Characterization of *Bacillus cereus* Isolates from Raw Milk
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**ABSTRACT**

*Bacillus cereus* is food spoilage and an emerging foodborne pathogen. It can produce several types of defects in milk e.g. bitty cream, sweet curdling etc. The present investigation was undertaken to study the presence and contamination level of *B. cereus* in raw milk samples obtained from instructional dairy farm, Pantnagar, Uttarakhand, India. *B. cereus* was isolated from 17 (11.3%) of the 150 raw milk samples analyzed. All the *B. cereus* isolates were strongly β-hemolytic and motile. *B. cereus* count in raw milk ranged from $2.2 \times 10^3$ to $7.9 \times 10^5$ cfu/ml. All isolates showed hemolytic action which indicates their pathogenic nature. It is thus suggested that consumption of such type of contaminated milk without proper treatment can pose a potential public health threat.

**Keywords:** *Bacillus cereus*, contamination, gyrB gene, raw milk

*Bacillus cereus* is Gram-positive, rod shaped, endospore-forming, facultative anaerobic organism of genus *Bacillus*. There are several closely related *Bacillus* species share a significant genetic similarity to *B. cereus*, thus are included in a heterogenous “*B. cereus* group” which consists of at least four species viz., *B. cereus, B. anthracis, B. thuringiensis* and *B. mycoides* (Park et al., 2007). *B. cereus* is a widespread foodborne pathogen because of the resistance of its spores to adverse environmental conditions. *B. cereus* can be detected in a variety of raw milk and milk products especially in vegetative form, which exposed directly in contact with the soil (Te Giffel and Beumer, 1998; Scheldeman et al., 2005). They can be introduced into milk from various sources during production, handling and storage. On dairy farms the major contamination sources are soil, straw and other fodder. Management practices like used bedding probably also participates in this contamination route when cows are housed in winter. The udder will be contaminated, finally resulting in the presence of *B. cereus* in raw milk (Bennett and Belay, 2001).

*B. cereus* is an important cause of food-borne illness in humans and is frequently involved in emetic or diarrheal type of food poisoning induced by an emetic toxin and enterotoxin, respectively (Hall et al., 2001; Kramer and Gilbert, 1989). Other toxins are also produced during growth, including phospholipases, proteases and cereolysin. These toxins may contribute to the pathogenicity of *B. cereus* in nongastrointestinal disease (Drobniewski, 1993).

Differentiation of members of genus *Bacillus* is difficult because *Bacillus* species cluster together within a very tight clade phylogenetically and are
indistinguishable from one another (Subramanian et al., 2006). Use of gyrB gene of B. cereus encoding the subunit B protein of DNA gyrase as specific probe targets for differentiation of B. cereus from closely related species and proved more differential than 16S rDNA sequence analysis (Park et al., 2007; La Duc et al., 2004). The objectives of this study were to determine the incidence and level of B. cereus contamination in raw milk and their biochemical and molecular characterization.

A total of 150 raw milk samples were collected from instructional dairy farm, Pantnagar, Uttarakhand. Samples were directly taken from the udder of dairy cows at the time of milking in sterilized test tubes and aseptically brought to the laboratory immediately maintaining the proper cold chain.

Samples were processed for isolation and enumeration of B. cereus as per the methodology of Rhodehamel and Harmon (1998). Serial dilutions (1:10) of milk with peptone water was prepared and 0.1 ml of each diluted sample was inoculated and spread over agar plates. Plates were incubated for 24 h at 30°C. The typical eosin pink colonies on mannitol egg yolk polymyxin agar (MYP), surrounded by precipitate zone indicating lecithinase production were presumptively identified to be B. cereus. All the presumptive colonies of B. cereus were subjected to morphological and biochemical tests.

One typical colony from each of the isolates was picked and inoculated in 5 ml of brain heart infusion (BHI) broth and incubated overnight at 35°C. The broth culture was then subjected for DNA extraction using the Hi-Pura Bacterial and yeast genomic DNA purification kit (Hi-Media, Mumbai) as per the instructions of manufacturer. The DNA samples were diluted to a concentration of 50 ng/µl prior to its amplification.

The primers (Forward: BCJH - 5'TCATGAAGAGCCTGTGTACG3'; Reverse: BCJH - 5'CGACGTGTCATTCAAGCCG3') for detection of gyrB gene (encoding the subunit B protein of DNA gyrase) for differentiation and confirmation of B. cereus used in this study were got synthesized from M/S Aldrich, USA. PCR was performed for molecular characterization of B. cereus by using reaction condition as described by Park et al. (2007) with suitable modifications.

Out of 150 raw milk samples, 17 samples (11.3%) were found positive for B. cereus. Similar findings were also reported by previous workers (Ziemann and Schutz, 1992; Odumeru et al., 1997; Ahmed et al., 1983). On the contrary, a higher percentage of B. cereus (37%) in raw milk was reported by Martin et al. (1962). In India, Bedi et al. (2005) found 66% contamination level in raw milk with more than 10^5 cfu/g counts of B. cereus in 16.6% samples. Low incidence rate reported in the current study indicates lower dust load in milking yard, where samples were collected. B. cereus contamination level in positive milk samples ranged from 2.2 x 10^3 to 7.9 x 10^5 cfu/ml in present study (Table 1). It supports the fact that milk is a very suitable media for the growth and multiplication of bacteria and within a short period of time the bacterial load may reach to dangerous levels. It indicates that post milk collection, storage and processing is a very critical from hygiene and safety point of view (Berthold, 2007).

Resistance to 0.001% lysozyme and PRDB test were positive for all isolates. All B. cereus bacteria produced β-hemolysis on 5% sheep blood agar. It indicates that all strains produced hemolysin toxin which is the virulence factor of bacteria and possibly having capacity to produce diarrheal syndrome and necrotic activities (Beecher and Wong, 1994). Almost similar reporting was done by Schiemann (1978) who reported that more than 96% isolates of B. cereus showed beta-hemolysis on blood agar.
Table 1. Cultural and biochemical characteristics of *B. cereus* isolates of raw milk origin

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Colony forming unit/ml</th>
<th>Motility</th>
<th>β-Hemolysis</th>
<th>PRDB</th>
<th>Biochemical tests</th>
<th>Modified VP test</th>
<th>Nitrate reduction</th>
<th>Lysozyme resistance</th>
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<td>1</td>
<td>2.92 x 10³</td>
<td>+</td>
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¹positive – change in color of broth from red to yellow; ᵃpositive- development of orange color; ᵃpositive - development of pink color; ᵃpositive- development of turbidity in broth

All isolates in the present study showed motility, resistance to 0.001% lysozyme and produced positive reaction for PRDB test, while modified VP and nitrate reduction test was found positive in 12 and 16 isolates respectively (Table 1). Out of 17 isolates, 15 (88%) and 10 (58.8%) showed positive reaction for nitrate and modified VP test, respectively, even after 48 h incubation. In contrast to this, higher positive VP reaction of 91% (Priest *et al*., 2004) and 100% (Wong *et al*., 1988) was reported to be produced by *B. cereus* isolates. Haque and Russell (2004) also found that all *B. cereus* isolates were VP negative and reduced nitrate, while Chopra *et al*. (1980) observed 87% isolates reducing nitrate which was similar to the present study, but higher level of isolates produced positive VP test as compared to present study.

All the biochemically positive isolates were subjected to PCR targeting *gyrB* gene by using species-specific primers. All isolates produced PCR product of 475 bp on agarose gel electrophoresis (Fig. 1.), which was specific to *B. cereus*. So the use of PCR targeting *gyrB* gene proved to be rapid and efficient for identification and confirmation of *B. cereus* in the present study.

Fig. 1. Molecular characterization of *B. cereus* by using *gyr B* gene (475 bp). L1- Positive control of *B. cereus*; L2, L3- *B. cereus* isolates of milk origin; L4 - Negative control; M - Marker (100 bp DNA ladder)
The occurrence of hemolytic phenomenon on blood agar suggests the pathogenic nature of the all recovered isolates. Moreover, an incidence rate of 11% with medium to high level of *B. cereus* in raw milk in dairy farms can pose a serious public health threat. It clearly indicates that proper hygienic measures before and after collection of milk should be employed to reduce the contamination of milk.

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References


