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ORIGINAL ARTICLE

Simultaneous detection of pathogenic *B. cereus, S. aureus* and *L. monocytogenes* by multiplex PCR

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Abstract Three important foodborne pathogens, *Bacillus cereus, Listeria monocytogenes* and *Staphylococcus aureus* are of major concern for food safety in terms of frequency and seriousness of the disease. The occurrence these three important pathogens and their coexistence in food matrices are predominant. Moreover, symptoms associated with *B. cereus* and *S. aureus* food poisoning not only closely resembles each other but can also be overlapping with other foodborne infections. In this context, detection of these three pathogens simultaneously in food samples by a single multiplex PCR (mPCR) would have advantages in terms of rapidity and cost saving, when compared with single organism specific PCRs. mPCR has been standardized by targeting three major diarrheal enterotoxin genes *hbl A*, *cyt K* and *nhe A* of *B. cereus*, virulence associated *nuc* and *Ent B* genes of *S. aureus* and virulence associated *hly* and *iap* genes of *L. monocytogenes* along with internal amplification control (IAC). The results showed that mPCR accurately identified all the three organisms individually or in combination without non-specificity. The mPCR was able to detect as low as 10 to 100 organisms per ml of growth following overnight enrichment of spiked food samples (vegetable biriyani and milk) and their presence in naturally contaminated samples also. The high throughput and cost effective multiplex PCR method developed in this study could provide a powerful tool for simultaneous, rapid and reliable detection of *B. cereus*, *S. aureus* and *L. monocytogenes* in food samples.

Keywords *Bacillus cereus* · *Listeria monocytogenes* · *Staphylococcus aureus* · mPCR · IAC · Pathogens

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Introduction

Foodborne outbreaks are of major concern worldwide. To date around 250 different foodborne diseases have been described and bacteria are the causative agents of two-thirds of foodborne disease outbreaks. Among bacteria that cause foodborne poisoning, some are particularly important in terms of frequency and seriousness of the disease. In this context, typical gram-positive pathogens such as *Bacillus cereus* [1], *Listeria monocytogenes* [2] and *Staphylococcus aureus* [3, 4, 5] have been categorized as both spoilage as well as pathogenic microorganisms. In detail, *B. cereus* is a gram-positive spore forming bacteria and contaminates nearly all agricultural products and plays a role in the contamination and spoilage of food products [1]. *B. cereus* causes emesis and diarrhea by secreting extracellular toxins such as hemolysin BL, non-hemolytic enterotoxin and cytotoxin K [6, 7]. *S. aureus*, a gram-positive pathogen with the ability to grow in a wide range of temperatures ranging from 7°C to 48.5°C and at pH 4.2 to 9.3, can adopt itself to grow in a wide variety of foods and causes food poisoning by secreting enterotoxins [3]. *L. monocytogenes* is another important gram-positive foodborne pathogen produces several exotoxins contributing to virulence and is capable of causing severe infections such as septicemia, encephalitis and meningitis, especially in immunocompromised individuals, newborns and pregnant women [8].

Combination of their ubiquitous nature and coexistence of these three organisms in food and environment is a major concern in terms of food safety. In addition to this, *S. aureus* food poisoning resembles *B. cereus* foodborne intoxication in its symptoms and incubation period. Synergism has also been observed that the combination of *S. aureus* sphingomyelinase and *B. cereus* phosphotidylcholine hydrolase resulted in a total lysis of an erythrocyte preparation in 14–60 min; neither enzyme alone affected greater than 2% lysis of the erythrocyte population over 180 min [9].

Single gene PCRs have been developed to detect these organisms individually by targeting toxin-specific or genus-specific genes. However, detection of only one organism by single PCR often misinterprets another organism's presence, thereby delaying medication and treatment procedures. In this context, detection of these three pathogens simultaneously by a single multiplex PCR (mPCR) would have advantages in terms of rapidity, convenience and cost saving. To achieve this, three major diarrheal enterotoxin genes *hbl A* (hemolysin BL) [6], *cyt K* (cytotoxin K) [10] and *nhe A* (non-hemolytic enterotoxin) [11] of *B. cereus*, virulence associated *nuc* (thermonuclease) [12, 13] and *ent B* (enterotoxin B) [3] of *S. aureus*, and virulence associated *hly* (listeriolysin O) [14] and *iap* (immune-associated protein) [15, 16] genes of *L. monocytogenes* were selected. Moreover, an internal amplification control (IAC), which has now become almost mandatory for diagnostic PCRs was also included [17]. Following standardization, the mPCR was evaluated with 20 *B. cereus*, 16 *S. aureus* and 3 *L. monocytogenes* strains individually and in combination to assess the sensitivity and the specificity. Spiking studies were also conducted in food samples to evaluate the efficacy of the system. The detection of all these three pathogenic bacteria simultaneously from food samples could be completed in less than 24 h with this mPCR method.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Reference strains were obtained from ATCC (Hi-media, Mumbai) and National Collection of Industrial Microorganisms (NCIM). *B. cereus* and *S. aureus* isolates used in this study were isolated from soil and food samples by classical cultural methods used for the evaluation of the mPCR method.

Primers and internal amplification control

All the primers used in this study were designed indigenously by using the GenBank database. An IAC was constructed for which both the forward and reverse primers had 5′ overhanging ends of *nhe A* gene, whereas, the 3′ ends of both primers were joined by complementary sequence of pUC 18 DNA. All the primer sequences and IAC sequences are listed in Table 2.

For generation of IAC DNA, the PCR reaction performed in 20 µl reaction mixture contained 300 nM of IAC primer, 200 µM concentrations of dNTP mix (MBI Fermentas), 0.5 units of Taq polymerase, 2.0 mM $MgCl_2$, in 1X PCR buffer (MBI Fermentas) with 375 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min and extension at 72°C for 1 min. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72°C. PCR product was purified using the PCR purification kit (Qiagen). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in 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 μl^{-1}) × (6.023×10^{23}) / (660 g mol l^{-1}X number of base pairs of PCR fragment) = the number of genomic copy per microlitre [18].

DNA extraction and mPCR

DNA was extracted by boiling method from the overnight cultures of bacteria grown in tryptic soy yeast extract (TSBYE) broth (Hi-media, Mumbai) [19]. Multiplex PCR was carried out in 20 µl reaction containing 400 nM of nheA F and nheA R, 200 nM of forward and reverse primers of cyt K and hly, 300 nM of hbl A and ent B primers, 160 nM of iap and nuc primers, 200 µM concentrations of dNTP mix (MBI Fermentas), 10^5 copies of IAC DNA, 1.2 unit of Taq polymerase, 2.0 mM $MgCl_2$ in 1 X PCR buffer (MBI Fermentas) with 1.5 µl of template DNA. Amplification consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min and extension at 72°C for 1.30 min followed by final 6 min extension at 72°C. The PCR products were analyzed on 2% (wt/vol) agarose gel.

Determination of specificity and sensitivity of mPCR

To further test the specificity of assay, mPCR was evaluated by using *A. hydrophila, Salmonella typhi*, *Yersinia enterocolitica,* and *E. coli* strains (Table 1). To assess the minimum amount of *B. cereus*, *L. monocytogenes* and *S. aureus* DNA detectable by mPCR, 10-fold serial dilutions of overnight growth of *B. cereus* ATCC 14579, *L. monocytogenes* ATCC 15313 and *S. aureus* NCIM 2150 were serially diluted at a concentration of 10^6–10^0 CFU ml^{-1}. DNA was prepared by boiling method as described earlier. A 1.5 µl aliquot of each dilution was added to five

Table 1 Bacterial strains used for the evaluation of specificity of mPCR and their toxin profile

Bacterial strain	*hbl* A	*nhe* A	*cyt* K	*nuc*	*ent* B	*hly*	*iap*	IAC
Bacillus cereus								
ATCC 14579	+	+	+	–	–	–	–	+
ATCC 10876	+	+	+	–	–	–	–	+
ATCC 11778	–	+	–	–	–	–	–	+
NCIM 2155	+	+	+	–	–	–	–	+
NCIM 2156	+	+	+	–	–	–	–	+
NCIM 2185	–	+	+	–	–	–	–	+
NCIM 2703	+	+	–	–	–	–	–	+
NCIM 2106	+	+	–	–	–	–	–	+
Isolate 1	–	–	–	–	–	–	–	+
Isolate 2	+	+	+	–	–	–	–	+
Isolate 3	–	+	–	–	–	–	–	+
Isolate 4	–	+	–	–	–	–	–	+
Isolate 5	–	+	+	–	–	–	–	+
Isolate 6	+	+	–	–	–	–	–	+
Isolate 7	–	+	+	–	–	–	–	+
Isolate 8	+	+	+	–	–	–	–	+
Isolate 9	+	+	–	–	–	–	–	+
Isolate 10	–	+	–	–	–	–	–	+
Isolate 11	–	+	–	–	–	–	–	+
Isolate 12	+	+	–	–	–	–	–	+
Staphylococcus aureus								
NCIM 2120	–	–	–	+	+	–	–	+
NCIM2010	–	–	–	+	+	–	–	+
NCIM2437	–	–	–	+	+	–	–	+
NCIM 2492	–	–	–	+	–	–	–	+
NCIM 2127	–	–	–	+	+	–	–	+
NCIM 2720	–	–	–	+	–	–	–	+
NCIM 2124	–	–	–	+	+	–	–	+
NCIM 2079	–	–	–	+	+	–	–	+
NCIM 2901	–	–	–	+	–	–	–	+
NCIM 2654	–	–	–	+	–	–	–	+
IVRI 1	–	–	–	+	–	–	–	+
DRDE 1	–	–	–	+	+	–	–	+
Isolate A	–	–	–	+	+	–	–	+
Isolate B	–	–	–	+	+	–	–	+
Isolate C	–	–	–	+	+	–	–	+
Isolate D	–	–	–	+	–	–	–	+
Listeria monocytogenes								
ATCC 13932	–	–	–	–	–	+	+	+
ATCC 15313	–	–	–	–	–	+	+	+
NCIM 835	–	–	–	–	–	+	+	+
Aeromonas hydrophila ATCC 49140	–	–	–	–	–	–	–	+
Salmonella typhimurium	–	–	–	–	–	–	–	+
Escherichia coli MTCC 730	–	–	–	–	–	–	–	+
Yersinia enterocolitica ATCC23715	–	–	–	–	–	–	–	+

Table 2 Primer sequences, accession nos. and anticipated sizes of PCR products for the *B. cereus* group specific primers used in this study

Primer	Target gene	Sequence (5′- 3′)	Product size	Accession no.
cyt K F	*cyt* K	GGCGCTAGTGCAACATTACG	482	AE016877
cyt K R		TCATACCAGGAGAGAAACCGC		
nhe A F	*nhe* A	AAGGCGAATGTACGAGAGTGG	553	NC_004722
nhe A R		CTTCTCTCGTTTGACTATCTGCAG		
hbl F	*hbl* A	TTACCTGGTAGAATCGTACAAGATC	164	AJ237785
hbl R		CCTGTATTAATCGCTTCTACCATTG		
nuc F	*nuc*	GCTGGCATATGTATGGCAATT	389	DQ399678
nuc R		GCTTCAGGACCATATTTCTCTACA		
ent B F	*ent*	CCAGATCCTAAACCAGATGAGTT	325	AY856382
ent B R		GTTTTTCGTTTGTCAGTTTGATG		
hly F	*hly*	TGCAAGTCCTAAGACGCCAA	753	AY750900
hly R		CCACACTTGAGATATATGCAGGA		
iap F	*iap*	TCGCACAAAGTAAAGGGACTA	663	AF532293
iap R		CTGGAGCTTCTTTTTTCACTTC		
IAC F	PUC 18	AAGGCGAATGTACGAGAGTGGTCCTGCAACTTTATCC	829	
IAC R		CTTCTCTCGTTTGACTATCTGCAGTGGTTTCTTAGACGTCAGGTG		

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

separate PCR tubes in the presence of 10^5 copies of IAC DNA.

Analysis of experimentally spiked food/milk samples

In order to validate the mPCR method for detection of *B. cereus, S. aureus* and *L. monocytogenes,* milk and vegetable biriyani were utilized for experimental inoculation. For this, samples of retort processed vegetable biriyani and pasteurized milk-based dishes were procured from the local market. Before spiking experiments, the food samples were checked for the presence of *B. cereus*, *S. aureus* and *L. monocytogenes*. But any of these organisms could not be detected by culture in these samples. Vegetable biriyani rinse was prepared by suspending 10 g of sample in 90 ml of TSBYE broth and macerated 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 milliliter each of vegetable biriyani rinse and the milk sample was inoculated with 100 µl of *B. cereus* (ATCC 14579), *S. aureus* (NCIM 2010) and *L. monocytogenes* (ATCC 13932) cultures separately, to achieve concentrations of 10^4 to 10^{-1} CFU ml^{-1}. Each food sample was diluted with TSBYE at the ratio of 1:10, mixed well and incubated overnight (18 h) at 37°C. One milliliter was taken at the end of the incubation period from all the samples and processed for DNA extraction by boiling method. The DNA (1.5 µl) was used as template in PCR assay.

Analysis of field samples

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

Results

Multiplex PCR

Multiplex PCR was optimized using the PCR conditions as indicated in Materials and methods. The annealing temperature of 57°C, and 2 mM $MgCl_2$ concentration were finally selected because of optimal intensity of amplification of all the bands observed at those conditions though the bands were visible at all temperatures (52°C to 62°C) and all concentrations of $MgCl_2$ (1.5 mM to 2.5 mM). The primer concentration of NheA F and NheA R primers were increased to 400 nM to get adequate resolution 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.

When mPCR was performed with *B. cereus* strains ATCC 14579 and ATCC 1087, three genes (*hbl A*, *nhe A* and *cyt K*) and IAC were amplified. When mPCR was conducted on *S. aureus* strains NCIM 2437 and NCIM 2120 DNA, two genes (*nuc* and *ent B*) and an IAC were amplified. Likewise when *L. monocytogenes* strains ATCC 13932 and 15313 DNA was used in mPCR, two genes (*hly* and *iap*) were amplified along with IAC. With DNA of mixed culture of *B. cereus* ATCC 14579, *S. aureus* NCIM 2437, *L. monocytogenes* ATCC 13932, all the eight PCR products (*hbl A*, *nhe A*, *cyt K*, *nuc*, *ent B*, *hly* and *iap* PCR products along with IAC) were obtained (Fig. 1).

Specificity and sensitivity of mPCR

There was no amplified product obtained except that of IAC with non *Bacillus* strains (Table 1). The detection limit of the assay was found to be 10^3 CFU ml^{-1} (30 CFU per reaction) of *B. cereus*, 10^4 CFU ml^{-1} (300 CFU per reaction) of *S. aureus* and 10^4 CFU ml^{-1} (300 CFU per reaction) of *L. monocytogenes*. In terms of pure genomic DNA the detection limit was 5 pg for *B. cereus*, 3 pg for *S. aureus* and 6 pg for *L. monocytogenes*.

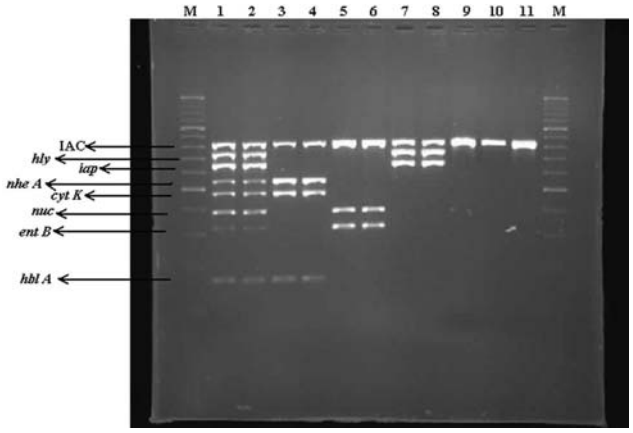

Fig. 1 Agarose gel electrophoresis patterns showing mPCR amplification products. Lanes M, DNA molecular size marker (100-bp ladder); Lanes: 1, *cyt* K, *nhe* A, *hbl* A, *hly*, *iap*, *nuc*, *ent B* and IAC simultaneously (mixed DNA of *B. cereus*, *L. monocytogenes* and *S. aureus*); 2, *cyt* K, *nhe* A, *hbl* A, *hly*, *iap*, *nuc*, *ent B* and IAC simultaneously (DNA of mixed culture *B. cereus*, *L. monocytogenes* and *S. aureus*); 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, *nuc*, *ent* B and IAC (*S. aureus* NCIM-2437); 6, *nuc*, *ent* B and IAC (*S. aureus* NCIM-2120); 7, *hly*, *iap* and IAC (*L. monocytogenes* ATCC-13932); 8, *hly*, *iap* and IAC (*L. monocytogenes* ATCC-15313); 9, negative control (*A. hydrophila*); 10, negative control (*Yersinia enterocolitica* ATCC-49140); 11, blank (distilled water)

Analysis of experimentally contaminated food/milk samples

Three independent experiments have been performed to detect sensitivity of mPCR assay and their mean values have been taken as result. The sensitive detection level for *B. cereus* is 12 CFU ml^{-1} in milk, 23 CFU ml^{-1} in vegetable biriyani, for *S. aureus*, 45 CFU ml^{-1} in milk, 82 CFU ml^{-1} in vegetable biriyani, for *L. monocytogenes*, 53 CFU/ml^{-1} in milk and 150 CFU ml^{-1} in vegetable biriyani rinse after 18 h of enrichment (Table 3).

Analysis of field samples

Out of 10 milk samples tested, two were identified to contain *B. cereus* and one sample was identified to contain both *B. cereus* and *S. aureus* and two samples contain *L. monocytogenes* by mPCR method. Out of 10 rice-based foods tested, one sample was identified to be containing *B. cereus* by mPCR. *L. monocytogenes* could not be detected in any of these samples either by mPCR or by culture, classical conventional biochemical tests. The same samples also yielded *B. cereus*, *S. aureus* and *L. monocytogenes* following culture and conventional biochemical identification.

Discussion

B. cereus, *S. aureus* and *L. monocytogenes* are major foodborne pathogens following their isolation from various sample sources [12, 20, 15]. The coexistence of these three important pathogens in food matrices along with their close resemblance in terms of symptoms and incubation period causes misinterpretation when we use single-gene PCR based assays. In this context, identification of these pathogens using multiplex PCR by multiplexing of pathogen specific genes is a definite help for food industries. In this study, we have described a multiplex PCR for the detection of these three pathogens by selecting major enterotoxin genes along with the conserved genes of respective organisms. In addition, to make the mPCR assay acceptable to the

Table 3 Sensitivity of mPCR with artificially contaminated samples (n=3)

Organism	Detection limits by mPCR	
	Milk CFU ml^{-1}	Vegetable biriyani CFU ml^{-1}
B. cereus	1.2×10^1	2.3×10^1
S. aureus	4.5×10^1	8.2×10^1
L. monocytogenes	5.3×10^1	1.5×10^2

present norms of a diagnostic PCR internal amplification control (IAC) has been included [17, 18].

The choice of major diarrheal enterotoxin genes namely *hbl A*, *cyt K* and *nhe A* of *B. cereus* to be reasonable as these toxins have been grouped as major toxins which cause diarrhea and other infections in humans [21]. For *S. aureus*, *nuc* and *ent B* genes were chosen because *ent B* is involved in food poisoning and *nuc* gene is conserved and it has been tested for specific detection of *S. aureus*. In case of *L. monocytogenes, iap* and *hly* genes were chosen as they encode a highly conserved surface protein gene and listeriolysin O which is involved in intracellular spreading of the organism in mammals respectively. Primers designed for this mPCR were chosen to maintain a near uniform annealing temperature and also care was taken to maintain 60 bp differences among different PCR products for clear resolution. The IAC incorporated was pUC 18 plasmid DNA flanked by *nhe A* primers. Optimum number of copies of IAC DNA was selected to give a good visible band in order to avoid competition between target DNA and IAC DNA for *nhe A* primers.

Detection of very low levels of bacterial contamination in food necessitates that these samples to be cultured for few hours in TSBYE broth 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 to 10^2 organisms per ml of growth of these pathogens following overnight enrichment in TSBYE. This detection sensitivity was adequate enough to precisely pick up 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 to 10^4 CFU per ml) due to overnight enrichment of spiked food samples. When evaluated on a few naturally occurring food and environmental samples (total 20 samples), the mPCR detected two samples positive for *B. cereus*, one sample positive for *S. aureus* and two samples positive for *L. monocytogenes* with identical results obtained following the conventional culture, isolation and biochemical identification procedures. This strengthens the claim of the reported mPCR as a viable and reliable alternative for simultaneous detection of these three organisms within 24 h. Though the naturally occurring food samples tested in the present study are low in number, the results obtained are highly promising and need to be evaluated further on a larger and diverse food sample sources. Multiplexing of toxin-specific as well as pathogen-specific genes along with IAC has enabled the system to identify pathogenic *B. cereus, S. aureus* and *L. monocytogenes* without giving 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 foodborne outbreaks where these organisms are involved.

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