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Direct Detection of Bacillus cereus and its Enterotoxigenic Genes in Meat and Meat Products by Polymerase Chain Reaction

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Abstract

This study was conducted to standardize a method to directly detect *B. cereus* (gyrB) and its enterotoxigenic genes (hblDAC, nheABC, cytK and entFM) from the meat and meat products by PCR, without going for isolation and identification procedures. The method employed was compared with the standard microbiological procedures to determine its efficacy. Among the 150 food samples (raw meats and meat products) analyzed, 60 (40%) were positive for isolation and 59 (39.33%) turned out positive by direct PCR (targeting sequence within gyrB gene). Food samples screened directly by multiplex-PCR showed almost similar enterotoxin gene profile as of isolates from these samples. Among the positive samples, only two samples from the meat products showed different enterotxigenic gene pattern compared to isolates from these samples. The incidences of various enteroxigenic genes hblD, hblA, hblC, nheA, nheB, nheC, cytK and entFM was 66.10%, 66.10%, 67.78%, 96.61%, 96.61%, 93.22%, 67.78% and 100%, respectively. Therefore, the current method can be employed for the direct detection of *B. cereus* and its enterotoxigenic genes in the meat and meat products without going for isolation and identification procedures and the method can be extended to other foods as well.

Keywords: Bacillus cereus, Direct Detection, Enterotoxigenic genes, PCR, Meat

Introduction

Bacillus cereus is a gram positive, spore forming bacterium belonging to the family Bacillaceae. Based on similarity in 16S rRNA sequence, *B. cereus*, *B. anthracis, B. thuringiensis* and *B. mycoides* make up the *B. cereus* group (Ash *et al.*, 1991). The species of the *B. cereus* group have a high level of DNA homology (Seki *et al*., 1978), but a specific sequence within gyrB gene can distinguish B. cereus from the other members of the group (Yamada *et al.*, 1999). *B. cereus* is wide spread in environment (soil, water and dust) from where it easily spreads to foods of plant origin and through cross contamination to other foods such as milk, meat and meat products (Granum, 1994, Larsen and Jorgensen, 1997). *B. cereus* is an important cause of foodborne illness in humans and is frequently involved in a number of foodborne outbreaks (Notermanns and Batt, 1998, Lund *et al*., 2000). *B. cereus* causes two distinct types of foodborne illnesses, the emetic and diarrheal syndromes, which involve emetic and diarrhegenic toxins, respectively (Kramer and Gilbert, 1989). B. cereus is also involved in a variety of local and systemic infections like meningitis, endopthalmitis, endocarditis, periodonitis, osteomyelitis, wound infection and septicemia (Kortiranta *et al*., 2000, Schoeni and Wong, 2005).

B. cereus produces several toxins, including an emetic toxin (Agata *et al.*, 1995b) and five different enterotoxins, namely: Hemolysin BL (Beecher and Mac Millan, 1990, 1991), non hemolytic enterotoxins (Ganum *et al*.,1996, 1999), cytotoxin K (Lund *et al*., 2000), enterotoxin FM (Asano et al., 1997) and enterotoxin T (Agata *et al*., 1995a). Hemolysin BL, non hemolytic enterotoxins and cytotoxin K are regarded as primary virulence factors involved in *B. cereus* diarrhea (Granum *et al*., 1999, Lund *et al*., 2000).

As the isolation and identification procedures are time consuming and laborious and currently no method is available to directly detect *B. cereus* and its enterotoxigenic genes from various foods. Therefore, the current work was undertaken to standardize a PCR for direct detection of *B. cereus* and its enterotoxigenic genes from meat and meat products.

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Materials and methods

Sampling

The samples (n=150) comprising raw meat and meat products were collected at random from different retail shops in and around different cities of India. Approximately 30 g of meat sample was collected in a clean plastic zip lock sachet and brought to laboratory and processed immediately or kept at 4°C till processed. Raw meat samples comprising chicken (50), mutton (25) and chevon (30) were collected from retail shops in Ludhiana and Srinagar cities (India), maintaining proper cold chain. A total of 45 meat products [Chicken seekh kabab (5), Mutton seekh kabab (5), Chicken curry (4), Chicken salami (4), Chicken lolypop (4), Chicken sausage (4), Chicken pickle (3), Chicken tika (3), Chicken pakoda (3), Chicken nugget (2), Chicken cutlet (2), Mutton shami kabab (2), Chicken fingers (2) and Chicken momo (2)] were collected from different retail shops and restaurants of the Ludhiana city (India).

Isolation and Identification

About 10 g from each sample was weighed aseptically and homogenized in 30 ml of Brain Heart infusion broth (BHIB). After an incubation period of 18 h at 37°C a loopful was streaked on Polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) media (Hi-Media, Mumbai-India). The plates were incubated for 24 h at 37°C (Shinagawa, 1990). All the presumptive isolates of *B. cereus* were subjected to morphological and biochemical tests as per the method described in Bacteriological Analytical Manual (Rhodehamel and Harmon, 2001).

Bacterial strains: Two reference strains of B. cereus were used - ATCC 14579 procured from Hi-Media (Mumbai, India) and NCTC 11143 (Kindly provided by Prof. M.M. Willayat, of SKUAST-K, J&K, India). The reference strains of *B. cereus* were used as positive control during the biochemical and molecular characterization of the isolates.
DNA extraction from food samples
Approximately 5 g of meat samples was cut into small pieces and inoculated into 20 ml nutrient broth containing polymyxin B (10000 U/100 ml, to inhibit growth of Gram negative bacteria) and incubated at 32°C for 16 h. Avoiding tissue pieces 1 ml of supernatant broth was taken into micro-centrifuge tube and centrifuged at 5000 g for 3 min, pellet was washed twice with 500 µl of sterile MilliQ water to remove the PCR inhibitors and finally dissolved in 100 µl of sterile MilliQ water. Dissolved pellet was heated for 10 min in boiling water bath, chilled immediately on crushed ice for 20 min and centrifuged at 7000 g for 5 min, the supernatant collected served as template.
Oligonucleotide Primers: The details and concentration of the primers used in the present study are provided in Table 1.

Detection of gyrB gene of B. cereus in food samples

The DNA amplification was carried as per the method described by Yamada *et al*. (1999) with slight modifications. The PCR was set up in a reaction volume of 50 µl containing 3 µl template DNA, 1X PCR buffer (10mM Tris-HCl pH 8.3 and 50mM KCl) 1.5 mM MgCl2, 200 µM of each dNTPs, 1 U Taq DNA Polymerase and primer concentration of 1 µM each. Amplification was carried out in thermocycler (Biometra, Germany) with initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 57oC for 1.5 min, 72°C for 2.5 min and a final extension of 72°C for 7 min.
Detection of eneterotoxigenic genes: The PCR conditions used for the detection of enterotoxigenic genes (hblADC, nheABC, cytK and entFM) in the current study are described elsewhere (Ngamwonsatit *et al*., 2008). The amplification was set up in a final reaction volume of 20 µl, containing 5 µl of template DNA, 1X PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 1.5 mM MgCl2, 200 µM of each dNTP, 5 U Taq DNA Polymerase (MBI-Fermentas, India) and primer concentration ranging from 0.2-0.4 µM. PCR reaction was carried out in thermocycler (Biometra, Germany) with initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 45 sec, 54oC for 1 min, 72°C for 2 min and a final extension of 72°C for 5 min.

Amplicons were separated on agarose gel (1.5%), stained with ethidium bromide (5 µg/ml), applying 5-6 Volts/cm and sizes were estimated using 100 bp DNA ladder (MBI-Fermentas, India).

Results

Among 150 samples, 60 were positive for isolation

Table 1. Primer Sequence for Enterotoxigenic Genes (hblCDA, nheABC, cytK and entFM) and gyrB gene specific for *B. cereus*.

Target gene	Primer	Sequence	Product size (bp)	Primer Conc. (μM)	References
hblC	FHblC	CCTATCAATACTCTCGCAA	695	0.4	Ngamwongsatit *et al.* 2008
	RHblC	TTTCCTTTGTTATACGCTGC			
hblD	FHD	GAAACAGGGTCTCATATTTT	1018	0.4	Thaenthanee 2004
	RHD2	CTGCATCTTTATGAATATCA			
hblA	FHblA	GCAAAATCTATGAATGCCTA	884	0.4	Ngamwongsatit *et al.* 2008
	RHblA	GCATCTGTTCGTAATGTTTT			
nheA	F2NheA	TAAGGAGGGGCAAACAGAAG	759	0.2	Ngamwongsatit *et al.* 2008
	RNheA	TGAATGCGAAGAGCTGCTTC			
nheB	F2NheB	CAAGCTCCAGTTCATGCGG	935	0.2	Ngamwongsatit *et al.* 2008
	RNheB	GATCCCATTGTGTACCATTG			
nheC	FNheC	ACATACCTTTTGCAGCAGAAC	618	0.2	Ngamwongsatit *et al* 2008
	R2NheC	CCACCAGCAATGACCATATC			
cytK	FCytK	CGACGTCACAAGTTGTAACA	565	0.3	Ngamwongsatit *et al* 2008
	R2CytK	CGTGTGTAAATACCCAGTT			
entFM	FEntFM	GTTCGTTCAGGTGCTGGTAC	486	0.2	Ngamwongsatit *et al.* 2008
	REntFM	AGCTGGGCCTGTACGTACTT			
gyrB	BC1	ATTGGTGACACCGATCAAACA	365	1	Yamada *et al.* 1999
	BC2r	TCATACGTATGGATGTTATTC			

of *B. cereus*, making an incidence of 40%. The percent isolation from the chicken, mutton, chevon and meat products was 32, 40, 36.67 and 51.11, respectively. About 59 (39.33%) samples turned out positive for amplification of *B. cereus* specific 365-bp amplicon (sequence within gyrB gene) by direct PCR. Of the 59 positive samples, 16 (32%), 10 (40%), 11 (36.67%), and 22 (48.89%), were from chicken, mutton, chevon and meat products, respectively. The only food sample turning negative for direct detection by PCR was from meat product (Chicken pickle-2), though it was positive for isolation of *B. cereus*. The food samples confirmed positive for gyrB gene, were screened directly by multiplex-PCR for the presence of eight enterotoxigenic genes (hblADC, nheABC, cytK and entFM). The eight fragments of enterotoxigenic genes namely, hblD, nheB, hblA, nheA, hblC, nheC, cytK and entFM, had a predicted size of 1018, 935, 884, 759, 695, 618, 565 and 486, respectively (Fig.1). The food samples screened directly showed almost similar enterotoxigenic gene pattern as that of isolates from these samples. Only two samples from the meat products (Chicken pickle and Chicken momo), showed different enterotoxigenic pattern when compared to isolates from these samples. Only entFM and nheA were detected in Chicken

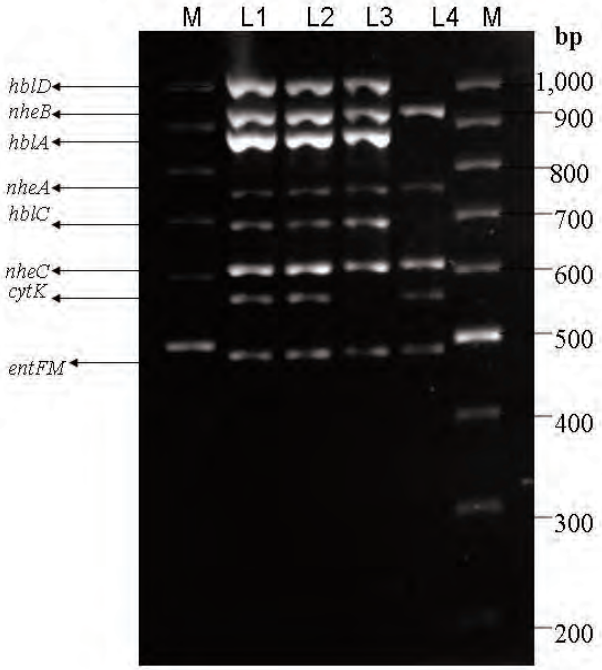

Fig.1. Agarose gel (1.5%) electrophoresis showing different enterotoxigenic genes of *B. cereus* from food samples.
M presents 100 bp DNA ladder.
L1 presents mutton sample experimentally contaminated with reference strain of *B. cereus* (ATCC 14579)
L2 presents *B. cereus* contaminated chevon sample (Group I)
L3 presents *B. cereus* contaminated meat product (Group III)
L4 presents *B. cereus* contaminated chicken sample (Group II)

Pickle-1, while as isolate from the sample harbored all the genes. The Chicken momo-2 showed the presence of hblD, nheA and entFM by direct screening, whereas, all the genes except cytK were present in the isolate from this sample. The incidences of various enterotoxigenic genes hblA, hblC, hblD, nheA, nheB, nheC, cytK and entFM in the samples was 66.10%, 66.10%, 67.78%, 96.61%, 96.61%, 93.22%, 67.78% and 100%, respectively.

According to the presence or absence of enterotoxigenic genes, the food samples were divided into six groups. The samples having all the eight genes were assigned Group I. Group II and Group III lacked hbl complex (hblCDA) and cytK, respectively. The isolates lacking both hbl complex and cytK were placed in Group IV. The Group V comprised the isolates in which, none, one, two or all the three genes of hbl complex were absent but cytK, entFM and at least one gene of nhe complex (nheABC) was present. The isolates which were similar to Group V but lacking cytK gene were placed in Group VI. Among the total samples positive for gyrB gene (59), 29 (49.15%), 6 (10.16%), 5 (8.47%), 10 (16.94%), 4 (6.78%) and 4 (6.78%) occupied Group I, Group II, Group III, Group IV, Group V and Group VI, respectively (Table 2).

Table 2. Distribution of Virulence Groups in Food Samples

Source	No. of Samples	Positive by PCR	Group I	Group II	Group III	Group IV	Group V	Group VI
Chicken	50	16	7	2	2	3	1	1
Chevon	25	10	4	1	1	3	1	0
Mutton	30	11	6	1	1	2	0	1
Meat Products	45	22	13	2	1	2	2	2
Total	**150**	**59**	**30 (50.84%)**	**6 (10.16%)**	**5 (8.47%)**	**10 (16.94%)**	**4 (6.78%)**	**4 (6.78%)**

Group I: Isolates having all eight genes were assigned
Group II and Group III: Isolates lacked hbl complex (hblCDA) and cytK, respectively.
Group IV: Isolates devoid of both hbl complex and cytK.
Group V: Isolates in which none, one, two or all the three genes of hbl complex were present but cytK, entFM and at least one gene of nhe complex (nheABC) were present.
Group VI: Similar to Group V but lacking cytK gene.

Discussion

The isolation and identification procedures are time consuming and cumbersome, therefore, a faster method was standardized for directly screening food samples for the presence of enterotoxigenic genes of B. cereus without need to go for lengthy isolation and identification procedures. By direct screening and isolation, 59 (39.33%) and 60 (40%) samples were positive, respectively. To test the efficacy of the method, meat samples negative for both isolation and direct detection (PCR) were artificially contaminated by standard strain of *B. cereus* (ATCC 14579). After which the meat samples turned out positive for both gyrB gene and all the enterotoxigenic genes by direct PCR. Among the food samples positive for presence of *B. cereus* specific gyrB gene, only two samples from the meat products (Cpik1 and Cmom2) showed different enterotoxigenic pattern when compared to isolates from these samples. This can be probably due to presence of PCR inhibitors (spices, flour) and/or due to less bacterial load in these samples (Ombui *et al*., 2008). The complete hbl complex was detected in 36 (61.01%) samples, Aragon-Algero *et al*. (2008) and Ngamwonsatit *et al.* (2008) detected hbl complex in 67.7% and 65.94% of *B. cereus* isolates, respectively. The complete nhe complex was detected in 54 (91.52%) samples. Aragon-Algero *et al*. (2008) and Vyeletelova and Banyko (2008) detected nhe complex in 99.4% and 98% of isolates, respectively. The cytK gene was detected in 40 (67.78%) samples, similarly Chitov *et al.* (2008) reported a prevalence of 70.40% for cytK gene. The entFM was detected in all the samples, suggesting it as the most common gene, likewise, Yang *et al.* (2005), Ngamwongsatit *et al.* (2008),

Vyeletelova and Banyko (2008) and Aragon-Algero *et al.* (2008) also detected entFM in all the isolates. These findings are similar to the current study, however, slight variations as reported by authors, could be due to the difference in the genotype of B. cereus strains in the different geographical locations.

There was a higher incidence of the enterotoxigenic genes in mutton and meat products as 54.54% and 59.01% of samples from mutton and meat products, respectively, harbored all the genes (Group I). The high incidence in mutton as compared to chevon and chicken may perhaps be due to different source of sampling, as the mutton samples were procured from the retail shops in Srinagar city (India), indicating adaptation of more pathogenic strains of B. cereus in foods from Srinagar, as some food poisoning outbreaks due to *B. cereus* have been reported from Srinagar City (Hussain *et al*. 2007). The higher incidence of virulence genes in meat products is in accordance to the findings of Baachil and Jaiswal (1988), and may be possibly due to contamination of meat products during processing or afterwards. In rest of the samples the enterotoxigenic gene pattern was almost similar (Table 2). Thus, the enterotoxigenic gene profile of food samples screened directly being almost similar to those of *B. cereus* isolates from same samples, indicate that the current method can be employed for the direct detection of enterotoxigenic genes of *B. cereus* from meat and meat products, without going for isolation. The method can also be extended for the screening of variety of other food samples.

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