only established characteristic that distinguishes *B. thuringiensis* from *B. cereus*. These crystal proteins show entomopathogenic properties to insects [13]. Even though *B. thuringiensis* is considered as safe to humans, occasionally these strains are responsible for human infections similar to those caused by strains of *B. cereus* [12, 14–17].

Bacillus anthracis is a human/animal pathogen causing acute fatal disease anthrax among mammals. It differs from *B. cereus*, by the presence of plasmids pXO1 and pXO2, which encode the lethal toxin complex and the poly- γ -D-

T. D. K. Kumar (✉) · H. S. Murali · H. V. Batra
Division of Microbiology,
Defence Food Research Laboratory, Sidhartha Nagar,
Mysore – 570011, Karnataka, India

E-mail: devikalyan@gmail.com

glutamic acid capsule, respectively [18]. According to recent reports, a non-*B. anthracis* strain containing anthrax toxin genes, with a potential to cause severe inhalation anthrax-like illness has also been isolated. Here in the presence of a circular plasmid, named pBCXO1, with 99.6% similarity with the *B. anthracis* toxin-encoding plasmid, pXO1 was identified [19]. Initially this isolate was identified as *B. cereus* by phenotypic and 16S rRNA analysis. Likewise some plasmids with high similarity to the pXO1 plasmid of *B. anthracis* have been found in strains of *Bacillus* species [20].

In order to address above mentioned points, there is a need to develop a system which can detect enterotoxigenic *B. cereus* and *B. thuringiensis* with discriminative potential for *B. anthracis*. However, the laboratory procedures for detection and identification of enterotoxigenic *B. cereus*, *B. thuringiensis* and *B. anthracis* by conventional methods are time consuming. Assays have been developed to detect enterotoxigenic *B. cereus*, *B. thuringiensis* and *B. anthracis* separately by individual gene polymerase chain reactions (PCRs) by targeting specific genes of the respective organisms. In this context, identification of enterotoxigenic *B. cereus* group organisms from environmental matrices, e.g. water/food, by multiplex PCR (mPCR) by targeting major enterotoxin toxin genes along with phenotype

specific genes can obviously be more reliable and cost-effective. To address above mentioned areas, we have described a robust and specific mPCR assay (mPCR) with internal amplification control (IAC) for the detection of enterotoxigenic *B. cereus* group organisms with discriminative potential for *B. anthracis* from water and food matrices.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Reference strains obtained from American Type Culture Collection (ATCC), National Center for Industrial Microorganisms (NCIM, India), Defense Research and Development Establishment (DRDE, Gwalior) and Microbial Type Culture Collection (MTCC, India) were used for evaluation of the mPCR method. Nineteen isolates of *B. cereus* group strains were collected from different soil samples of Karnataka region as well as spoiled food samples were also examined by the mPCR standardized. All the isolates and standards were identified till *B. cereus* group level by 16S rRNA sequencing by using reported primers.

Table 1 Toxin profile of *B. cereus* and non-*B. cereus* group organisms during mPCR

S. No.	mPCR pattern (<i>hbl A/nhe A/cyt K/cry I A/pag</i> /IAC)	Organism
1	+, +, +, -, +	<i>B. cereus</i> ATCC 14579, <i>B. cereus</i> ATCC 10876, <i>B. cereus</i> NCIM 2155, <i>B. cereus</i> NCIM 2156, isolate 2, isolate 8, isolate 14 and isolate 18
2	-, +, -, -, +	<i>B. cereus</i> ATCC 11778, isolate 3, isolate 4, isolate 10 and isolate 11
3	-, +, +, -, +	<i>B. cereus</i> NCIM 2185, isolate 5, isolate 7 and isolate 15
4	+, +, -, -, +	<i>B. cereus</i> NCIM 2703, NCIM 2106, isolate 6, isolate 9, isolate 12, isolate 16, isolate 17 and isolate 20
5	+, +, +, +, -, +	<i>B. thuringiensis</i> ATCC 10872, <i>B. thuringiensis</i> NCIM 5109 and <i>B. thuringiensis</i> NCIM 5108
6	+, +, -, +, -, +	<i>B. thuringiensis</i> NCIM 5110
7	+, -, -, -, +	<i>B. mycoides</i> ATCC 11778, <i>B. mycoides</i> NCIM 2106 and isolate 19
8	-, +, -, -, +	<i>B. anthracis</i> sterni DRDE
9	-, -, -, -, +	<i>B. mycoides</i> MTCC 645, <i>A. hydrophila</i> ATCC 49140, <i>Salmonella typhimurium</i> ATCC 13331, <i>Escherichia coli</i> MTCC 730 and <i>Yersinia enterocolitica</i> ATCC23715

Primers and IAC

Five pairs of primers were designed to detect genes *hbl A*, *nhe A*, *cyt K*, *cry I A* and *pag* using the Gene Bank database sequences (Table 2). Conserved regions were selected and primers were designed with Gene runner software. All primers used in the study were synthesized by MWG

Biotech Pvt. Ltd., Bangalore. To check the presence of inhibitors within PCR mixture, IAC was constructed based on pUC 18 DNA. The IAC primers used in this reaction had 5' overhanging ends, which were identical to the primers used in mPCR specific for *nhe A* (*nhe A F* and *nhe A R*), whereas 3' ends were complementary to a DNA sequence of Plasmid University of California (pUC) 18 (Table 2).

Table 2 Primer sequences, Accession No.'s, and anticipated sizes of PCR products for the *B. cereus* group specific primers used in this study

Primer	Sequence (5' - 3')	Product size	Target gene	Accession No.
<i>cyt K F</i>	GGCGCTAGTGCAACATTACG	482	<i>cyt K</i>	AE016877
<i>cyt K R</i>	TCATACCAGGAGAGAAACCGC			
<i>nhe A F</i>	AAGGCGAATGTACGAGAGTGG	553	<i>nhe A</i>	NC_004722
<i>nhe A R</i>	CTTCTCTCGTTTGAATCTATCTGCAG			
<i>hbl F</i>	TTACCTGGTAGAATCGTACAAGATC	164	<i>hbl A</i>	AJ237785
<i>hbl R</i>	CCTGTATTAATCGCTTCTACCATTG			
<i>pag F</i>	CAGGAGAACCGGTTATTAAATG	1419	<i>pag</i>	AY700758
<i>pag R</i>	GTTCGAGCCTGTATCCAC			
<i>cry F</i>	AAGATGGGCACGCAAGACTAG	413	<i>cry I A</i>	DQ285666
<i>cry R</i>	GTTCCAGCAGGATAAGCCATT			
IAC F	AAGGCGAATGTACGAGAGTGGTCCTGCAACTTTATCC	829		
IAC R	CTTCTCTCGTTTGAATCTATCTGCAGTGGTTTCTTAGACGTCAGGTG			

IAC primers are flanked by *nhe A* primers on 5' end

The PCR reaction mixture for generation of IAC DNA contained 300 nM of each primer, 200 μ M each dNTP, 0.5 units of *Taq* polymerase, 2.0 mM $MgCl_2$ in 1 $\times$ PCR buffer with 375 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min in Eppendorf master cycler gradient thermal cycler. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72°C. PCR product was purified using commercially available kit (Qiagen). The concentration of IAC DNA was determined spectrophotometrically at 260 nM and was stored in de distilled water (DDW) at –20°C. The following equation was used to calculate the copy number of the PCR product concentration: weight of PCR fragment (in g/ μ l) $\times$ $(6.023 \times 10^{23}) / (660 \text{ g mol/l} \times \text{number of base pairs of PCR fragment}) = \text{the number of genomic copy per } \mu\text{l}$ [21].

DNA extraction and PCR

For DNA extraction, bacteria were placed in Luria Bertani broth and incubated at 37°C. Template DNA from each bacterial strain was extracted by boiling method [22]. One milliliter of cells was removed and centrifuged at 10,000 rpm for 3 min. The pellet was resuspended in 50 μ l of distilled water, boiled for 12 min, and centrifuged again for 3 min at 12,000 rpm. The supernatant was transferred to a new microfuge tube and stored at –20°C for use.

Multiplex PCR was carried out in 50 μ l reaction containing 400 nM of forward and reverse primers of *nhe A*, 200 nM of forward and reverse primers of *cyt K*

and *pag*, 300 nM of *hbl A* and *cry I A* primers, 200 μ M concentrations of each dNTP (MBI Fermentas), 10^5 copies of IAC DNA, 1.2 unit of *Taq* polymerase (MBI Fermentas), 2.0 mM $MgCl_2$ in 1 $\times$ PCR buffer (MBI Fermentas) with 1.5 μ l (~300 pg of DNA) of template DNA. Various concentrations of IAC DNA were tried before choosing 10^5 copies per reaction. Amplification consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 9°C for 1 min, primer annealing at 57°C for 1 min and extension at 72°C for 1.30 sec followed by a final 6 min extension at 72°C in Eppendorf master cycler gradient thermal cycler. The PCR products were analyzed on a 2% (wt/vol) agarose gel.

Determination of specificity mPCR

To further test the specificity of assay, DNA templates from *Aeromonas hydrophila*, *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Escherichia coli* strains were used in this assay.

Determination of sensitivity of mPCR

To assess the minimum amount of *B. cereus*, *B. thuringiensis* and *B. anthracis* Sterne DNA detectable by mPCR, 10-fold serial dilutions of overnight growth of *B. cereus* ATCC 14579, *B. thuringiensis* ATCC 10872 and *B. anthracis* Sterne were serially diluted at a concentration of 10^6 – 10^0 CFU/ml. DNA was prepared by boiling method as described earlier. A 1.5 μ l aliquot of each dilution was added to five separate PCR tubes in the presence of 10^5 copies of IAC DNA.

Analysis of artificially contaminated food/water samples

In order to validate the mPCR method for detection of *B. cereus*, milk and food samples were collected and artificially inoculated. Samples of rice-based dishes (vegetable biriyani) and milk (two each) were procured from the local market. Vegetable biriyani rinse was prepared by suspending 10 g of sample in 100 ml of buffered peptone water (BPW) and mascerated thoroughly with the medium using stomacher. The rinse was centrifuged at 1,500 g to get rid of the particulate debris and supernatant was stored in aliquots of 10 ml at -20°C for further use. Ten milliliters each of vegetable biriyani rinse and milk sample was inoculated with 100 μl of *B. cereus* (ATCC 14579), *B. thuringiensis* (ATCC 10872) and *B. anthracis Sterne* cultures to achieve concentration of 10^4 – 10^5 CFU/ml. Inoculated food samples were 10-fold diluted with BPW, mixed well and incubated overnight (18 h) at 37°C . One milliliter of BPW was taken at the end of incubation period from all samples and processed for DNA extraction by boiling method. The 300 pg of DNA (1.5 μl) was used as template in PCR assay.

Analysis of field samples

Twenty samples each of milk (25 ml) and rice-based dishes (25 g) were collected as described earlier. All of these above samples were subjected to mPCR and cultural methods after 18 h enrichment in BPW broth.

Results

Multiplex PCR

The reaction conditions for the mPCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The annealing temperature of 57°C , and 2 mM MgCl_2 concentration were selected even though optimal amplification could be achieved at annealing temperatures ranging from 52°C to 62°C and concentrations of MgCl_2 ranging from 1.5 mM to 2.5 mM. In the final standardization the concentration of *nhe A F*, *nhe A R* primers were increased to 400 nM because faint bands were seen in the presence of IAC DNA. The IAC co-amplified with target DNA and had amplicon size of 829 bp. Inclusion of varying concentrations of IAC DNA in mPCR mix did not change the detection limit of the assay and 10^5 copies were found to be optimum.

The mPCR was carried out by the simultaneous addition of primer pair for *hbl A*, *nhe A*, *cyt K*, *cry I A* and *pag* genes along with IAC. The results obtained with the mPCR are

given in Table 1. Variation in the diarrheal enterotoxin genes profile could be noticed for the *B. cereus* standard strains and suspected *B. cereus* strains for the validation. All the *B. thuringiensis* standard strains are positive for the genes *hbl A*, *nhe A*, *cyt K* other than *cry I A* gene. *B. anthracis Sterne* strain was positive for *nhe A* as well as *pag* (Fig. 1). Further testify, mPCR was performed on other related bacteria produced negative results for the genes screened. IAC was uniformly amplified in all the PCR reactions. To confirm the results obtained with mPCR, eight reference strains and 11 isolates of *B. cereus*, three reference strains of *B. thuringiensis* and *B. anthracis Sterne* strain were tested by individual PCR assays for genes which were targeted. Full agreement of the two methods was observed.

Sensitivity and specificity of mPCR

The detection limit of the assay was found to be 10^3 CFU/ml (30 CFU per reaction) of *B. cereus*, 10^4 CFU/ml (300 CFU per reaction) of *B. thuringiensis* and 10^4 CFU/ml (300 CFU per reaction) of *B. anthracis Sterne*. In terms of pure genomic DNA the detection limit was 5 pg for *B. cereus*, 3 pg for *B. thuringiensis* and 6 pg for *B. anthracis Sterne*. To further test the specificity of assay, DNA templates from *A. hydrophila*, *S. typhi*, *Y. enterocolitica* and *E. coli* strains were used in this assay. There was no amplified product obtained except that of IAC.

IAC and detection probability

The IAC co-amplified with target DNA and had amplicon size of 826 bp. Inclusion of varying concentrations of IAC DNA in mPCR mix did not change the detection limit of the assay and 10^5 copies were found to be optimum.

Analysis of experimentally contaminated food samples

B. cereus group could be detected in all artificially inoculated milk and food samples after overnight enrichment in BPW. Detection of *B. cereus* in milk and vegetable biriyani samples showed that mPCR could detect 15 CFU/ml in milk and 69 CFU/ml in vegetable biriyani samples. For *B. thuringiensis* mPCR detection limits were 38 CFU/ml in milk and 83 CFU/ml vegetable biriyani samples. Detection limits of mPCR for *B. anthracis* are 59 CFU in milk and 72 CFU/ml in vegetable biriyani samples (Table 3).

Analysis of field samples

When evaluated on a total of 40 naturally occurring samples of rice-based dishes and milk together, the mPCR detected four samples positive for *B. cereus* and one sample positive

Fig. 1 Agarose gel electrophoresis patterns showing mPCR amplification products for the *Bacillus cereus* group genes. Lanes M, DNA molecular size marker (100 bp ladder; Fermentas); Lanes: 1, *cyt K*, *nhe A*, *hbl A*, *pag*, *cry I A* and IAC simultaneously (mixed DNA of *B. cereus*, *B. thuringiensis* and *B. anthracis Sterne*); 2, *cyt K*, *nhe A*, *hbl A*, *pag*, *cry I A* and IAC simultaneously (DNA of mixed culture of *B. cereus*, *B. thuringiensis* and *B. anthracis*); 3, *cyt K*, *nhe A*, *hbl A* and IAC (*B. cereus*, ATCC 14579); 4, *cyt K*, *nhe A*, *hbl A* and IAC (*B. cereus* ATCC 10876); 5, *cyt K*, *nhe A*, *hbl A* and IAC (*B. cereus* NCIM 2155); 6, *cyt K*, *nhe A*, *hbl A*, *cry I A* and IAC (*B. thuringiensis* ATCC 10872); 7, *cyt K*, *nhe A*, *hbl A*, *cry I A* and IAC (*B. thuringiensis* NCIM 5109); 8, *nhe A*, *pag* and IAC (*B. anthracis Sterne*); 9, *nhe A*, *pag* and IAC *B. anthracis Sterne* 1; 10, *nhe A*, *pag* and IAC (*B. anthracis Sterne*); 11, Blank.

Table 3 Sensitivity of mPCR in spiking experiments

Organism	Detection limits by mPCR	
	Milk CFU/ml	Vegetable biriyani CFU/ml
<i>Bacillus cereus</i>	1.5×10^1	6.9×10^1
<i>Bacillus thuringiensis</i>	3.8×10^1	8.3×10^1
<i>Bacillus anthracis Sterne</i>	5.9×10^1	7.2×10^1

for *B. thuringiensis*. The identical results were obtained from among these samples following the conventional culture, isolation and biochemical identification. The individual gene PCRs performed on these isolates also found the same results.

Discussion

Numerous methods, both the conventional as well as rapid systems including PCRs have been attempted for the identification of toxigenic *B. cereus*, *B. thuringiensis* and *B. anthracis* strains following their isolation from various sample sources [23–26]. These *Bacilli* being close

to each other chromosomally often create problems to investigators for their specific identification. Recent evidences indicating the involvement of *B. thuringiensis* strains positive for the diarrheal enterotoxins in food poisoning incidences and isolation of *B. anthracis* strains exhibiting the properties of *B. cereus* presented challenges even for developing specific PCR assay [17, 20]. PCR-based assays have been developed to detect enterotoxin genes of *B. cereus* group individually [27, 28], or by multiplexing [24–26, 29]. On other side, detection of enterotoxin genes alone is not sufficient for proper surveillance in the context of bio warfare agents like *B. anthracis*. So there is a need to develop a system, which is having potential to detect enterotoxigenic nature of *B. cereus* group strains along with the discriminative potential for *B. anthracis* strains. In this study, we have described a multiplex PCR for the detection of enterotoxigenic *B. cereus* group strains in food matrices as well as to discriminate *B. cereus* group organisms preliminarily by selecting major enterotoxin genes and plasmid borne phenotype specific genes. Further to make the mPCR assay acceptable to the present norms of a diagnostic PCR [21, 30] and to avoid false negatives, IAC was included in the assay.

Among *B. cereus* group, *hbl A*, *nhe A*, *cyt K* and *ent Fm* (Enterotoxin FM) are broadly distributed enterotoxin genes [25]. However, we have selected only *hbl A*, *nhe A* and *cyt K* toxin genes for mPCR in view of their prevalence and toxicity as these toxins associated with ocular toxicity and necrotic enteritis in addition to their role in food poisoning. Along with these, *cry I A* and *pag* genes have been selected as specific plasmid borne phenotypic markers of *B. thuringiensis* and *B. anthracis* strains for preliminary identification and differentiation from *B. cereus* strains. Primers for this mPCR were designed based on the conserved regions using NCBI database. Care was taken to maintain a minimum of 60 bp differences among different PCR products for clear resolution and also to obtain a relatively high stringency with a near uniform annealing temperature, in order to avoid non-specificity. As was the observation of Henegariu and coworkers [31], we also found that the relative concentrations of the primers was an important factor in determining approximate equal yields of amplification products from each of the organisms in a single reaction. The mPCR assay was standardized with DNA extracted by boiling lysis method in order to save time and expenditure. Minimum no of copies of IAC DNA that gave good visible band was selected in order to avoid competition between target DNA and IAC DNA for *nhe A* primers particularly at low concentration of target DNA.

Detection of very low levels of bacterial contamination in food necessitates that these samples to be cultured for few hours in BPW for providing conditions for growth and multiplication of bacterial pathogens to a detectable level. The mPCR had a reasonably high level of sensitivity in experimentally spiked vegetable biriyani and milk samples and able to detect as low as 10^1 – 10^2 organisms per ml of growth of these pathogens following overnight enrichment in BPW. This detection sensitivity was adequate enough to precisely pickup the presence of these pathogens from among the natural food samples. However, the detection sensitivity of spiked food samples is higher when compared with pure reference culture (10^3 – 10^4 CFU/ml) is due to overnight enrichment of spiked food samples. When evaluated on a total of 40 naturally occurring samples of rice-based dishes and milk together, the mPCR detected four samples positive for *B. cereus* and one sample positive for *B. thuringiensis*. In this study, total 35 strains of *B. cereus* group including 16 reference strains and 19 isolates have been used to validate the mPCR. The identical results were obtained from among these samples following the conventional culture, isolation and biochemical identification. The individual gene PCRs performed on these isolates also found the same results. This strengthens the claim of this mPCR as a viable and reliable alternative for simultaneous detection of these organisms from different kinds of samples within 24 h.

Conclusion

Multiplexing of toxin specific genes as well as plasmid borne phenotypic specific genes has enabled the system to identify enterotoxigenic *B. cereus* group strains with discriminative potential for *B. anthracis* and *B. thuringiensis* strains. IAC further enhanced the sensitivity of the mPCR system by avoiding false negatives. Considering the low cost involved and relatively much shorter time needed to detect these important organisms of *B. cereus* group, this tool is useful for investigation of food borne outbreaks where these organisms are involved.

Acknowledgement This work has been supported by institutional core funds from the Defense Research and Development Organisation, Ministry of Defense, Government of India.

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