Toxigenic Gene Profiling of *Bacillus cereus* in Traditional Fermented Foods of Lateritic West Bengal

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Submitted by

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CERTIFICATE

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Declaration

I do hereby declare that the present Master thesis entitled **'Toxicogenic Gene Profiling of *Bacillus cereus* in Traditional Fermented Foods of Lateritic West Bengal'** embodies the original research work carried out by me in the Department of Biological Sciences, Midnapore City College, Paschim Medinipur, West Bengal, India under the supervision of 'Dr. Kuntal Ghosh' Assistant Professor and Teacher-in-Charge, Department of Biological Sciences, Midnapore City College at Kuturiya, Bhadutala, 721129, Paschim Medinipur, West Bengal, India.

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Abstract

Foodborne illness has become a major public health problem in both developed and developing countries. In India, traditional fermented foods and beverages (*Haria, Idli,* and *Curd*) are being produced in the household and small cottage industries where the qualities of the products are not checked properly. In this study, we found 66.8% of traditional fermented foods of West Bengal were contaminated by *B. cereus* and 33.2% samples exceeded the regulatory limit (10^4 CFU/g). For the first time, heat-resistant spore contents were measured and detected in all the above-mentioned tainted samples ranging from 61.1% to 72.1%. A total of 50 *B. cereus* strains were isolated and categorized into 15 different patterns based on the toxin-related genes. Most prevalent toxin was *entFM* (100%), followed by *nheB* (87.4%), *nheC* (86.6%), *nheA* (82.7%), *hblA* (50.4%), *hblD* (49.6%), *hblC* (47.2%), *bceT* (14.2%), and *EM* (3.9%) using multiplex PCR. Most of the strains were highly susceptible to erythromycin (86.6%), tetracycline (75.6%) and resistant to ampicillin (70.9%), spectinomycin (70.1%), and chloramphenicol (57.5%). Clearly, the high contamination level of this pathogen in traditional fermented foods of West Bengal is a matter of concern and requires constant attention.

Keywords: Fermented food, toxigenic gene, antibiotics, *Bacillus cereus*, prevalence

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Chapter 1. Introduction

1. Introduction

Food fermentation is one of the oldest and most economical methods for preserving food materials. Fermented foods constitute a substantial part of the diet in worldwide (Steinkraus, 1995) and are estimated to provide 20-40% of the energy of an individual (Campbell-Platt, 1994). However, most of the traditional fermented foods in India are being prepared in the household and small cottage industries where the qualities of the products are not being evaluated. Therefore, the incidences of food borne illnesses have increased globally, and it becomes more important in developing countries where food products are exposed to contaminated environments during food processing and temperature abuse during transportation and storage at retail outlets (WHO, 2015). In India, majority of outbreaks of food borne disease are unreported, unrecognized or un-investigated and may only be noticed after major health or economic damage. The reported bacterial food borne disease outbreaks in India during 1980–2009 indicated that 24 outbreaks have occurred involving 1,130 persons (Tewari & Abdullah, 2015).

Among the food borne pathogens, *Bacillus cereus* is one of the pathogens. *B. cereus* is a gram-positive, rod-shaped, spore-forming bacterium that is ubiquitous in natural environment. This bacterium is the causative agent of diarrheal and emetic food poisoning associated with the food products (EFSA, 2005). Diarrheal syndrome is caused by the production of enterotoxins including hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE), single protein enterotoxins cytotoxin K (CytK), and enterotoxin FM (EntFM) (Schoeni & Wong, 2005). The Nhe complex is composed of NheA, NheB, and NheC proteins,which are encoded by nheA, nheB, nheC (Yim et al., 2015). The emetic foodborne illness is induced by a small heat and acid-stable cyclic dodecadepsipeptide toxin cereulide (Agata et al., 1994). The onset of diarrheal syndrome is within 8-24 h after consumption of food, and includes abdominal pain, cramps, and diarrhea, whereas, emetic type of illness is characterized by vomiting and nausea, which appear about 1-5 h after the consumption of food containing toxin cereulide (Ceuppens et al., 2011). Several studies already suggested that the prevalence of *B. cereus* in Indian foods were 15% to 80% with a contamination level as high as 10^8 CFU/g or CFU/ml (Bachhil & Negi, 1984; Bedi et al., 2005; Chopra et al., 1980; Das et al., 2009; Kumari & Kalimuddin, 2004; Kumari & Sarkar, 2014; Willayat et al., 2007; Yadava, 2004). The high prevalence of *B.*

cereus is a matter of concern from the point of public health. However, most of the above-mentioned studies did not focus on the fermented foods. Hence, the prevalence and contamination level of *B. cereus* in the Indian fermented foods are almost unknown. Moreover, the toxigenic gene profiling of *B. cereus* isolates has rarely studied in India. Clearly, there is a need of characterization of *B. cereus* isolates.

Chapter 2. Literature Review

2. Literature of Review

2.1. International Status:

B. cereus is one of the foodborne pathogens and causes diarrheal and emetic food poisoning associated with the food products (EFSA, 2005). In many developed countries, the exact contamination level of *B. cereus* in traditional fermented foods has been regularly monitoring. For example, in South Korea, the prevalence and contamination level of *B. cereus* in soybean based fermented foods have been well established. A number of studies showed high prevalence of *B. cereus* (24-80%) with a contamination level of (10^2 - 10^8 CFU/g) in the Korean traditional soybean-based fermented foods including *deonjang* (fermented soybean paste), *gochujang* (fermented hot red pepper paste) and *cheonggukjang* (fast-fermented soybean paste) (Kim et al., 2015; Kim & Kim, 2012; Lee et al., 2009; Yim et al., 2015). Moreover, fewer studies genetically characterize the isolates, which are a matter of great concern to the regulatory authority and related-industry (Kim et al., 2015; Yim et al., 2015). The high prevalence of *entFM* (100%) and *nhe* (97.6%) genes were observed among the isolates isolated from Korean *deonjang*, *gochujang*, *cheonggukjang* (Moravek et al., 2006; Moravek et al., 2004; Yim et al., 2015). The prevalence of *hbl* genes was 50.4% and *cytK* was 48.0% in the report of Yim et al. (2015) in the Korean soybean based fermented products.

Several attempts have been made on the isolation and characterization of the *B. cereus* infecting phages. Some of the studies applied the phage to control the *B. cereus* in fermented foods. Till date 59 *B. cereus*-infecting phages have been reported in the NCBI database. However, most of the studies have not focused on the application of phage to control *B. cereus* in fermented foods.

2.2. National Status:

The reported bacterial food borne disease outbreaks in India during 1980–2009 indicated that 24 outbreaks have occurred involving 1,130 persons (Tewari & Abdullah, 2015). Among those outbreaks, *B. cereus* were one of the contaminating bacteria in Indian raw foods (milk, fish, chicken, meat products, fried rice) and fermented foods (dairy based products such as cheese; legume-based foods such as amriti, dhokla, dosa, *idli*, papad, wadi) (Kamat et al., 1989; Kumari & Sarkar, 2014; Roy et al., 2007a; Yadava, 2004). Kumari and Sarkar (2014) reported that 33-55% of different dairy based fermented foods

and beverages in India are contaminated by *B. cereus* with a contamination level up to 10^8 CFU/g or CFU/ml. Moreover, the authors have isolated 144 multidrug resistant (at least five antibiotics) *B. cereus* from those fermented foods. Moreover, 20% of Indian legume-based fermented foods (out of 105 samples) were found contaminated by *B. cereus*(Roy et al., 2007b). Roy et al. (2007a) reported that 48 multidrug resistant (at least nine antibiotics) *B. cereus* isolated from 6 different kinds of legume-based Indian fermented foods (*amriti, dhokla, dosa*, *idli*, *papad* and *wadi*). Although the toxigenic gene profiles and cytotoxicity level of *B. cereus* were reported from different countries, only one study genetically characterized the *B. cereus* isolated from Indian fermented foods [Rather et al. (2011) characterized the enterotoxigenic genes of *B. cereus* isolated from different milk samples].

Chapter 3. Aims and Objectives

3. Aims and Objectives

The aim of this study was to observe the pattern of toxigenic genes in *Bacillus cereus* isolates.

The objectives are:

i. Prevalence of *Bacillus cereus* in Indian traditional fermented foods (*Idli*, *haria*, *curd*, and *paneer*).
ii. Phenotypic and molecular characterization of *Bacillus cereus* isolated from traditional fermented foods.

Chapter 4. Materials and Methods

4. Materials and Methods

4.1 Sample collection: Indian fermented food samples (n=50, cereal based fermented foods, *Idli* and *haria*, & dairy based fermented foods, *curd* and *paneer*) were collected randomly from West Bengal, India. The samples were collected in sterilized container and transported into the laboratory in ice box.

4.2 Isolation and quantification of *B. cereus* strains: Isolation and enumeration *of B. cereus* was performed by standard spread plate techniques using *Bacillus cereus* agar media (supplemented with polymyxin B and egg yolk emulsion). The colonies showing different morphologies were selected and sub-cultured on Tryptone soya agar. The strains were stored at – 20 °C for further use.

4.3 Spores contamination levels determination: *B. cereus* spores contamination level of each sample was determined by heat treatment at 80°C for 15 min. The heat-treated samples were quickly cooled down. The spores were enumerated by spreading onto the surface of *Bacillus cereus* agar base supplemented with polymyxin B and egg yolk emulsion, and the plates were incubated at 37°C for 24 h (Cazemier et al., 2001).

4.4 Detection of enterotoxin and emetic toxin genes by multiplex PCR amplification: Genomic DNA isolation kit was used to isolate bacterial genomic DNA following manufacturer's instructions. The oligonucleotide primers used by Yang et al. (2005) for the detection of various toxins and *B. cereus* group genes (Internal transcribed gene, *ITS*) were used in this study. Then Multiplex PCR was applied for detection of enterotoxin and emetic toxin genes in *B. cereus* isolates following the method described by Yang et al. (2005). The PCR products were analyzed on a 2% (w/v) agarose gel, and electrophoresis was performed for 45 min at 100v.

4.5 Antibiotic sensitivity test: To analyze the antibiotic susceptibility/resistance patterns of *B. cereus* isolates against a panel of 5 antibiotics, the Kirby-Bauer Disk diffusion assay method was adopted (Park et al., 2009).

4.6 Statistical analysis: All of the experiments were performed at least three times and the results were expressed as mean ± standard deviation (SD).

Chapter 5. Results

5. Results

5.1 Prevalence of *B. cereus* in traditional fermented foods of West Bengal: Fifty fermented food samples were collected from different markets of Paschim Midnapore (20 no.), Bankura (10 no.), and Jhargram (20 no.) districts. The contamination levels of *B. cereus* in those products were evaluated by plating on *B. cereus* differentiation agar plates. *B. cereus* was detected in 34 (66.8%) samples and 16 samples (33.2%) exceeded the regulatory limit (10^4CFU/g) recommended by FAO/WHO (Table 1). *Haria* samples (84.6%) were mostly tainted by *B. cereus* followed by *Idli* (72.1%), and *Curd* (67.2%). *B. cereus* was ranged between 10^3 - 10^8 CFU/g in the tested foods (Table 1). A total of 50 *B. cereus* strains were isolated from those products. The isolates were sub-cultured twice and preserved in -20 °C for further use.

Table 1. Prevalence of *B. cereus* in various traditional fermented foods of West Bengal.

Source (no.)	No. of positive samples (%)	Counts (CFU/g)	Samples outside regulatory limit (%) *
Haria (30)	21 (67.2)	$6.5 \times 10^3 – 2.4 \times 10^6$	6 (22.0)
Idli (10)	8 (72.1)	$2.0 \times 10^3 – 9.0 \times 10^7$	5 (54.8)
Curd (10)	7 (61.1)	$7.8 \times 10^3 – 3.2 \times 10^8$	6 (59.1)
Total (50)	**36 (66.8%)**		

* Regulatory limit of *B. cereus* is $\leq 10^4$ CFU/g, constituted by Korean Food Regulatory Body in 2007.

5.2 Heat-resistant *B. cereus* spore contamination levels in traditional fermented foods

For enumeration of heat-resistant spores, the samples were heated at 80 °C for 15 min before spreading on *B. cereus* selective agar plates. All of the 50 *B. cereus* containing samples shown above were contaminated with *B. cereus* spores. The counts ranged from 2.4×10^3 to 4.0×10^3 CFU/g in *Haria*, 2.0×10^2 to 2.2×10^3 CFU/g in *Idli*, and 2.8×10^3 to 3.2×10^3 CFU/g in *Curd* (Table 2).

Table 2. Prevalence of *B. cereus* spores in different traditional fermented foods of West Bengal.

Source (no.)	No. of positive samples (%)	Spore count (CFU/g)
Haria (30)	21 (67.2)	$2.4 \times 10^3 – 4.0 \times 10^3$
Idli (10)	8 (72.1)	$2.0 \times 10^2 – 2.2 \times 10^3$
Curd (10)	7 (61.1)	$2.8 \times 10^3 – 3.2 \times 10^3$
Total (50)	**36 (66.8)**	

5.3 Toxigenic gene profiles in the *B. cereus* isolates

Fifty *B. cereus* isolates were characterized by multiplex PCR to determine the distribution of toxin genes (*hblCDA*, *nheABC*, *bceT*, *entFM*, and *EM*) (Fig. 1 & Table 3). All of the strains carried at least one enterotoxin gene. Most prevalent toxin was *entFM* (100%), followed by *nheB* (87.4%), *nheC* (86.6%), *nheA* (82.7%), *hblA* (50.4%), *hblD* (49.6%), *hblC* (47.2%), *bceT* (14.2%), and *EM* (3.9%).

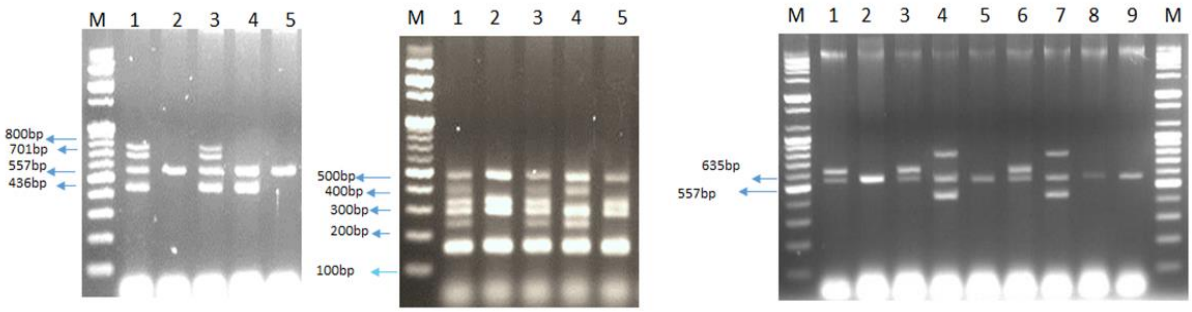

Fig. 1. Agarose gel (2.0%) electrophoresis showing different enterotoxigenic genes in *B. cereus* isolates. nheA – 475bp, hblC – 386bp, nheB – 328bp, entFM – 290bp, hblA – 237bp, cytK 1800 bp and bceT – 701 bp, nheC 557bp, em 1 -635bp and hblD – 436.

The isolates were categorized into fifteen different toxigenic patterns (I - XV) according to the presence or absence of enterotoxins genes (Table 3). The toxigenic pattern I contained all nine enterotoxin genes except *EM*, while pattern II harbored eight enterotoxin genes except *bceT* and *EM*. Pattern III and IV covered all of the Nhe components and cytotoxin K but produce one or two *hbl* genes. Likewise, isolates of toxigenic pattern types VIII, X, XI, XIII, XIV, and XV did not produce the product for

hbl genes but yielded one or all three PCR products for Nhe components. Emetic toxin gene was identified in 5 isolates and they are unable to produce Hbl toxin (Table 3).

Table 3. Toxigenic gene patterns of *B. cereus* isolates from traditional fermented foods of West Bengal as determined by multiplex PCR.

Pattern	*hblC*	*hblD*	*hblA*	*nheA*	*nheB*	*nheC*	*entFM*	*bceT*	*Em*[a]
I	+	+	+	+	+	+	+	+	-
II	+	+	+	+	+	+	+	-	-
III	-	+	+	+	+	+	+	-	-
IV	-	+	-	+	+	+	+	-	-
V	+	+	+	+	+	-	+	-	-
VI	+	+	+	+	-	+	+	-	-
VII	+	-	+	+	+	-	+	-	-
VIII	-	-	-	+	+	+	+	-	-
IX	+	-	+	+	-	+	+	-	-
X	-	-	-	+	+	+	+	-	-
XI	-	-	-	+	+	+	+	-	+
XII	-	+	-	-	-	+	+	-	-
XIII	-	-	-	-	-	+	+	-	-
XIV	-	-	-	-	+	-	+	-	-
XV	-	-	-	-	-	-	+	-	-

+: present, -: absent, [a]Emetic specific sequence

5.4 Antibiotic susceptibility of the *B. cereus* isolates

Antibiogram of *B. cereus* isolates is shown in Table 4. The strains were categorized as susceptible, intermediate and resistant according to the previously developed methods (Chon et al., 2012; Kim et al., 2010). Most of the strains were highly susceptible to erythromycin (86.6%) and tetracycline (75.6%). They were resistant to ampicillin (70.9%), spectinomycin (70.1%), and chloramphenicol (57.5%).

Table 4. Antibiotic susceptibility of *B. cereus* isolates.

Antibiotics	**Antibiotic concentration per disk**	**No of Strains (%)**		
		Resistance	**Intermediate (%)**	**Susceptible (%)**
Ampicillin	10 µg	35 (70.9)	10 (19.7)	5 (9.4)
Chloramphenicol	30 µg	29 (57.5)	10 (19.7)	11 (22.8)
Erythromycin	15 µg	5 (9.4)	2 (3.9)	43 (86.6)
Spectinomycin	10 µg	35 (70.1)	4 (7.9)	11 (22.0)
Tetracycline	30 µg	7 (15.0)	5 (9.4)	38 (75.6)

Chapter 6. Discussion

6. Discussion

B. cereus contamination is a major problem in Indian traditional foods and beverages (Gajamer and Tiwari, 2014). In this study, we first evaluated the prevalence of *B. cereus* in such foods (samples were collected between April 2022 and May 2022). About 66.8% of the samples were contaminated by *B. cereus* with a level of 10^3 - 10^8 CFU/g and 33.2% exceeded the boundary (< 10^4 CFU/g) (Table 2). The incidence rate of *B. cereus* was consistent with the previous studies (71%) (WHO, 2015). On the other hand, contamination levels determined in this study (10^3 - 10^8 CFU/g) were higher than that of the previous report (10^2 - 10^3 CFU/g) where the samples were collected between May 2013 and November 2013 (Yim et al., 2015). This variation might be due to the different sampling period.

Nowadays, the contamination level of *B. cereus* is routinely checked in the food industries by using fast detection methods (targeting the vegetative cells, not spores) such as ELISA, PCR etc. (Law et al., 2014). As *B. cereus* can easily form spores (Kim et al., 2009; Kramer & Gilbert, 1989), it can be assumed that the rapid detection methods (targeting the vegetative cells) may not provide the actual contamination level of this pathogen. In this study for the first time, we are reporting the content of heat resistant *B. cereus* spores (10^2 - 10^3 CFU/g) in Indian traditional fermented foods (Table 2). The higher number of spores (10^2 - 10^3 CFU/g) along with the vegetative cells may easily exceed the regulatory limit (< 10^4 CFU/g). Total *Bacillus cereus* counts range, spore counts range, total to spore ratio needs to be further studied.

Toxigenic gene profiles were highly diverse among the isolated *B. cereus* strains. The findings of fifteen different toxigenic arrangements (Table 3) among *B. cereus* isolates contrasted with the previous report (six different toxigenic patterns) of Yim et al. (2015) (*Doenjang*, *Kochujang*, *Ssamjang*, and *Cho-Kochujang*), whereas, in good agreement with the report of Kim et al. (2009) (15 toxigenic patterns of *B. cereus* were observed in Korean rice, red peppers, and Sunsik).

The high prevalence of *entFM* (100%) and *nhe* (97.6%) genes were in good agreement with previous studies (Moravek et al., 2006; Moravek et al., 2004; Yim et al., 2015). The

prevalence of *hbl* genes was found higher (50.4%) and *cytK* was slightly lower (48.0%) than the previous report of Yim et al. (2015) [*hbl* (34.5%) and *cytK* (57.5%)].The variances in results could be due to the sample collection at different time period. Nevertheless, the high prevalence and diversity of toxin possession genes may display large variability in pathogenesis, should be studied further.

Antibiogram of these isolates showed their resistant/susceptible properties against different antibiotics. Most of the *B. cereus* strains in this study were found susceptible to erythromycin (86.6%) and tetracycline (75.6%) (Table 4). On the other hand, the high prevalence of β-lactam antibiotics resistance to ampicillin (70.9%) and spectinomycin (70.1%) suggests that the strains were able to produce β-lactamase (Table 4) (Park et al., 2009).

In spite of the high-level contamination, no *B. cereus* food poisoning case has been reported possibly due to the low daily consumption rate of these foods. Hence, the relationship between *B. cereus* contamination level and actual hazardous level should be clarified and further investigated. However, high contamination level of this pathogen in Indian traditional fermented foods is a matter of concern and requires constant attention.

Chapter 7. Conclusion

7. Conclusion

The present study showed that the Indian traditional fermented food products were contaminated by *B. cereus* and their spores, leading up to a proposal that the spore counts should be considered as a critical parameter for evaluating the qualities of these products. The isolates from 36 positive samples exhibited various toxigenic profiles and most of the strains harbored multiple toxin genes. Virulence genes of diarrheal type were more broadly distributed than emetic type, among the tested *B. cereus* isolates. The antibiogram of *B. cereus* isolates revealed that most of the strains were highly susceptible to erythromycin (86.6%), tetracycline (75.6%) and resistant to ampicillin (70.9%), Spectinomycin (70.1%), and Chloramphenicol (57.5%). However, fifteen different toxigenic patterns suggest that the strains might display the large variability in pathogenicity and in causing diseases. Therefore, a proper hazard analysis critical control point should be defined properly to maintain the quality of such foods.

Chapter 8. Future scope

8. Future scope

It is clear from this study that the isolated *B. cereus* are diverse according to toxigenic gene profiling. Since, we have tested only 50 isolates, genomic characterization of more strains should be done to get a complete portrait. Moreover, a continuous monitoring of pathogenic loads in the Indian fermented foods should be performed to reduce health hazards.

Chapter 9. References

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