



Enterotoxigenic profile of *Bacillus cereus* strains isolated from raw and pasteurized milk

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ABSTRACT

The present investigation was undertaken to study the incidence of *Bacillus cereus* in milk, its source of contamination and to detect the enterotoxigenic genes (*hblCDA*, *nheABC*, *cytK* and *entFM*) in isolates by a multiplex-PCR. *B. cereus* could be isolated from 2 (4%) of the 50 raw milk samples taken aseptically directly from the animals, while among 36 raw pooled milk samples from dairy farms and 74 pasteurized milk samples, 12 (33.33%) and 28 (37.83%) turned out positive, respectively. The levels of *B. cereus* in contaminated milk samples ranged from 0.5×10^2 to 2.6×10^5 CFU/ml. The other related *Bacilli* like *B. mycoides* and *B. thuringiensis* were isolated from 10 (6.25%) and 6 (3.75%) milk samples, respectively. The multiplex-PCR of *B. cereus* isolates revealed the incidence of various enterotoxigenic genes *hblD*, *hblA*, *hblC*, *nheA*, *nheB*, *nheC*, *cytK* and *entFM* as 73.80, 69.04, 71.42, 95.23, 90.45, 95.23, 66.7 and 100%, respectively. A higher incidence of *B. cereus* in pasteurized milk and a large proportion (54.76%) of isolates harbouring all the enterotoxigenic genes, most of them being among the isolates from pasteurized milk samples (73.91%) pose a potential public health threat. Moreover, the genes in the operons (*hblCDA* and *nheABC*) can occur independently from each other.

Key words: *Bacillus cereus*, Enterotoxigenic genes, Milk, Multiplex-PCR

Bacillus cereus is ubiquitous in nature and was isolated from infant food (Rowan *et al.* 1996), pasteurized milk (Andersson *et al.* 1995), spices, meat products and dairy products (Te Giffel *et al.* 1997), where it may represent a serious health hazards. Increased number of psychrotolerant strains, especially in the dairy industry has lead to greater surveillance of *B. cereus* (Granum *et al.* 1993, Van Netten *et al.* 1990). *B. cereus* causes early emetic syndrome and a late onset diarrhoeal syndrome (Kramer and Gilbert 1989). The illness associated may be mediated by synergistic effects of one or more enterotoxin (s). The strains of *B. cereus* produced 6 different toxins (Andersson *et al.* 1998) including an emetic toxin (Agata *et al.* 1995b) and at least 5 enterotoxins, namely hemolysin BL (Beecher and MacMillan 1990), non hemolytic enterotoxin (Lindback *et al.* 2004), cytotoxin K (Lund *et al.* 2000) and enterotoxin FM (Asano *et al.* 1997). Enterotoxin T, a protein, was also identified (Agata *et al.* 1995a), however, its enterotoxic activity was questioned by Hansen *et al.* (2003). The illness associated with *B. cereus* is often under

reported because the illness is relatively mild and usually lasts for less than 24 h (Kramer and Gilbert 1989). However, severe forms of illness resulting in deaths were also reported (Lund and Granum 1996, Lund *et al.* 2000).

A very limited data is available pertaining to the occurrence of *B. cereus* in pasteurized and raw milk and also about the enterotoxigenic profile of isolates in India. It is also important to determine the source of *B. cereus* contamination in milk, to take necessary measures to prevent the contamination in milk. There is a serious threat regarding the high incidence of *B. cereus* in pasteurized milk and of various enterotoxigenic genes. Keeping in view the above facts the incidence and enterotoxigenic gene profile of *B. cereus* from raw and pasteurized milk was carried out.

MATERIALS AND METHODS

Sampling: Milk samples (160–86 raw and 74 pasteurized milk samples) were collected from different retail shops and dairy farms (17) of Punjab (India). Accordingly, among 86 raw milk samples, 50 samples were taken aseptically directly from individual animals and the rest 36 were milk samples pooled from cows of one dairy farm (milk canes, storage tanks etc.). Approximately 30 ml of milk sample was collected in sterile vials.

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Isolation and Identification: Milk sample (1 ml) was mixed with 9 ml of buffered peptone water to make a 1 in 10 dilution for the total count of *B. cereus*. 10 Serial dilution was made in 9 ml of buffered peptone water to a dilution of 10^{-3} . From the each dilution, 0.1 ml was surfaced on polymyxin-pyruvate-egg yolk-mannitol-bromothymolblue agar (PEMBA) media and the plates were incubated at 37°C for 24 h (Chitov *et al.* 2008). All the presumptive colonies of *B. cereus* and other related species were collected and subjected to morphological and biochemical tests for identification of species as per Rhodehamel and Harmon (2001). The organisms were identified on the basis of tests like: gram reaction, cell morphology, spore production, motility, nitrate reduction, catalase production, tyrosine decomposition, VP reaction, hemolysis, rhizoid growth, protein-toxin crystal formation, aerobic and anaerobic utilization of glucose and mannitol fermentation.

Enterotoxigenic profile of *B. cereus* and other species of *B. cereus* group

Bacterial strains: Two reference strains of *B. cereus* were used - ATCC 14579 (procured commercially) and NCTC 11143.

DNA template preparation: After biochemical confirmation, the individual colony from PEMBA media was inoculated in 5 ml nutrient broth and incubated in shaker incubator (150 rpm) for 5 h at 32°C. Cells were collected from 1 ml of culture broth by centrifugation at 5000 *g* for 3 min. The cell pellet was washed once with 500 µl of sterile MilliQ water, the pellet was resuspended in 100 µl of sterile MilliQ water, boiled for 10 min and tubes were snap chilled in crushed ice for 20 min. The chilled samples were centrifuged at 6000 *g* for 5 min and the supernatant collected served as template. The concentration and purity of the prepared template was checked spectrophotometrically.

Multiplex PCR protocol: The primers employed (Table 1) in the current study were used as in an earlier study (Ngamwongsatit *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 dNTPs, 5 U *Taq* DNA polymerase and primer concentration ranging from 0.2–0.4 µM. PCR reaction was carried out in thermocycler with initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 45s, 54°C 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%), applying 5–6 volts/cm and sizes were estimated using 100 bp DNA ladder.

RESULTS AND DISCUSSION

All the presumptive isolates showing egg yolk reaction on PEMBA medium were collected and subjected to morphological and biochemical tests for the identification of species. The isolates were confirmed as *B. cereus* when they were gram positive, spore formers, rod shaped, devoid of protein toxin crystals in spores, motile, hemolytic, g were able to reduce nitrate, positive for VP and catalase production utilized citrate and t decomposed tyrosine. The isolates fermented glucose both aerobically and anaerobically and none of the isolates fermented mannitol. There was some variability regarding catalase production and tyrosine decomposition as 2.5 and 3.75% of isolates, respectively, showed negative reaction. Schiemann (1978) had also reported some strains of *B. cereus* negative for catalase production and tyrosine decomposition.

Milk samples screened in this study comprised 86 raw milk samples and 74 pasteurized milk samples. Among the 36 pooled milk samples, 12 (33.33%) were positive for presence of *B. cereus*. On the contrary, among the 50 raw milk samples taken aseptically from individual animals, only

Table 1. Primer sequence of enterotoxigenic genes (*hblCDA*, *nheABC*, *cytK* and *entFM*)

Target gene	Primer	Sequence	Product size (bp)	Primer Conc. (µM)
<i>hblC</i>	FHblC	CCTATCAATACTCTCGCAA	695	0.4
	RHblC	TTTCCTTTGTTATACGCTGC		
<i>hblD</i>	FHD	GAAACAGGGTCTCATATTTT	1018	0.4
	RHD2	CTGCATCTTTATGAATATCA		
<i>hblA</i>	FHblA	GCAAAATCTATGAATGCCTA	884	0.4
	RHblA	GCATCTGTTCGTAATGTTTT		
<i>nheA</i>	F2NheA	TAAGGAGGGGCAAACAGAAG	759	0.2
	RNheA	TGAATGCCAAGAGCTGCTTC		
<i>nheB</i>	F2NheB	CAAGCTCCAGTTCATGCGG	935	0.2
	RNheB	GATCCCATTGTGTACCATTTG		
<i>nheC</i>	FNheC	ACATACCTTTTGCAGCAGAAC	618	0.2
	R2NheC	CCACCAGCAATGACCATATC		
<i>cytK</i>	FCytK	CGACGTCACAAGTTGTAACA	565	0.3
	R2CytK	CGTGTGTAAATACCCAGTT		
<i>entFM</i>	FEntFM	GTTCGTTTCAGGTGCTGGTAC	486	0.2
	REntFM	AGCTGGGCCTGTACGTACTT		

Table 2. Incidence and levels of *B. cereus* in milk samples analyzed

Sample	No. of samples screened	Positive sample	Incidence (%)	Levels of <i>B. cereus</i> (CFU/ml)
Raw milk (udder)	50	2	4	$2.8 \times 10^2 - 5 \times 10^3$
Raw milk (pooled)	36	12	33.33	$1.3 \times 10^3 - 2.6 \times 10^5$
Pasteurized milk	74	28	37.83	$0.5 \times 10^2 - 1.7 \times 10^4$
Total	160	42	26.25	

2 (4%) turned out positive, clearly indicating that environment (utensils, improper milking, soiled teats etc.) rather than the interior of udder act as a main source of *B. cereus* contamination in milk. Presence of *B. cereus* in milk samples taken aseptically may be due to animal suffering from mastitis of *B. cereus* origin (Schiefer *et al.* 1974). Among the 74 pasteurized milk samples 28 (37.83%) turned out positive, Te Giffel *et al.* (1997) and Zhou *et al.* (2008) reported a incidence of 40% and 33%, respectively, in pasteurized milk samples, which is in accordance to the present findings. The incidence is higher than that of pooled raw milk because the *B. cereus* spores survive the pasteurization temperature (Andersson *et al.* 1995), moreover, milk processing lines may act as source of contamination (Lin *et al.* 1998). The results and level of *B. cereus* in each type of milk sample is given in Table 2. Though the levels of *B. cereus* population decreased in pasteurized milk samples the incidence increased, because of survival of *B. cereus* spores in raw milk. Besides, *B. cereus* other species of *Bacilli* were also isolated during the present study. *Bacillus thuringiensis* (identified by the production protein toxin crystals) was isolated from 6 (3.75%) and *Bacillus mycoides* (identified by typical rhizoidal growth) from 10 (6.25%) milk samples.

The 41 isolates of *B. cereus* were screened by multiplex PCR for the presence of eight enterotoxigenic genes. All the eight fragments of enterotoxigenic genes *hblD*, *nheB*, *hblA*, *nheA*, *hblC*, *nheC*, *cytK* and *entFM*, having the predicted size of 1018, 935, 884, 759, 695, 618, 565 and 486 respectively, were amplified in a single tube PCR (Fig. 1). The sizes of different fragments were measured against 100 bp DNA ladder and *B. cereus* ATCC 14579 was used as positive control. According to the presence or absence of enterotoxigenic genes all the isolates (42) were divided into 5 groups (Table 3). The isolates having all the 8

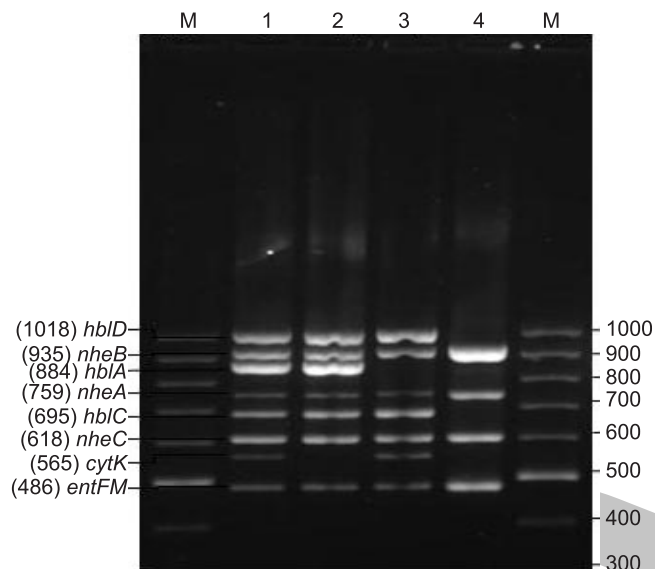


Fig. 1. Agarose gel electrophoresis showing the various enterotoxigenic genes of *Bacillus cereus*. M, presents 100bp DNA ladder; lane 1, *B. cereus* ATCC 14579 strain; L2-L4, present various strains of *B. cereus* isolated from milk. The numbers in parenthesis indicate the size of PCR products and bp indicates base pair.

genes were assigned group 1. Group 2 and group 3 lacked *hbl* complex (*hblCDA*) and *cytK*, respectively. The isolates lacking both *hbl* complex and *cytK* were placed in group 4. The group 5 comprised the isolates which lacked none, 1, 2 or all the 3 genes of *hbl* complex but *cytK*, *entFM* and at least 1 gene of *nhe* complex (*nheABC*) were present.

A varying incidence of enterotoxigenic genes in *B. cereus* was reported by various authors (Yang *et al.* 2005, Ngamwonsatit *et al.* 2008, Chitov *et al.* 2008). The complete *hbl* complex could be detected in a 30 (71.42%) of isolates Casale *et al.* (2008) and Ngamwonsatit *et al.* (2008) detected *hbl* complex in 67.7% and 65.94% of isolates. The *entFM* was detected in all the *B. cereus* isolates, suggesting it as the most common gene as Yang *et al.* (2005), Ngamwonsatit *et al.* (2008) and Casale *et al.* (2008) also detected *entFM* in all the isolates. The complete *nhe* complex was detected in 38 (90.45%) isolates. The results are supported by the findings of Casale *et al.* (2008) and Vyeletelova and Banyko (2008) who detected *nhe* complex in 99.4% and 98% of isolates, respectively. While as Chitov *et al.* (2008) detected *nhe* complex in 45.6% of isolates, which is significantly less than

Table 3. Distribution of virulence groups in *B. cereus*

Source of Isolates	No. of isolates	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Raw milk (udder)	2	0	1 (50%)	0	0	0	1 (50%)
Raw milk (pooled)	12	6 (50%)	0	2 (16.67%)	2 (16.67%)	2 (16.67%)	0
Pasteurized milk	28	17 (60.71%)	2 (7.14%)	4 (14.28%)	4 (14.28%)	0	1 (3.57%)
Total	42	23 (54.76%)	3 (7.14%)	6 (14.28%)	6 (14.28%)	2 (4.76%)	2 (4.76%)

Table 4. Occurrence of enterotoxigenic genes in *Bacillus cereus* and other related species

Isolate	No. of isolates	<i>hblD</i>	<i>hblA</i>	<i>hblC</i>	<i>nheA</i>	<i>nheB</i>	<i>nheC</i>	<i>cytK</i>	<i>entFM</i>
<i>B. cereus</i>	42	31 (73.80)	29 (69.04)	30 (71.42)	40 (95.23)	38 (90.45)	40 (95.23)	28 (66.7)	42 (100)
<i>B. thuringiensis</i>	6	4 (66.67)	4 (66.67)	4 (66.67)	6 (100)	4 (66.67)	6 (100)	4 (66.67)	6 (100)
<i>B. mycoides</i>	10	6 (60)	4 (40)	6 (60)	8 (80)	8 (80)	6 (60)	6 (60)	8 (80)

The numbers in parenthesis indicate the incidence (%) of various enterotoxigenic genes.

the present study. In the present study *cytK* was detected in 66.7% of isolates, while as Ngamwongsatit *et al.* (2008) reported a higher incidence (88.88%). The varying incidence reported by different authors, clearly indicate that distribution of enterotoxigenic genes vary among the strains from different geographical locations.

As the members of *B. cereus* group (*B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. mycoides*) have 99% of DNA homology (Ash *et al.* 1991, Jensen *et al.* 2003). The incidence of enterotoxigenic genes *hblCDA*, *nheABC*, *cytK* and *entFM* was also studied in the isolated species (*B. thuringiensis* and *B. mycoides*). All the *B. thuringiensis* isolates showed *entFM* and at least one gene of *nhe* complex, while some isolates of *B. mycoides* showed absence of *entFM*. A relatively higher incidence was observed in *B. thuringiensis* compared to *B. mycoides* (Table 4).

The overall incidence of various enterotoxigenic genes: *hblD*, *hblA*, *hblC*, *nheA*, *nheB*, *nheC*, *cytK* and *entFM* in *B. cereus* were 73.80%, 69.04%, 71.42%, 95.23%, 90.45%, 95.23%, 66.7% and 100%, respectively (Table 4). The incidence is higher than reported by Chitov *et al.* (2008), which could be due to difference in the genotype of isolates in different geographical locations. Besides a relatively higher incidence was detected in isolates of pasteurized milk as compared to isolates from raw milk, indicating adaptation of more pathogenic strains in processing lines and hence contamination of milk during processing.

So in order to control the contamination of milk we must follow the hygienic practices right from primary production i.e. the udder should be properly cleaned, milk should be collected hygienically, utensils and all other equipments should be clean. Only by reducing the contamination in raw milk, we can reduce the bacterial load of pasteurized milk. Moreover, in present scenario in India the incentives are given on the basis of fat and SNF, neglecting the microbiological quality. The measures should be taken to include microbiological standards only then the farmers will give emphasis on hygienic production of milk. There was a higher incidence of enterotoxigenic genes in pasteurized milk than in raw milk, which indicates that contamination may have occurred during processing or post-pasteurization during packaging of milk. So the processing lines should be sanitized on regular basis and packaging should be carried out properly to render consumption of safe milk for human consumption.

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REFERENCES

- Agata N, Ohta M, Arakawa Y and Mori M. 1995a. The *bceT* gene of *Bacillus cereus* encodes an enterotoxin protein. *Microbiology* **141**: 983–88.
- Agata N, Ohta M, Mori M and Isobe M. 1995b. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiological Letters* **129**: 17–20.
- Andersson A U, Granum P E and Ronner P. 1995. What problems does the food industry have with the spore forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *International Journal of Food Microbiology* **28**: 145–56.
- Andersson M A, Mikkola R, Helin J, Anderesson M C and Salkinoja S M. 1998. A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin related depsipeptide ionophores. *Applied and Environmental Microbiology* **64**: 1338–43.
- Asano S I, Nukumizu Y, Bando H, Iizuka T and Yamamoto T. 1997. Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **63**: 1054–57.
- Ash C, Farrow J A, Dorsch M, Steckebbrandt E and Collins M D. 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus* and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Journal of Systemic Bacteriology* **41**: 343–46.
- Beecher D J and Mac Millan J D. 1990. A novel bicomponent hemolysin from *Bacillus cereus*. *Infection and Immunity* **58**: 2220–27.
- Casale A A L, Gabriela P, Volzgraciela L, Buccelli R V, Maiza L and Teresa D M. 2008. Enterotoxigenic and genetic profiles of *Bacillus cereus* strains of food origin in Brazil. *Journal of Food Protection* **71**: 2115–18.
- Chitov T, Dispan R and Kasinrer W. 2008. Incidence and diarrhegenic potential of *Bacillus cereus* in pasteurized milk and cereal products in Thailand. *Journal of Food Safety* **28**: 467–81.
- Granum P E, Brynestad S and Kramer J M. 1993. Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. *International Journal of Food Microbiology* **77**: 269–79.
- Hansen B M, Høiby P E, Jensen G B and Hendriksen N B. 2003.

- The *Bacillus cereus* *bceT* enterotoxin sequence reappraised. *FEMS Microbiological Letters* **223**: 21–24.
- Jensen G B, Hansen B M, Eilenberg J and Mahillon J. 2003. The hidden lifestyles of *Bacillus cereus* and other relatives. *Environmental Microbiology* **5**: 631–40.
- Kramer J M and Gilbert R J. 1989. *Bacillus cereus* and other *Bacillus* species. *Foodborne Bacterial Pathogens*. Pp 27–70. (Ed.) Doyle M P. Marcel Dekker Inc., New York.
- Lin S, Scraft H, Odumeru J A and Griffiths M W. 1998. Identification of contamination sources of *Bacillus cereus* in pasteurized milk. *International Journal of Food Microbiology* **43**: 159–71.
- Lindback T, Fagerlund A, Roland M S and Granum P E. 2004. Characterization of *Bacillus cereus* non-hemolytic enterotoxin (NHE). *Microbiology* **150**: 3959–67.
- Lund T and Granum P E. 1996. Characterisation of a non-hemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiological Letters* **141**: 151–56.
- Lund T, Debuyser M L and Granum P E. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology* **38**: 254–61.
- Ngamwongsatit P, Buasri W, Pianariyanon P, Pulsrikarn C, Ohba M, Assavanig A and Panbangred W. 2008. Broad distribution of enterotoxins genes (*hblCDA*, *nheABC*, *cytK* and *entFm*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel Primers. *International Journal of Food Microbiology* **121**: 352–56.
- Rhodehamel E J and Harmon S M. 2001. *Bacillus cereus*. *Bacteriological Analytical Manual*. (Eds) Jackson G J, Merker R I and Bandler R. Centre for Food Safety and Applied Nutrition, U S Food and Drug Administration, College Park, MD. (Retrieved from: <http://www.cfsan.fda.gov/~ebam-14.html>.)
- Rowan N J, Anderson J G and Anderton A. 1996. The bacteriological quality of hospital-prepared infant food. *Journal of Hospital Infections* **39**: 259–67.
- Schiefer B, Macdonald K R, Klavano G G and Van Dreumel A A. 1976. Pathology of *Bacillus cereus* mastitis in dairy cows. *Canadian Veterinary Journal* **17**: 239–43.
- Schiemann D A. 1978. Occurrence of *Bacillus cereus* and the bacteriological quality of Chinese 'Take out' foods. *Journal of Food Protection* **41**: 450–54.
- Te Giffel M C, Beumer R R, Granum P E and Rombouts F M. 1997. Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in the Netherlands. *International Journal of Food Microbiology* **34**: 307–18.
- Van Netten P, Van De Moosdijk A, Van Hoensei, Mossel D A A and Perles I. 1990. Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* **69**: 73–79.
- Vyletelova V and Banyko J. 2008. Detection of *HBL* and *NHE* enterotoxin genes in *Bacillus cereus* using multiplex PCR. *12th International Symposium on Microbial Ecology (ISME12)*. Cairns, Australia.
- Yang C I, Shih D Y C, Huang T P, Huang Y P, Wang J Y and Pan T M. 2005. Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. *Journal of Food Protection* **68**: 2123–30.
- Zhou G, Liu H, He J, Yuan Y and Yuan Z. 2008. The occurrence of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* in Chinese pasteurized full fat milk. *International Journal of Food Microbiology* **121**: 95–200.