DETECTION OF TOXIGENIC STRAINS OF *Bacillus cereus* IN MILK AND DAIRY PRODUCTS THROUGH POLYMERASE CHAIN REACTION

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**ABSTRACT**

Fifty nine samples of raw milk, 60 samples of pasteurized milk, 24 cream, 39 butter, 27 curd, 19 peda, 21 paneer and 20 skim milk powder samples were analyzed for the presence of different *Bacillus* species and a total of 182 isolates were obtained. The isolates were identified as *Bacillus subtilis* (51), *Bacillus megaterium* (39), *Bacillus cereus* (28), *Bacillus licheniformis* (32), *Bacillus coagulans* (7), *Bacillus sphaericus* (8), *Bacillus pumilus* (9) and *Bacillus circulans* (8). All the 28 *Bacillus cereus* isolates obtained in the study were subjected to polymerase chain reaction amplification for the presence of 365 nucleotides and all the isolates formed a distinct PCR product of 365 bp, which confirmed that these isolates were *Bacillus cereus*. Only 2 out of 28 *Bacillus cereus* isolates were found to be enterotoxigenic. One isolate harboured all the five genes of B, B', L1, L2 and ET of the HBL-ET complex whereas the other isolate contained B, L1, L2 and ET genes and the B' component was missing. Both the toxigenic strains of *Bacillus cereus* produced similar discontinuous haemolysis pattern on blood agar and cytotoxic effect on Vero cell line.

**Key words:** *Bacillus cereus*, Dairy products, Milk, PCR, Toxigenic

**INTRODUCTION**

The genus *Bacillus* is widely distributed in the environment and present in many food products; it constitutes a significant proportion of microbial flora of raw milk, easily contaminate various dairy products, causing spoilage because of their proteolytic and lipolytic activity. These organisms survive heat treatment and high temperature used for processing of the products, activate spore germination and outgrow resulting in spoilage of the products.

However, strains that are capable multiplying below 7°C and above 45°C are not the most common. Generally, spores can germinate and multiply in humid, low acid foods, from 4°C to 55°C. Most of the reported outbreaks of *Bacillus cereus* poisoning are related to consumption of heat treated foods, mainly cooked dishes containing pasta and rice. However, milk and dairy products do act as carrier for these organisms.

Certain strains of *Bacillus cereus* are capable of producing a large array of potentially pathogenic substances including four haemolysins, three different types of phospholipase C, the emetic toxin (cereulide) and at least four enterotoxins. The role of haemolysin BL (Hbl) and of the non-haemolytic enterotoxin (Nhe) in diarrhoeal outbreaks has been elaborately studied. (Stenfors-Arnesen et al., 2008). The genes coding for Nhe, unlike those coding for Hbl, are present in most, if not all strains of *Bacillus cereus* (Guinebretiere et al., 2010).

**MATERIALS AND METHODS**

**Collection of samples:** Fifty nine raw milk, 60 pasteurized milk, 24 cream, 39 butter, 27 curd, 19 peda, 21 paneer and 20 skim milk powder samples were collected aseptically from the farm, processing dairies, local vendors and markets and brought to laboratory for further analysis. Samples of raw milk were also collected from the research farm in the

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**Bacterial strains used in the study:** Bacillus cereus MTCC 1272 obtained from Institute of Microbial Technology, Chandigarh was used as a reference strain for enterotoxigenic studies, which was found to contain all the genes for haemolysin BL enterotoxin complex. The Bacillus species isolated from different milk and milk products were used to study all the parameters.

**Morphological and cultural characteristics of organisms:** Morphological, cultural and biochemical tests were carried out to identify the isolates as per the Buchanan and Gibbons (1994) and the isolates were assigned serial numbers.

**Polymerase chain reaction (PCR)**

**DNA isolation by high salt method:** Two ml of test culture was taken into a 2 ml eppendorf tube, washed (5000 rpm, 10 min) with phosphate buffered saline (PBS). The supernatant was discarded and the pellet thus obtained was washed twice with TKM-1 (Tris – Potassium chloride – Magnesium chloride) solution after centrifugation at 10,000 rpm for 10 min as described by Maniatis et al. (1982).

**PCR protocol for the detection of Bacillus cereus:** PCR assay was performed in a DNA thermal cycler (Perkin-Elmer, US). Reaction volumes of 100 µl contained 100 ng of genomic DNA, deoxyribonucleoside triphosphates at a concentration of 200 µM each and primers at 1µM each in the reaction buffer (TRIS-HCl, 100mM; MgCl₂, 15mM; KCl, 500mM; pH 8.3) as described by Yamada et al. (1999) were used. The PCR reaction conditions were 30 cycles each consisting of 1 min at 94°C, 1.5 min at 58°C and 2.5 min at 72°C, with a final extension step at 72°C for 7 min.

**Agarose gel electrophoresis:** Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis unit. Two percent agarose gel in TBE buffer containing ethidium bromide (0.5 – 1.0 mg) was used for electrophoresis. After the mixture of PCR product with the tracking dye, each sample was loaded on to the gel. Electrophoresis was carried out at 90V at room temperature for 30 min to 1 h depending upon the length of the gel or till the bromophenol blue migrated more than the half the length of the gel and the gel was visualized using ultraviolet light. 100 kb ladder was used as molecular weight marker.

**Haemolysis of blood agar:** The method as described by Beecher and Wong (1994) was followed to detect the discontinuous haemolysis pattern produced by Bacillus cereus isolates.

**Culturing of Bacillus cereus for enterotoxin assays:** Bacillus cereus cultures were inoculated into 10 ml of brain heart infusion with 0.1% glucose (BHIG). The broths were then placed in a rotary shaker at 30°C, 150 rpm for 24 h. Following incubation, 100 µl of this broth was used to inoculate a fresh tube of BHIG. The culture was then incubated in a rotary shaker at 30°C, 150 rpm for 24 h. Following incubation, the sample was centrifuged at 5000 g for 5 min. The supernatant was then filtered through a 220 nm syringe filter (Millipore, USA) and added to a fresh tube.

**Vero cell cytotoxicity assay:** Vero cells were maintained in DMEM with 10% foetal bovine serum at 37°C. Cells were treated with antibiotic Trypsin Versene and diluted to 10⁶ cells per ml in normal growth medium. Falcon 96-well flat bottom cell-culture plates were seeded with 100 µl of this dilution. Plates were incubated for approximately 24 h or until the cells formed a confluent monolayer as described by Buchanan and Schultz, (1994).

The culture supernatant of B.cereus was added (100 µl / well) and a twofold serial dilution was carried out across the plate. The plates were then incubated at 37°C for 18 h. Following incubation, plates were examined under a light microscope for damage to the Vero cells. Reactions were considered positive if more than 50% of Vero cells had detached from the plate.

**Statistical analysis:** The data were subjected to statistical analysis as per Snedecor and Cochran (1989).
**RESULTS AND DISCUSSION**

**Detection of Bacillus cereus isolates using PCR:** The gyrB genes that encode the subunit B protein of DNA gyrase (topoisomerase type II) as targets of highly specific probes was used to distinguish the Bacillus cereus from other closely related Bacillus species. The 28 Bacillus cereus isolates obtained in the study were subjected to polymerase chain reaction amplification for the presence of 365 nucleotides and all the isolates formed a distinct PCR product of 365 bp. The results are presented in Plate 1 and Table 1. The findings of the present study agreed with the results of Yamada et al. (1999) who in their effort to design a specific primer for different Bacillus species obtained a PCR product of 365 bp for Bacillus cereus.

**Detection of enterotoxigenic strains of Bacillus cereus using PCR:** Of the 28 isolates of Bacillus cereus, only two were found to be harbouring genes responsible for enterotoxin production (HBL-ET group). The amplification of the bands is presented in Plate 2. The source of one Bacillus cereus isolate was from butter obtained from local vendor. The isolate contained 4 genes of B, L1, L2 and ET of the HBL-ET complex in which, the amplicon for the B’ component was missing. The other toxigenic isolate was from peda sourced from local vendor; harboured all the five genes of B, B’, L1, L2 and ET of the HBL-ET complex. This finding is in agreement with that of Veld et al. (2001) who detected all three genes (B, L1, and L2) for the HBL enterotoxin complex in 55% of the 86 strains tested. They also detected the enterotoxin-T gene in 62% of the strains.

However, Svensson et al. (2007) found that out of total 156 isolates, less than 3 per cent of them were positive for harboring NheA enterotoxin component. Rowan et al. (2003) concluded that all five B. cereus strains tested revealed the presence of DNA sequences encoding hemolysin BL (HBL) enterotoxin complex and B. cereus enterotoxin T (BceT). Torkar and Mozina (2001) found that 96% of B. cereus produced nonhaemolytic diarrhoeal enterotoxin and 78% of the strains produced both enterotoxin types.

### Table 1: PCR amplification of species specific and enterotoxigenic genes of Bacillus cereus

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primers</th>
<th>Primers Sequence</th>
<th>Product Size</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Species specific Bacillus cereus primer</strong></td>
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<tr>
<td>gyrB</td>
<td>BC1</td>
<td>ATTTGTTGACACCGATCAAACA</td>
<td>365</td>
<td>Yamada et al. (1999)</td>
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<tr>
<td></td>
<td>BC2r</td>
<td>TCATACGTATGGATGTTATTC</td>
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<td>232</td>
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<td></td>
<td></td>
<td></td>
<td>810</td>
<td>Veld et al. (2001)</td>
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<td></td>
<td></td>
<td>977</td>
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<td>428</td>
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</table>

**Entero-**

Agarose gel showing species specific amplification of 365 bp fragment of Bacillus cereus in Lane 1 to 6; Lane 7 negative control Lane 8 Marker Gene Ruler 100 bp.
PLATE 2: Detection of enterotoxigenic Bacillus cereus by polymerase chain reaction.

Haemolysis of blood agar: The production of discontinuous haemolysis pattern on sheep blood agar is characteristic of haemolysin BL produced by enterotoxigenic strains of Bacillus cereus. Both the toxigenic strains of Bacillus cereus produced similar discontinuous haemolysis pattern on blood agar. Fermanian and Wong (2000) found that 74 to 78 percent of the total Bacillus strains tested were found to be positive for discontinuous haemolysis. Torkar and Mozina (2001) concluded that 80% of 82 B. cereus strains produced haemolysis and Radhika et al. (2002) found that 11 out of 29 isolates were haemolytic.

Cell cytotoxicity assay: The two Bacillus cereus isolates positive for enterotoxin production were used to study their toxin producing capabilities on Vero cell line. Both the toxigenic strains produced similar cytotoxic effect on Vero cell mono layer. Christiansson et al. (1989) found that ninety four strains of Bacillus cereus produced HEL cytotoxicity out of 136 strains tested. 26% of the 37 Bacillus cereus isolates were found to be positive for enterotoxin production by Vero cell assay by Giffel et al. (1996). In this study, 7.14% of the Bacillus cereus tested showed cytotoxic changes on Vero cell line.

CONCLUSION

The aerobic spore formers, especially the Bacillus species are commonly found in milking barns, bedding materials and dry fodders like hay and straws. However, they gain entry into milk and subsequently into milk products due to inadequate hygienic practices being followed during dairy cattle management, milking, milk collection, transportation, storage and value addition. The widespread distribution of Bacillus cereus and the ability of the spores to survive long-term storage in dried products, and the thermal resistance of spores help to explain the wide variety of foods that have been implicated in B. cereus foodborne illness outbreaks.

Although most of the Bacillus species are spoilage causing in nature, some of them are found to be toxigenic and pose danger to consumers. The present study revealed the presence of two enterotoxigenic strains of Bacillus cereus sourced from butter and peda samples. Although butter is not very much prone to contamination by aerobic spore formers, presence of toxigenic strain of Bacillus cereus indicate their ubiquitous presence in the dairy environment and their resistant nature. Control measures include keeping the holding time of heat treated dairy product between preparation and consumption to a minimum and if the dairy product has to be stored, it has to be rapidly cooled to prevent multiplication of the organism. The results of the present study highlight the importance of producing milk under hygienic conditions till it reaches the consumers.

REFERENCES


