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Advances in Life Science and Biotechnology

Vol. 4 (1), pp.4 - 11, January, 2016

Commonness of toxigenic attributes in local food confines of *Bacillus cereus* in the city of Mysore, Southern India

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Abstract

In the growing concern for microbial food safety of traditional foods, the present study has attempted to characterize toxigenic profile of native food isolates of *Bacillus cereus*. In a total of 65 traditional foods, 26 isolates were characterized by morphological, cultural and biochemical attributes as *B. cereus*. Of these, 12 isolates (46%) were confirmed as *B. cereus* by PCR with 16S rDNA and phosphatidylinositol phospholipase C primers in PCR. Among *B. cereus* isolates, 8 (67%) and 6 (50%) were positive for binding and lytic components of haemolysin (hbl complex) and sphingomyelinase (sph) in PCR. Besides, 67% of the isolates exhibited discontinuous haemolytic pattern in blood agar. The toxigenic food isolates of *B. cereus* CFR 1529, 1530, 1534 and 1536 revealed a high degree of sequence homology with selected strains of *B. cereus* and other species of *B. cereus* cluster as evidenced in sequence homology of partial nucleotide sequences of respective PCR amplicons of selected target genes. The study does indicate that toxigenic traits appear to be well spread within *B. cereus* cluster and have become stable traits among food isolates of *B. cereus* prevalent in the food chain.

Keywords: *Bacillus cereus*, PCR, phosphatidylinositol phospholipase C, haemolysin BL, sphingomyelinase, toxigenic, sequence homology, microbial diversity.

INTRODUCTION

The practices of hygiene and sanitation which prevail during preparation and marketing of traditional foods provide ample opportunities for contamination with food-borne pathogenic bacteria. Among the several important bacterial pathogens, strains of *Bacillus cereus* are of significance as they are opportunistic organisms and can dominate in any given situation, because of its ubiquitous nature and able to occur in a diverse range of foods (Varadaraj et al., 1992; Warke et al., 2000; Oguntoyinbo and Oni, 2004; Hanashiro et al., 2005; Murindamombe et al., 2005; Rosenquist et al., 2005; Reyes et al., 2007; Roy et al., 2007; Das et al., 2009). The study of *B. cereus* in relation to foods has gained significance in the light of its ability to form heat resistant endospores and capacity to grow and produce toxins in a wide variety of foods (Andersen et al., 2001; Agata et al., 2002; Ehling-Schulz et al., 2004a; Ouoba et al., 2008). Strains of *B. cereus* cause two different types of foodborne illnesses in humans, namely diarrhoeal and emetic types, which are attributed to few of the established toxins produced by this bacterial species (Schoeni and Wong, 2005; Ehling-Schulz et al., 2006b). Bacillus cereus food poisoning is underreported, as both types of illnesses are relatively mild and usually last for less than 24 h. On a few occasions, illnesses have become severe leading to hospitalization and/or even death (Dierick et al., 2005). The unique properties of *B. cereus* like heat resistant endospore forming ability, toxin production and psychrotrophic nature gives ample scope for this organism to be a prime cause of public health hazard (Griffiths and Schraft, 2002).

At present, the toxins related to illnesses caused by *B. cereus* have been characterized at molecular level, wherein diarrhoeal syndrome is linked to non-haemolytic enterotoxin (NHE), haemolytic enterotoxin (HBL) and cytotoxin K (CytK) and that of emetic type results from the action of cereulide (Ces) toxin (Tsen et al., 2000; Ehling-Schulz et al., 2006b). Molecular diagnostic methods based on polymerase chain reaction have been described for the detection of potent toxigenic cultures of *B. cereus* targeting enterotoxin complexes. Molecular approaches in understanding toxigenicity of *B. cereus*

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strains have revealed a certain degree of diversity (Hsieh et al., 1999; Hansen and Hendriksen, 2001; Guinebretiere et al., 2002; Radhika et al., 2002; Ehling-Schulz et al., 2004b; Dietrich et al., 2005; Abriouel et al., 2007; Ngamwongsatit et al., 2008).

The objective of present study was to assess the prevalence of toxigenic traits in isolates of *B. cereus* from selected food samples. Through sequence analysis of selected toxigenic native food isolates of *B. cereus* and its comparison with few other well characterized *B. cereus* strains, the genetic relatedness of native isolates in traditional foods was evidenced.

MATERIALS AND METHODS

Enumeration and characterization of *B. cereus* in food samples

A total of 65 food samples being sold in various sale points in the local markets of Mysore, a southern City in India were enumerated for the viable counts of *B. cereus.* The food samples comprised processed rice-based foods with added spices and vegetable salads (traditional fast foods), ice cream, raw milk, traditional milk-based sweets, processed wheat based foods and spiced cooked rice-based foods, which were analyzed over a period of 6 months (November to April). Requisite quantities of samples based on the general consumption pattern in a single serving were collected in sterile, disposable polythene bags and brought to laboratory in insulated icebox. Analysis of samples was performed within 60 min of collection. A homogeneous sample was prepared in 0.85% sterile saline to give an initial 1:10 dilution. Aliquots of 0.1 ml from appropriate dilutions were surface plated on sterile Polymyxin B sulphate pyruvate egg yolk mannitol bromothymol blue agar (HiMedia Laboratories, Mumbai, India) plates. The inoculated plates were incubated at 37°C for 24 - 48 h. Presumptive colonies of *B. cereus* were randomly selected based on characteristic colony features (peacock blue coloured colonies with a surrounding zone of egg yolk precipitation). Then, the presumptive isolates were purified by streaking on Brain Heart Infusion (HiMedia Laboratories, Mumbai, India) agar plates and the individual purified colonies were selected and isolated. Prior to the use of these isolates in experimental trials, they were grown successively twice in BHI broth for 20 h at 37°C in an orbital shaker incubator (Alpha Labs., Bangalore, India) at 120 rpm.

The presumptive isolates were identified by morphological, cultural and biochemical characteristics. The morphological tests included appearance of cells, Gram's reaction, motility and presence and position of spores. Isolates were tested for their growth in anaerobic agar (HiMedia Laboratories, Mumbai, India) and under anaerobic condition, 7% NaCl and at 45, 55 and 65°C. The biochemical characteristics included production of catalase and lecithinase, Voges- Proskauer reaction, nitrate reduction, citrate utilization, acid and gas from glucose, hydrolysis of starch, gelatin and casein and acid from glucose, mannitol, xylose and arabinose (Varadaraj et al., 1992; Cappucino and Sherman, 2004).

As reference cultures, strains of *B. cereus* F 4810 obtained through the courtesy of Dr. J. M. Kramer, Central Public Health Laboratory, London, UK and food isolates of *B. cereus* CFR 1410 and 1462 (Radhika et al., 2002) maintained in the culture collection stock of this Department were included as positive controls in the experimental protocols.

Haemolysin BL activity in isolates of *B. cereus*

Haemolysin BL activity in the individual culture supernatants of characterized native isolates of *B. cereus* obtained after 6 h growth at 30°C was detected by the gel diffusion assay of Beecher and Wong (1994) as previously described (Radhika et al., 2002).

PCR detection for species specificity and toxigenic traits

In this experimental trial, the potential toxigenic target genes selected were those of 16S rDNA (universal eubacterial primers), phosphatidylinositol phospholipase C (pi-plc), haemolysin BL (hbl) and sphingomyelinase (sph). A duplex PCR to detect *B. cereus* isolates with species-specific oligonucleotide Pi-PLC and haemolysin BL primers and a uniplex PCR with 16S rDNA and Sph primers were performed. The nucleotide sequences and amplification conditions of these PCR primers are presented in Table 1. The genomic DNA of individual isolates was extracted from 1.5 ml aliquots of culture broth by Phenol -chloroform method and ethanol precipitation (Schraft and Griffiths, 1995). The dried DNA was then dissolved in Tris ethylene diamine tetra acetic acid (TE) buffer of pH 8.0 and used as template DNA for amplifying the target genes using the respective primers. PCR amplification was performed in an automated DNA Thermal Cycler (Eppendorf, Master Cycler, Cedex, France) following the conditions as detailed in Table 1 and the PCR products analyzed in 1.2% agarose gels (Sambrook and Russel, 2001) and documented in Gel Documentation System (Vilber Lourmat, France).

Nucleotide sequence analysis of amplified PCR product and alignment

Considering the positive results with PCR primers and activities of haemolysis, four native isolates of *B. cereus* namely CFR 1529, 1530, 1534 and 1536 were subjected for PCR amplification with the respective 16S rDNA, Pi -PLC, Ha-1 and Sph primers. The resultant PCR amplified products were purified using commercially available PCR purification Spin Kit (HiPur A, HiMedia Laboratories, Mumbai, India) and subjected to sequence analysis (Sigma Aldrich, Bangalore, India). The partial nucleotide sequences of these specific target genes were subjected to BLAST programme of NCBI (Altschul et al., 1997) to assess the per cent homology with closely related strains and species of *B. cereus* cluster documented in Gene Bank database. A similarity network tree was constructed based on Force Type of Neighbour-Joining method from the same programme. Multiple alignment sequences was performed using partial nucleotide sequences obtained for the respective toxigenic target genes in the four native food isolates of *B. cereus* against selected strains of *B. cereus* / B. anthracis / B. mycoides / *B. thuringiensis* showing higher homology using Multalin version 5.4.1 (Corpet, 1988).

Statistical analysis

All the experimental trials were performed in triplicates and mean values were presented for applicable results after carrying out requisite statistical analysis using the software programme of Microsoft Excel (Microsoft Office 2003, Microsoft Redmond, WA, USA).

RESULTS

Prevalence and characterization of food isolates of *B. cereus*

The viable populations of *B. cereus* enumerated from

Table 1. Nucleotide sequence of specific primers and PCR conditions used in the detection of *B. cereus*.

Target gene	Primer designation	DNA sequences (5'-3') and PCR conditions	PCR product (bp)
16S rDNA Universal eubacterial	16S rDNA	GAGAGTTTGATCCTGGCTCAG CTACGGCTACCTGTTACGA 94°C 5′; 94°C 1′; 57°C 1′; 72°C 1′; 72°C 10′; (35 cycles)	1468
Phosphatidylinositol Phospholipase C (*pi-plc*) NCBI Acc. No. M30809	Pi-PLC	AGTATGGGGAATGAC ACAATTTTCCCACGA 94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 8′; (35 cycles)	342
Haemolysin BL (*hbl*) NCBI Acc. No. L20441	Ha-1	TGCGAGGTGAAATTCAACAA GAACGCCCGAATATTGAGAA 94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 8′; (35 cycles)	489
Sphingomyelinase (*sph*) NCBI Acc. No. M20194	Sph	CGTGCCGATTTAATT GGGC CAATGTTTTAAACATGGATGCG 94°C 5′; 94°C 20 s; 58°C 20s; 72°C 20s; 72°C 8'; (35 cycles)	558

Table 2. Viable counts and distribution pattern of isolates of *B. cereus* in foods.

Food sample	No of samples analyzed (n)	*Bacillus cereus*			
		Samples positive for *B. cereus*		Viable count (log 10 CFU/G) Mean± SD	Character
		(n)	(%)		
Processed rice based foods added with spices and vegetable salads (Traditional fast foods)	30	05	16.6	5.1 ± 1.3	5/CFRb 1
Milk and milk based foods	10	04	40.0	3.4 ± 0.2	4/CFR 15
Processed wheat based foods	10	01	10.0	4.3 ± 1.5	1/CFR 15
Spiced cooked rice based	15	02	13.0	4.5 ± 0.7	2/CFR 15

from diverse range of foods are summarized in Table 2. The mean viable counts range from a minimum of 3.4 $\log_{10}$ CFU/g in case of milk based foods to a maximum of 5.1 $\log_{10}$ CFU/g in spice based traditional fast foods. It was of relevance to present the incidence pattern of only *B. cereus* cultures against the prevailing background flora of non-*B. cereus* cultures in different types of traditional fast foods. The per cent incidence of *B. cereus* isolates in relation to samples analyzed range from a highest of 40% in milk based foods to a lowest of 10% in samples of processed wheat based foods. M mical character that from a tota belong to *B.* distinct features not shown). Th

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Table 3. Distribution pattern of *B. cereus* isolates for reactions with primers for toxigenic traits in PCR and haemolytic activity by plate assay.

B. cereus isolates[a]	PCR reactions[a]			Haemolytic activity
	Uniplex		Duplex	Discontinuous pattern
	Ha-1	Sph	(Pi-PLC and Ha-1	
CFR 1506	+	-	+	-
CFR 1508	+	-	+	-
CFR 1521	+	-	+	+
CFR 1525	+	-	+	+
CFR 1529	+	+	+	-
CFR 1530	+	+	+	-
CFR 1532	-	+	-	+
CFR 1533	-	-	-	+

(Table 1) designed specifically to detect *B. cereus* isolates (Padmapriya et al., 2004), 12 isolates (46%) of this cluster group gave a positive amplification in PCR.

The focus of present study was to detect food isolates of *B. cereus* (Pi-PLC confirmed), which could harbour potent toxigenic factors. The pattern of characterized *B. cereus* isolates exhibiting positive PCR reactions and haemolytic activity are presented in Table 3. All the 12 isolates showed positive amplification with 16S rDNA and Pi- PLC primers in PCR, which confirmed the species of *B. cereus* cluster. Among these 12 isolates of *B. cereus*, 8 (67%) and 6 (50%) isolates showed positive amplification of haemolysin BL (hbl) and sphingomyelinase (sph) genes, respectively, by uniplex PCR. Besides, the use of duplex PCR with Pi-PLC and Ha-1 primers showed positive amplification in 8 (67%) isolates, which were positive with these respective primers in uniplex PCR (Table 3). A representative pattern of duplex (Pi-PLC and Ha- 1) and uniplex (Sph) PCR in only four isolates of *B. cereus* is shown in Figure 1. Amongst isolates tested for haemolysin BL activity, 67% (8 out of 12 isolates) exhibited discontinuous haemolytic pattern on blood agar.

Molecular basis for relatedness / diversity of food isolates of *B. cereus*

Force type neighbour-joining phylograms in the case of selected native isolates of *B. cereus* CFR 1529, 1530, 1534 and 1536 were generated based on the blast analysis of partial nucleotide sequences of respective PCR amplicons of target genes (16S rDNA, Pi-Plc, hbl and sph), so as to assess the degree of sequence homology. In the background of using partial nucleotide sequences of toxin genes, only the representative phylograms of the four selected isolates in respect of 16S rDNA analysis are shown in Figure 2. The homology between the native isolates of *B. cereus* and other cultures within *B. cereus* cluster in respect of other target genes of this study are highlighted (data not shown).

In the case of 16S rDNA analysis, a high degree of se-

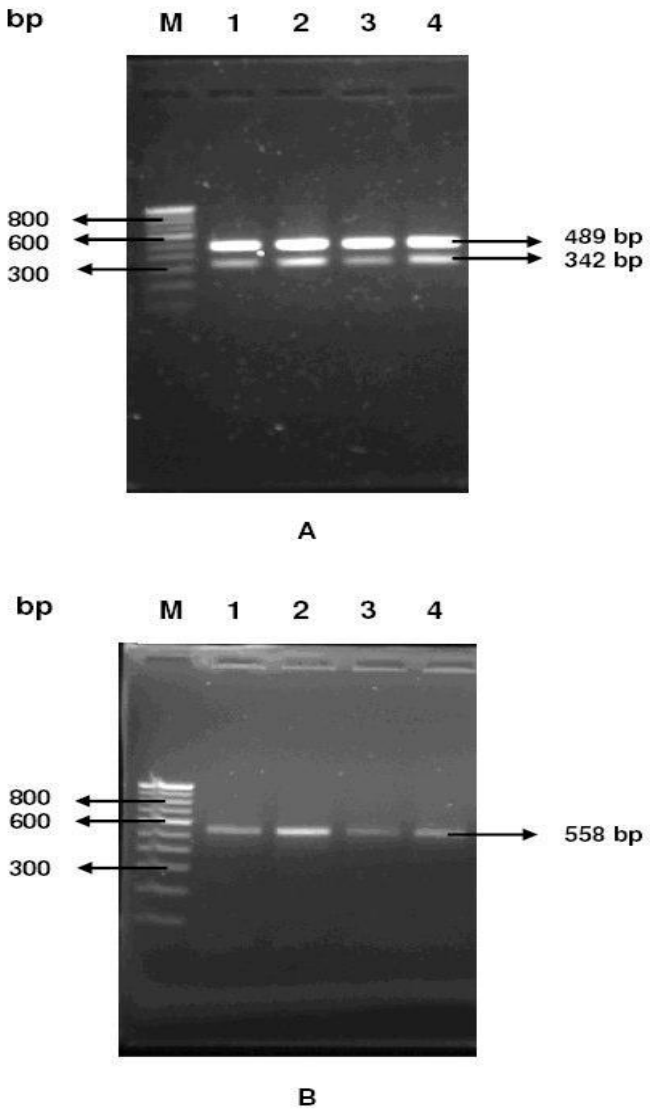

Figure 1. Agarose gel electrophoretic pattern of PCR products showing amplicons with Pi-PLC and Ha- 1 primers in duplex [**A**] and uniplex with Sph primers [**B**] in cultures of *B. cereus*; Lane M, 100 bp marker; Lanes 1 - 4, Native isolates of *B. cereus* CFR 1529, CFR 1530, CFR 1534 and CFR 1536.

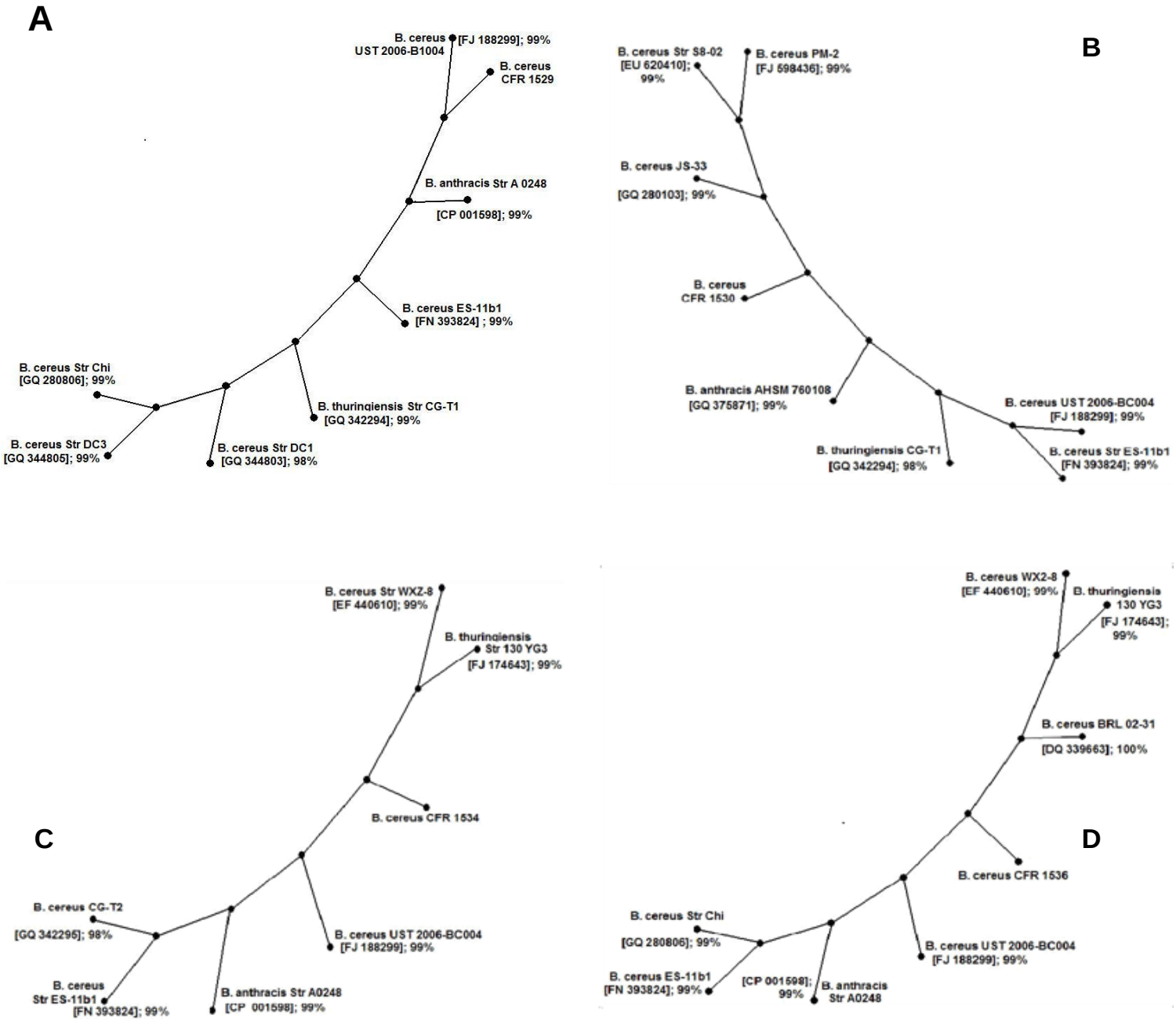

Figure 2. Neighbour-joining phylogram of native isolates of *B. cereus* CFR 1529 [**A**], 1530 [**B**], 1534 [**C**] and 1536 [**D**] based on nucleotide sequence analysis of PCR amplified product with primers of 16S rDNA.

quence homology (99%) was observed with 3 strains of *B. cereus.* Similarly, sequence analysis pi- plc showed a highly related homology (99 - 94%) with strains of *B. cereus* ATCC 14579, E 33L and ATCC 10987. Further, the sequence homology for *B. thuringiensis* and B. anthracis was 97 and 94%, respectively. The sequence analysis of hbl gene revealed a homology of 98 - 95% with strains of *B. cereus* as well as strains of *B. thuringiensis* WS 2734 and B. mycoides WSBC 10256 (95%) used in multiple alignment analysis. In an almost related manner, there existed a slightly lower (93 - 87%) homology in the nucleotide sequences of sph gene between the native isolates of *B. cereus* and 3 strains of *B. cereus* as well as *B. thuringiensis* and *B. anthracis* selected for sequence homology.

DISCUSSION

Potential toxigenic isolates of *B. cereus* in foods

The public health significance of *B. cereus* is of high concern in view of this organism being implicated in large

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number of food poisoning outbreaks, worldwide. In the present study, the prevalence of *B. cereus* recorded in a diverse range of foods was more or less similar to those reported by other researchers (Oguntoyinbo and Oni, 2004; Hanashiro et al., 2005; Murindamombe et al., 2005; Rosenquist et al., 2005; Reyes et al., 2007; Roy et al., 2007; Das et al., 2009). Despite product profile, which could be unfavourable for survival and multiplication of this organism, in the present study, the occurrence of *B. cereus* in milk and rice based foods may be the result of prevailing environmental conditions combined with post processing practices. In general, isolates of *B. cereus* tend to behave as mesophiles. However, changes in environmental conditions could bring in the predominance of psychrotrophic strains of *B. cereus* (Warke et al., 2000; Valero et al., 2007). Although identified *B. cereus* isolates exhibited a diversified pattern in respect of toxigenic traits being harboured by them, it is of importance to observe that all the characterized isolates of *B. cereus* were positive for amplification of Pi-Plc gene. Phospholipase is known to cause degradation of cell and mucous membranes, which are rich in phospholipids leading to necrosis and considered as a virulence factor (Kotiranta et al., 2000). Another virulence factor associated with *B. cereus* isolates was that of sphingomyelinase activity (Beecher and Wong, 2000), which was recorded in 50% isolates by PCR in our study.

Although diarrhoeal food poisoning arising out of *B. cereus* is due to 3 enterotoxins namely, haemolysin BL (hbl), non- haemolytic enterotoxin (nhe) and cytotoxin K (cytK), the major health hazard attributed to *B. cereus* is mostly related with hbl enterotoxin complex (Hsieh et al., 1999). In the present study, nearly 67% isolates of *B. cereus* were positive with Ha- 1 primers in PCR. The percent incidence observed in the present study was almost same as that of 71% recorded among isolates of *B. cereus* occurring in samples of tropical fish, shrimp and clam (Das et al., 2009). In comparison with other earlier studies, the incidence has been higher than those reported between 10 and 40% (Tsen et al., 2000; Radhika et al., 2002). Among the positive isolates, only 50% of them exhibited haemolytic activity (discontinuous pattern), wherein a definite relationship could not be arrived between the presence of hbl gene and haemolytic activity. A similar observation has also been reported in an earlier study, wherein despite the presence of gene, there was the absence of haemolysis, as the primers designed may be highly strain specific (Veld et al., 2001). Often, distinct mutations in plcR can drastically reduce the haemolytic activity in the cultures to a level beyond detection by qualitative methods.

The enterotoxigenicity among isolates of *B. cereus* has been established to the presence of all 3 components namely, a binding component B and two lytic components L_1 and L_2 in hbl complex (Schoeni and Wong, 2005) . In the present study, PCR primers used were targeted for the B component of haemolysin BL gene complex. In this background, it is quite likely that among the PCR positive isolates of B. cereus, there may be absence of either one or both (L_1 and L_2) lytic components of hbl complex. (Hsieh et al., 1999; PrüB et al., 1999; Radhika et al., 2002; Abriouel et al., 2007; Ouoba et al., 2008). Recent studies have shown that the transcription of potential virulence factors (haemolysin, sphingomyelinase and phospholipase C) are under the control of pleiotropic regulator plcR and are expressed simultaneously. There exists a complex interaction among these virulence factors, which may be cooperative, synergistic and antagonistic (Agaisse et al., 1999; Slamti et al., 2004). In the present study, the results of duplex PCR with 2 primer sets does indicate the suitability of an approach used in a very recent study, wherein 8 sets of novel PCR primers were designed to detect the wide distribution pattern of toxigenic genes among isolates of *B. cereus* and group them among 4 clusters based on the presence or absence of specific genes (Ngamwongsatit et al., 2008).

Phylogeny and relatedness among *B. cereus* cluster

Although molecular biology studies of many strains of *B. cereus* cluster are documented and available in public domain, the same is not true with the identity and traceability of culture to its source / origin (habitat and geographical distribution). This aspect is of high importance in microbial diversity and a large number of studies have viewed this lacuna to be of serious concern in conclusively establishing the genetic relatedness and/or diversity among pathogenic cultures. In our study, attempts were made to search for the linkages of sources and habitats of closely related cultures with the four native isolates of *B. cereus* used in comparative evaluation of sequence homology. The phylogram generated and multiple alignment sequences (Data not shown) for the respective target genes - 16S rDNA, pi-plc, hbl and sph revealed high degree of homology of the native isolates with strains of *B. cereus* and other species of *B. cereus* cluster.

In the case of 16S rDNA, all the four native isolates of *B. cereus* exhibited 99% homology with three strains of *B. cereus,* which had different isolation sources and geographical location. In a similar manner, a higher degree of homology in respect of pi-plc was observed for isolates of *B. cereus* CFR 1530, 1534 and 1536 with three strains of *B. cereus* and one strain each of *B. thuringiensis* and *B. anthracis*. These native isolates were obtained from rice and milk based foods and appears to be related with non- lethal strains of *B. cereus* associated with soil, dairy and carcass of zebra. The soil dwelling non-emetic strain of *B. cereus* ATCC 14579 has been studied for its complete genome sequence and identifies genes that are conserved between *B. cereus* and other species in the cluster (Ivanova et al., 2003).

The sequence homology of hbl gene revealed a close relatedness of the four native isolates of *B. cereus* with selected strains of *B. cereus* and species of *B. cereus* cluster. In comparison to the native isolates obtained from food based sources, the related strains of *B. cereus* for hbl gene appear to be predominantly associated with ill health conditions of human being, a strong causative factor for the prevalence of this organism in foods through personnel hygienic practices. This highly diversified genetic relatedness does indicate that the haemolysin toxin trait is not only confined to strains of B. cereus, but also is prevalent among other species of *B. cereus* cluster (PrüB et al., 1999). In an almost parallel manner, sequence homology observed with closely related strains of *B. cereus* reveal that sph gene har-bouring *B. cereus* cultures tend to be linked with animal based habitats. Although one of the closely related strains of *B. cereus* ATCC 10987 is documented as non-lethal dairy isolate, the presence of sph gene does not rule out the potential of such strains to be a cause of risk factor to human health. Considering the complexicity of *B. cereus* cluster, a complete comparative genomic analysis has been established, wherein most of the pathogenic traits have been attributed to the presence of plasmids (Rasko et al., 2005; Ehling-Schulz et al., 2006a; Rasko et al., 2007).

Conclusion

The present study has attempted to establish that potentiality of isolates of B. cereus, generally referred to as opportunistic foodborne pathogen, did harbour toxigenic traits as evidenced by PCR. The findings based on molecular biology approach indicate the stability of these toxigenic traits in isolates of closely related species, irrespective of the isolation sources and geographical boundaries.

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