VOLUME 2 ISSUE 1

ISSN: 2230-4818

JOURNAL OF SCIENTIFIC & INNOVATIVE RESEARCH

ORIGINAL RESEARCH ARTICLE

Molecular Characterization of Enterotoxin Genes from Food-borne Pathogen, *Bacillus cereus*

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ABSTRACT

Bacillus cereus is an opportunistic Food borne Pathogen that contaminates a wide variety of foods. There are mainly two types of illness caused by *B. cereus* in human beings namely diarrhoeal and emetic types. The emetic type is due to a single heat-stable toxin, and the diarrheal type to 3 or 4 heat-instable enterotoxins. In this research work, *B. cereus* spp were isolated from fermented rice noodle (Myanmar Traditional Food). For emetic toxin production, toxin of three isolated *B. cereus* were extracted by methanol extraction and subjected to LC-MS. Three isolated strains were genetically characterized by using gene specific primer for enterotoxin production. None of these strains showed emetic toxin production. But, all three strains tested had all the components of Haemolytic BL toxin (HBL), a haemolytic enterotoxin complex made of three proteins and Non Haemolytic Enterotoxin (NHE), a non-haemolytic enterotoxin complex made of three proteins and enterotoxin T genes. In addition, genes for the cytotoxin K were also found in two isolates, one isolate (E4) showed negative. From these results, the three isolates may be virulence because they have all HBL genes and all NHE genes. So, these strains seem to be enterotoxin producing strains.

Keywords: *Bacillus cereus*, Emetic toxin, Enterotoxin, Fermented rice noodle.

INTRODUCTION

Food-borne diseases, one of the major causes of malnutrition, are common in most countries of the South-East Asia Region.

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A large number of people suffer from communicable diseases caused by contaminated foods, including drinking water, which can be a major cause of cholera and other forms of epidemic diarrhoeal diseases.

Major Food-borne Pathogens are *Bacillus cereus*, *Escherichia coli*, *Shigella*, *Staphylococcus aureus*, *Salmonella* and *Clostridium botulinum* etc. Among

them, *Bacillus cereus* is responsible for contamination of rice and rice-based foods. Fatal and severe cases have been reported within the last few years. Most strains are presumably innocuous or mildly Pathogenic whereas few are presumably highly virulent. So, this research proposes to investigate virulence mechanisms of *B. cereus* in order to identify determinants specific of highly virulent strains. Then, mechanisms of the expression of the toxin genes will be investigated to explain the difference between high and low toxin producing strains.

All Myanmar people consume rice and rice-based products as staple food. Fermented rice noodle is one of local delicious foods consumed mainly as breakfast and snack. In this production, non-sterilized mill rice grains are soaked in water for 3 days for fermentation before wet-milling, in which contamination of *Bacillus cereus* may take place. However, no research has been done on this local fermented rice noodle, the knowledge of which is essential to upgrade status of the traditional local food and to increase its market and profitable.

B. cereus is the etiologic agent of two types of Food-borne disease, namely a toxico-infection with diarrhea as major clinical symptom and an intoxication causing vomiting. The diarrhoeal type of disease is caused by *B. cereus* species that are able to produce enterotoxins. So far, four toxins have been described: 1) Haemolytic BL toxin (HBL), a haemolytic enterotoxin complex made of three proteins, 2) Non Haemolytic Enterotoxin (NHE), a non-haemolytic enterotoxin complex made of three proteins, 3) enterotoxin T, a single protein, and 4) cytotoxin K, also a single protein. Three of these toxins are related to food borne outbreaks; the fourth, enterotoxin T, is not.[1-3]

Besides the discrimination of *B. cereus* on the basis of toxin-production, also growth temperature is a means of discrimination. *B. cereus* species may be subdivided according to their thermo tolerance: the psychrotrophes and the mesophiles.[4]

Most of the food poisoning cases caused by *B. cereus* are occurred in rice-based food. As the staple food in Myanmar is rice, it is very important to study this microorganism and its virulence genes.

The objective of this work is to study the enterotoxin genes of *B. cereus* by molecular methods.

MATERIALS AND METHODS

All bacteriological media used were those of dehydrated media procured from Hi-Media Lab, India. The specific requisites in respect of PCR specific reagents and fine biochemicals such as Taq polymerase, base pair ladder molecular markers, buffers, reagents, agarose, gel stain (ethidium bromide) used in this experimental study have been of molecular biology grade and obtained from

Sigma-Aldrich, Bangalore, India, and Bangalore Genei, Bangalore, India. Primer used for molecular characterization of Enterotoxin Genes were shown in Table 1.

A total of 8 rice-based food samples were collected from local market of Yangon, Myanmar.

Isolation of food isolates of *B. cereus* by Spread Plate Method

Isolates of *Bacillus cereus* were isolated from individual food samples by plating on a selective medium namely Mannitol Egg Yolk Polymyxin Agar (MYP agar). A homogeneous sample was prepared in 0.1% peptone broth to give an initial 1:10 dilution. Each of the sample weighing 10g was mixed with 90 ml 1% peptone broth.

Presumptive colonies of *B. cereus* were randomly selected based on characteristic colony features and purified by streaking on Nutrient agar plates. The individual purified colonies were selected and maintained in culture slants at 4°C.

Characterization of presumptive native food isolates of B. cereus

The presumptive native food isolates of *B. cereus* and reference cultures of *B. cereus* were identified individually by morphological, cultural and biochemical characteristics according to the documented procedures.[5]

Haemolytic activity

To test for hemolytic activity, the bottom of a blood agar plate is marked into 4 equal segments. Each segment is labeled and inoculated near its center by gently touching the agar surface with a 2mm loopful culture. The plate is then incubated at 30°C for 24 hr and checked for hemolytic activity as indicated by a zone of complete hemolysis surrounding the growth. *B. cereus* is usually strongly hemolytic, whereas *B. thuringensis* and *B. cereus* var mycoides are often weakly hemolytic, or produce hemo-lysis only under the growth. *B. anthracis* is usually non-hemolytic.

Study on Emetic Toxin Production of isolated strains

Selected strains and growth conditions

Three strains (A3, E3 and E4) were selected to study the emetic toxin production. Rice noodles were used as substrates. Cultures were grown overnight in nutrient broth at 30°C. Appropriate dilution in (0.85%) normal saline was performed for each overnight culture to provide inoculum for rice noodle at a level of approximately 105 CFU/g.

Bacterial inoculation of cooked noodle and incubation conditions

Rice noodles, purchased from a supermarket in Yangon, Myanmar, were autoclaved at 121°C for 20 min. 25ml of autoclaved water was added to

15g of autoclaved rice noodles in 250ml conical flask and placed in boiling water for 5 min. Then, cooked noodles were inoculated with ca. 105 CFU/g with appropriate dilution of selected strains. Non-inoculated rice noodle was used as negative control. All samples were incubated at 30°C for 7 days.

Cereulide extraction

The emetic toxin (cereulide) was extracted in duplicate: 6 ml methanol was added to 3 g of sample and then mixed with sterile spatulas. The homogenized liquid was collected in glass tubes and placed in boiling water 15 min, followed by evaporation under N2 atmosphere until dryness. The residue was resuspended and diluted in 3 ml methanol, vortexed and centrifuged. The supernatant was collected and stored at -20°C prior to analyses.

LC-MS analysis

Cereulide content of each extract was analyzed by LC-MS method as described by Haggblom et al. (2002) with the following modifications. The LC-MS analysis was performed on an LCQ Deca-XP Plus ion trap mass analyzer. Chromatographic separation was done on the Symmetry C8 column, 2.1mm x 150 mm, 5 um (Waters, USA). The mobile phase of the isocratic method was a mixture of 95% acetonitrile, 4.9% water, 0.1% trifluoroacetic acid at a flow rate of 0.2 ml min-1 with a sample injection volume of 5ul. Because, cereulide standard was not commercially available, valinomycin (Sigma) dissolved in pure methanol was used as an external standard. A full mass spectrum was recorded from 500 to 1300 m/z in positive electronspray mode (ESI+). The total ion chromatogram was smoothed with a Gaussian function. For detection of cereulide, the m/z values for adduct ions 1170.5 (NH4+ adduct) and 1191.5 (K+ adduct) were monitored.

PCR Detection of Selected Potent Toxigenic Traits among the identified native food isolates of B. cereus

The total genomic DNA of individual isolates was extracted by a relatively simple process (Melody, 1997). Identified isolates of *B. cereus* were subjected to uniplex PCR with species specific primers as detailed in Table 1. The reaction components for PCR amplification included the following: (i) Template DNA, (ii) Gene specific primers, (iii) Taq DNA polymerase , (iv) 10 X Reaction buffer: 100mM Tris pH 9.0, 500 mM KCL, 15 mM MgCl2 and 0.1% Gelatin, (v) Nuclease free water, (vi) dNTP mix (10mM of each dNTP). For a reaction volume of 25 µl, the following reaction components were combined in a thin-walled 0.2 ml PCR reaction tube to make a final volume of 25 µl. The content of the tubes were mixed by a brief spin in a microcentrifuge.

PCR amplification was performed in an automated DNA Thermal cycler (Eppendorf, Master cycler, Cedex, France) following the PCR conditions as detailed. The resultant PCR amplified products were analysed by agarose gel electrophoresis following the method.[6]

Table 1: Primers' names and sequences

Name	Sequence
L1A	ATATTCACCTTAATCAAGAGCTGTCAGG
L1B	CCAGTAAATCTGTATAATTTGCGCCC
L2A	TATCAATACTCTCGCAACACCAATCG
L2B	GTTTCTCTAAACATCTAAATATGCTCGC
B1F	ACGAACAATGGAGATACGGC
B2R	TTGGTAGACCCAAAATAGCACC
A-45-2F	GCTCTATGAACTAGCAGGAAAC
A-45-3R	GCTATTTACTTGATCTTCAACG
B-39-1F	CGGTTCATCTGTTGCGACAGC
B-39-2R	GATCCCATTGTGTACCATTGG
C-39-F	CCTTATAAAGAGAATAGGTG
C-39-2R	CGACTTCTGCTTGTGCTCCTG
BCET-F	TTAGTTTCAACAGCGTATCGGT
BCET-R	ATACACATGCAAATGCTCCGGAC
BC-CYTK-FC	GTAACTTTCATTGATGATCC
BC-CYTK-RC	GAATACATAAATAATTGGTTTCC

RESULTS AND DISCUSSION

Isolates from MYP agar that had lecithinase production as indicated by a zone of precipitate surrounding growth and mannitol fermentation negative were provisionally identified as *B. cereus* group. 41 strains were isolated from 12 rice-based food samples. After studying the growth characteristics and microscopic morphology, the positive 27 isolates were selected to study the biochemical characteristics.

Among these 27 strains, 10 strains were compatible with the biochemical characteristics of *B. cereus* spp according to the literature such as large gram positive bacilli that produce acid from glucose anaerobically, reduce nitrate to nitrite, decompose L-tyrosine, grow in the presence of 0.001% lysozyme, exhibit motility,are hemolytic, and do not produce endotoxin crystals or rhizoid growth .

3 strains were selected to study the emetic toxin production by LC-MS method. Toxin were extracted by methanol extraction and measured with LC-MS. But, none of these strains can produce emetic toxin.

To study the molecular characteristics of enterotoxin genes of isolates, 3 strains were selected. Genomic DNA of these isolates was extracted. And 16 primers were used for 8 genes concerned with these enterotoxins.

All three strains tested had all the component of HBL and NHE. A high occurrence of protein components and/or genes involved in diarrhoeal disease has previously been described for *B. cereus* from food. In addition, genes for the cytotoxin CytK were found in two isolates, one isolate (E4) showed negative. The importance of this frequent occurrence of CytK in B. cereus-like organisms is unknown though, as the role of CytK in food borne disease is not yet fully understood. All three isolates showed positive in enterotoxin T genes.

Based on the present knowledge (Wijnands et al. in preparation)[7], food, contaminated with B.cereus, must be considered hazardous to health in case of consumption if 1) not only, more or less, large numbers of vegetative cells or spores are present, but also if one or more of the following tests is positive: PCRs for the presence of all HBL genes, PCR for the presence of all NHE genes, PCR for the presence of cytotoxin-K gene, and LC-MS analysis for the detection of the interference of the emetic toxin with the metabolic action of mitochondriae; and 2) small numbers of microorganisms are present and the detection of the emetic toxin is positive.

Only when the strains contain all three genes of the HBL or the NHE enterotoxin, they have the potency to cause Food-borne illness. This is also the case when they have the gene for cytotoxin K and the ability to produce the emetic toxin. At this moment it is still unknown under what conditions the genes are expressed and the toxins formed.

When strains do not possess all genes of the HBL or the NHE enterotoxin, or do not possess the gene for cytotoxin K or the potential to produce emetic toxin, strains cannot be regarded as Pathogenic for Food-borne disease.

In all comments, the enterotoxin T has been omitted as a potential Pathogenic property as this enterotoxin has never yet been related to any Food-borne outbreak. Within a given strain, the presence of toxic components or genes encoding them does not necessarily lead to food borne disease following ingestion. Therefore, the exact influence of *B. cereus* on human disease cannot be estimated from the present results.

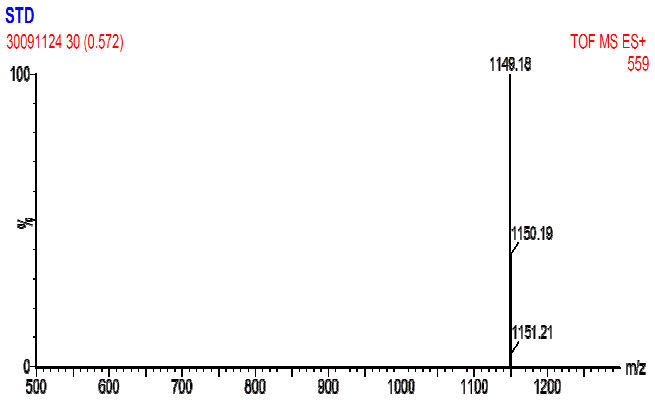

Fig. 1 MS profile of valinomycin used as standard for cereulide production of B. cereus

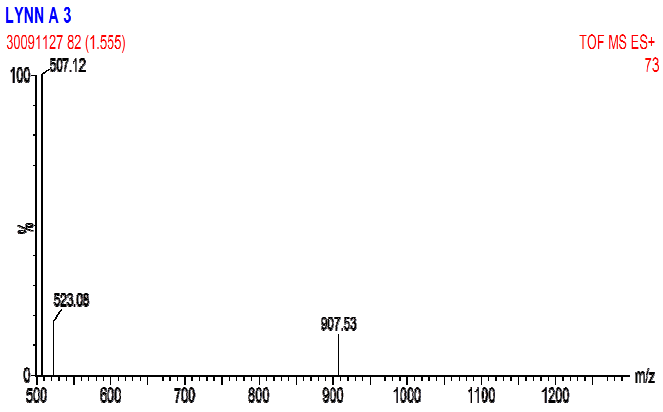

Fig. 2 MS profile of A3 islolate

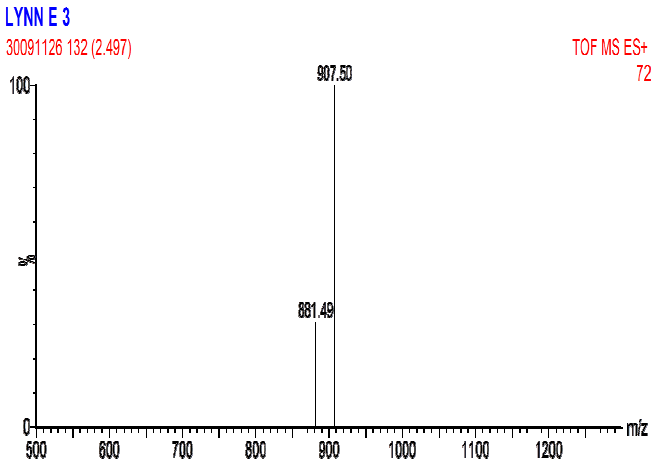

Fig. 3 MS profile of E3 islolate

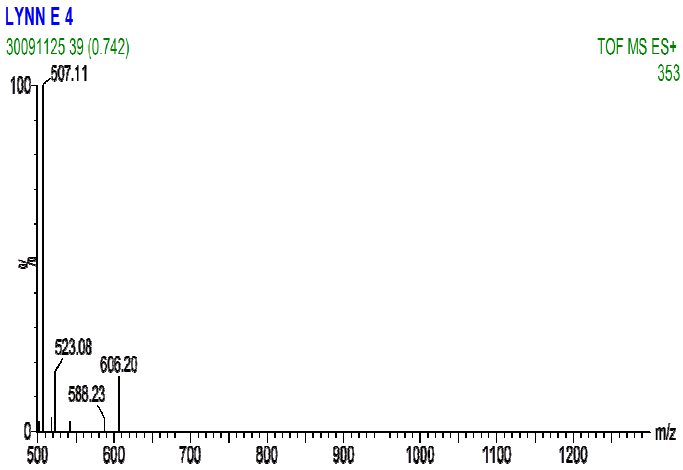

Fig. 4 MS profile of E4 islolate

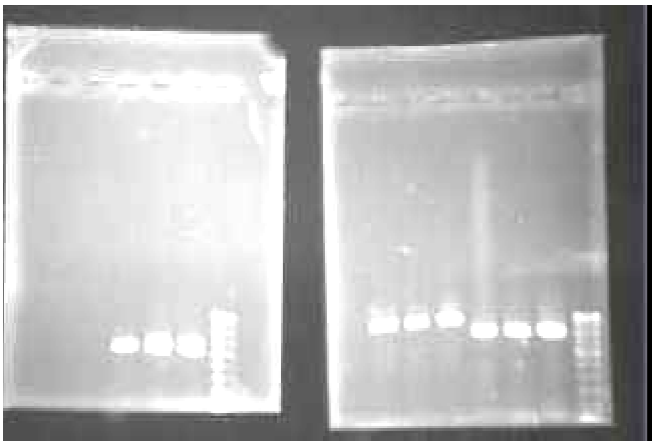

Fig 5 Detection of gene encoding for L-components of HBL (haemolytic BL enterotoxin)

1 - A3
2 - E3
3 - E4
M - 100 bp ladder Marker

Amplicon size
L1-gene: 809 basepair (bp)
L2-gene: 976 bp
B-gene: 600 bp

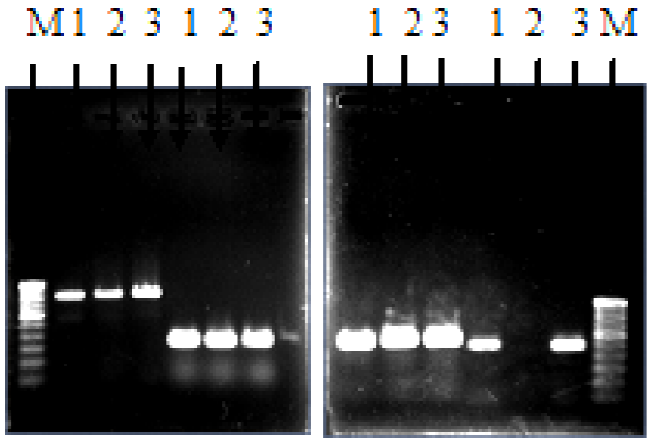

Fig. 6 Detection of genes encoding for NHE (non haemolytic enterotoxin) and cytotoxin k

Amplicon size
nheA: 540 bp
nheB: 312 bp
nheC: 834 bp
cytk: 480 bp
Based on Granum et al.(1999)

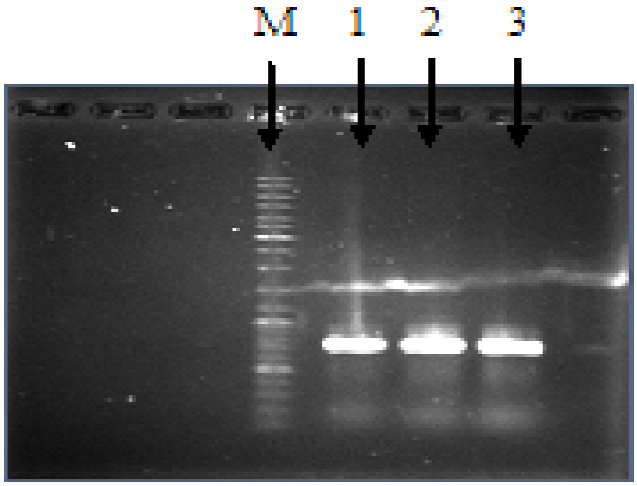

Fig. 6 Detection of genes encoding forEnterotoxin T

Amplicon size
bce-T: 741 bp
Based on Ombui et al. (1997)

CONCLUSION

From these results, the three isolates may be virulence because they have all HBL genes, all NHE genes. Two strains show positive in cytotoxin K gene. So, these strain can be said that enterotoxin producing strain.

With the methods described here, it is possible to distinguish between Pathogenic and non Pathogenic strains. For definitive discrimination into Pathogenic and non-Pathogenic *B. cereus* strains, it remains of importance to develop methods that can establish the transcription from the enterotoxin genes into messenger RNA and methods that can determine the formation of the enterotoxins.

ACKNOWLEDGEMENT

The authors would like to thank Central Food Technological Research Institute (CFTRI) and NAMS&T Science Center for financial support of this research work.

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