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PCR-based detection of enterotoxigenic isolates of *Bacillus cereus* from tropical seafood

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***Background & objectives*: *Bacillus cereus* is an important enterotoxigenic food borne pathogen. The present study was undertaken to assess the occurrence of *B. cereus* in tropical fish and evaluation of virulent gene specific PCR for differentiation of diarrhoeal enterotoxin producing isolates of *B. cereus* from non enterotoxigenic isolates.**

***Methods*: Selective plating on polymixin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA) was used for isolation of *B. cereus* from finfish, prawn and clams. Enterotoxin producing ability of all 42 isolates obtained from the samples was judged by reverse passive latex agglutination (RPLA) test and the presence of different virulent genes *i.e. hbla*, *bceT* and *entFM* was screened by PCR.**

***Results*: *B. cereus* and enterotoxigenic *B. cereus* were found to be in 36.7 and 29.41 per cent of fish samples, respectively. All the diarrhoeal enterotoxin producing isolates showed the presence of *hbla* gene, but *hbla* gene was not present in any of the non-enterotoxigenic isolates tested in this study.**

***Interpretation & conclusions*: Our findings indicated that *hbla* gene specific PCR can be employed for differentiation of enterotoxigenic *B. cereus* isolates from non-enterotoxigenic isolates.**

Key words *Bacillus cereus* - enterotoxin - PCR - RPLA - virulent gene

Bacilus cereus is an important food borne pathogen, which causes two distinct types of food poisoning *i.e.* diarrhoea and emesis caused by two different types of toxins[1,2] and also fatal meningitis[3]. This organism is also responsible for spoilage of different food products[4]. As *B. cereus* is a spore former organism, there is a risk of its transmission through heat-treated and processed food products. Some isolates of *B. cereus* can grow at refrigerated temperature[5,6] and spore can survive at high temperature. This organism is known to resist even pasteurization process of milk[7]. In India, presence of this organism has been reported in various food products including fish[8].

Till date, three different genes *viz. hbla*, *bceT* and *entFM* responsible for diarrhoeal enterotoxin production in *B. cereus* have been characterized[9-11]. Reverse passive latex agglutination (RPLA) test is a reliable assay for the detection of *B. cereus* diarrhoeal enterotoxin[12]. So far no study has been carried out to relate the presence of virulent genes and enterotoxin production in case of isolates of *B. cereus* from tropical seafood. Therefore, the present work was carried out to investigate the occurrence of enterotoxigenic *B. cereus* in tropical fish and shellfish, and also to study the relationship between diarrhoeal enterotoxin

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production and presence of different virulent genes in those isolates.

Material & Methods

Samples and reference bacterial strains: Fish samples were procured from local markets of Cochin, India. A total of 68 samples were tested during a period of 13 months from January, 2004 to February, 2005 in the Laboratory of Microbiology, Fermentation and Biotechnology Division, Central Institute of fisheries Technology, Cochin, Kerala. The samples included 50 finfishes, 10 prawns and 8 clams.

In addition to our *B. cereus* isolates, *B. cereus* NCIM 2106 was used as a standard strain. Standard strains of other bacteria like *B. subtilis* NCIM 2545, *Salmonella* Dublin ATCC 15480, *Escherichia coli* NCIM 2068 and *Staphylococcus aureus* NCIM 2079 were also used for PCR. *B. cereus* NCIM 2106, *Bacillus subtilis* NCIM 2545 and *E. coli* NCIM 2068 were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. *Salmonella* Dublin ATCC 15480 was obtained from American Type Culture Collection and maintained in our laboratory.

Isolation and identification of B. cereus: Isolation of *B. cereus* was done by selective plating on polymixin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA)[13]. Fish sample (25 g) was macerated in 225 ml of normal saline in a stomacher blender (Seward Ltd, U.K.) and serial 10-fold dilutions were made on normal saline. 0.5 ml from each dilution was surface plated on pre-dried PEMPA media. Plates were incubated at 37°C for 24 h. Typical suspected *B. cereus* colonies showing lecithinase reaction were picked up, purified and stored on nutrient agar slant for further confirmation, which was carried out by Gram's staining and standard biochemical reactions[14].

The ability to hydrolyze starch was tested by inoculating on starch agar[15]. After growth, the plates were exposed to iodine vapour to find clear zone around the colony in the case of positive reaction.

Test for diarrhoeal enterotoxin and haemolytic activity: The ability for the production of enterotoxin was tested by reverse passive latex agglutination (RPLA) test. Brain heart infusion (BHI) broth was inoculated with one loopfull of culture and was incubated overnight at 37°C with constant shaking at 150 rpm. The culture was centrifuged at 4000 X *g* at 4°C. Cell free supernatant was subjected to detection of enterotoxin by RPLA test using BCET-RPLA kit (Oxoid, UK) as per manufacturer's instruction.

Test for haemolysis production was done on 5 per cent sheep blood agar, both by stab inoculation and surface inoculation. Preparation of blood agar was done on Columbia blood agar base (Oxoid, UK) using sheep blood following the procedure as mentioned by Collins and co-workers[15]. In case of stab inoculation, the method as mentioned in Rhodehamel and Harmon[14] was followed. For surface inoculation, the organisms were simply streaked on 5 per cent sheep blood agar.

Preparation of lysate for PCR: Template of PCR was prepared by boiling method as described by Phelps and McKillip[16] with slight modification. Overnight growth of bacterial culture in BHI broth was centrifuged at 7000 x *g* for 10 min at 4°C. Bacterial pellet was washed once in normal saline and suspended in 150 µl of autoclaved millipore (Milli-Q, France) purified water with vortexing. The bacterial suspension was kept on boiling water bath for 10 min and frozen immediately at -70°C. After freezing, the lysate was thawed and centrifuged at 4000 x *g* to pellet the debris. Five µl of the supernatant was used as template in the PCR reaction.

Polymerase chain reaction: For screening of enterotoxigenic genes *viz*. *hbla*, *entFM* and *bceT* in all 42 isolates of *B. cereus*, primers specific for those genes were employed in PCR assays. Four sets of primers were employed in the PCR reactions (Table I). Primer pair BalF/BalR[17] is specific for *B. cereus* group. Primer pairs HblA1/HblA2[18], ENTA/ENTB[11] and BceT1/BceT2[18] were employed to screen the presence of *hbla*, *entFM* and *bceT* genes, respectively. 5µl of template was amplified in 25 µl of reaction mixture consisting of 10mM Tris-HCl (*p*H 9), 50 mM KCl, 1.5 mM $MgCl_{2,}$ 200 µM of each dNTP (Finnzyme, Finland), 1 U *Taq* DNA polymerase (Bangalore Genei, India) and 10 pmol of respective primers. Oligonucleotide primers were procured from Integrated DNA Technologies (IDT), USA. PCRs were performed using a thermocycler (Mastercycler, Eppendorf, Germany). PCR reaction using BalF and BalR primers consisted of 30 cycles of 94°C for 45 sec (denaturation), 55°C for 45 sec (annealing) and 72°C for 45 sec (extension). PCR reaction condition for the primers HblA1 / HblA2 were 30 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 1 min. On the other hand, a cycling condition consisting of 30 cycles of 94°C for 45 sec, 65°C / 55°C for 45 sec and 72°C for 45 sec was used in the PCR reaction using BceT1/BceT2 primers. In this PCR, two annealing temperature *i.e.* 55 and 65°C

Table I. Primers used in the study

Primer	Sequence	Target/specificity
BalF	5'- TGCAACTGTATTAGCACAAGC T -3'	*B. cereus*
BalR	5'- TACCACGAAGTTTGTTCACTACT -3'	group
HblA1	5′- GCTAATGTAGTTTCACCTGTAGCAAC- 3′	*hbla* gene
HblA2	5′- AATCATGCCACTGCGTGGACATATAA- 3′	
ENTA	5′- ATGAAAAAAGTAATTTGCAGG- 3′	*entFM* gene
ENTB	5′- TTAGTATGCTTTTGTGTAACC- 3′	
BceT1	5′- GAATTCCTAAACTTGCACCATCTC G- 3′	*bceT* gene
BceT2	5′- CTGCGTAATCGTGAATGTAGTCAAT- 3′	

Table II. Presence of enterotoxigenic *B. cereus* in finfish, prawn and clam samples

Source	Samples	Positive for *B. cereus* No. (%)	Positive for enterotoxigenic *B. cereus* No. (%)
Finfish	50	11 (22)	9 (18)
Prawn	10	7 (70)	7 (70)
Clams	8	7 (87.5)	4 (50)
Total	68	25 (36.7)	20 (29.41)

were tried, separately. PCR cycling condition for the primers ENTA / ENTB was 94°C for 45 sec, 52°C for 45 sec and 72°C for 1 min. In each PCR, total number of cycles was adjusted to 30. In all the PCR reactions, an initial denaturation was performed at 95°C for 3 min and after completion of 30 cycles, final extension was carried out at 72°C for 5 min.

The PCR products were analyzed in 1.5 per cent agarose gel prepared in 1X TAE buffer (40mM Tris-acatate and 1mM EDTA, *p*H 8.0) containing ethidium bromide (0.3 µg/ml). Electrophoresis was carried out at 7 volts/cm for 1 h 30 min in a submarine gel electrophoresis system (Bangalore Genei, India). Finally, the gel was photographed by an UV gel documentation system (Alpha Innotech Corporation, USA).

Results

Isolation of B. cereus: A total of 68 fish, shrimp and clam samples procured from local markets of Cochin were screened for the presence of *B. cereus.* Of these, 25 samples were found to be positive for *B. cereus* (Table II) and 42 isolates of *B. cereus* were recovered from those positive fish samples.

Enterotoxin production and haemolytic activity: Of the 42 isolates tested for the production of diarrhoeal enterotoxin by RPLA technique, 30 were shown to be enterotoxigenic. These 30 isolates were recovered from 20 different fish samples. So, the number of samples positive for enterotoxigenic *B. cereus* was 20

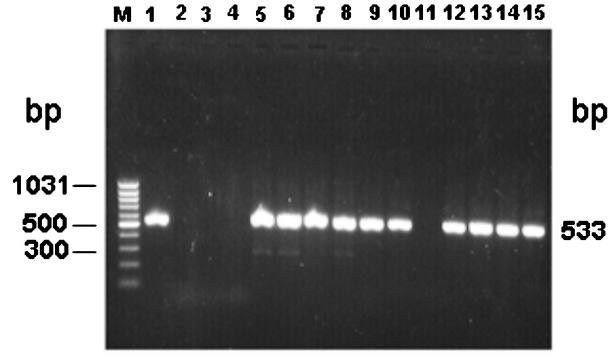

Fig. 1. *B. cereus* group specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *B. cereus* NCIM 2106, Lane 2: *B. subtilis* NCIM 2545, Lane 3: *Salmonella* Dublin ATCC 15480, Lane 4: *E. coli* MCIM 2068, Lanes 5-10: *B. cereus* isolates, Lane 11: *Staphylococcus aureus* NCIM 2079, Lanes 12-15: *B. cereus* isolates.

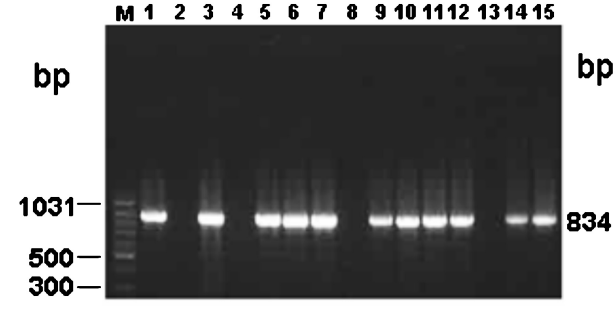

Fig. 2. *hbla* gene specific PCR. Lane M: 100 bp DNA ladder, Lane 1: *B. cereus* NCIM 2106, Lanes 2-15: *B. cereus* isolates.

(Table II). It has been found that all enterotoxigenic isolates were positive to starch hydrolysis. More samples of prawn and clam were positive for *B. cereus* and enterotoxigenic *B. cereus* compared to finfish (Table II).

All but one isolate showed β-haemolysis on sheep blood agar. The extent of haemolysin production varied within different isolates as evidenced by the zone of clearance on blood agar.

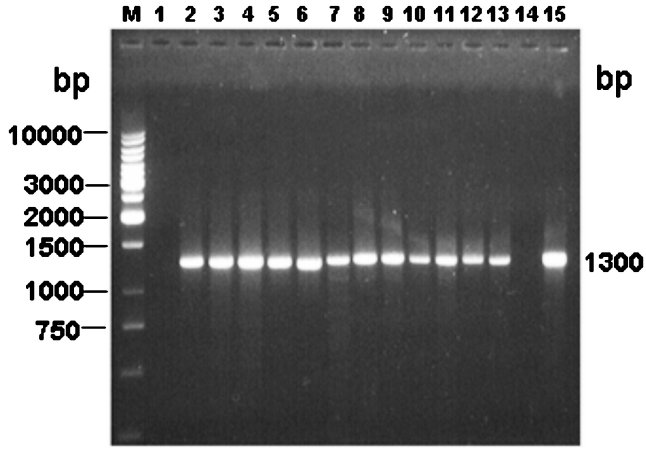

Fig. 3. *entFM* gene specific PCR: Lane M: 1 kb DNA ladder, Lane 1: *B. subtilis* NCIM 2545, Lanes 2-15: *B. cereus* isolates.

Polymerase chain reaction: PCR assay using *B. cereus* group specific primer pair BalF/BalR yielded 533 bp amplified product in all the 42 isolates and *B. cereus* NCIM 2106. Standard strains of other bacteria *viz*. *B. subtilis* NCIM 2545, *Salmonella* Dublin ATCC 15480, *E. coli* NCIM 2068 and *S. aureus* NCIM 2079 were also employed in this PCR. But none of them yielded any amplified product in this PCR reaction (Fig. 1). *hbla* gene specific PCR using primer pair HblA1/HblA2, yielded amplified product of 834 bp only in 30 enterotoxigenic isolates (Fig. 2). None of the non-enterotoxigenic isolates yielded any amplified product in this PCR. Primer pair ENTA/ENTB, which is specific for *entFM* gene, showed 1.3 kb amplified products in all isolates with exception of one (Fig. 3). No relationship was found between enterotoxin production by RPLA result and the presence of *entFM* gene. On the other hand, none of our isolates yielded any amplified product in *bceT* gene specific PCR using primer BceT1/BceT2 irrespective of annealing temperatures *i.e.* 55 or 65°C.

Discussion

The objectives of the study were to find the occurrence of enterotoxigenic *B. cereus* in fishes sold in local markets of Cochin and to evaluate the virulent genes specific PCR technique for differentiation of enterotoxigenic *B. cereus* from non-enterotoxigenic *B. cereus*. We found 29.4 per cent samples positive for enterotoxigenic *B. cereus* while 5 more fish samples were shown to be positive for non-enterotoxigenic *B. cereus*. All isolates yielded 533 bp amplified product using primers BalF/BalR, which is specific for *B. cereus* group[17]. In this study, we found 4 of our isolates had starch-hydrolyzing property but were non-enterotoxigenic. All enterotoxigenic *B. cereus* isolates possessed starch-hydrolyzing property. Agarwal and co-workers[19] also reported the relationship between starch hydrolysis and enterotoxin production in *B. cereus* isolates from meat, fish, milk products, cooked rice and dal. All our 30 enterotoxigenic isolates were positive to *hbla* gene specific PCR. Standard strain *B. cereus* NCIM 2106 was also enterotoxigenic as evidenced by RPLA test and was also positive to *hbla* gene specific PCR. On the other hand, all non-enterotoxigenic isolates were negative in *hbla* gene specific PCR. This indicated that the results of *hbla* gene specific PCR agreed with the results of RPLA assay. This observation was in agreement with that of Mäntynen and Lindstorm[18], who also found all enterotoxigenic *B. cereus* isolates positive to *hbla* gene specific PCR. The *bceT* gene was not been found in any of our isolates. We found that all but one of our isolates possessed *entFM* gene but no relationship could be established between the presence of *entFM* gene and enterotoxin production. All isolates with exception of one were β-haemolytic on 5 per cent sheep blood agar irrespective of whether they were enterotoxigenic or not.

A rapid and prompt method for detection of presence of enterotoxigenic *B. cereus* in food is important to ensure that food products are safe for consumption. The conventional method of differentiation of enterotoxigenic and non-enterotoxigenic strains of *B. cereus* by RPLA test takes long time to yield result. But, PCR is considered as a rapid and reliable method for the detection of presence of specific organism even when the organism is present in low number and the sample is contaminated with some other organisms[20]. In this present study, *hbla* gene specific PCR showed promising result in differentiation of enterotoxigenic and non-enterotoxigenic isolates of *B. cereus*. So, PCR using primers targeting *hbla* gene can be used as an alternative of RPLA in differentiation of enterotoxigenic *B. cereus* isolates from non-enterotoxigenic isolates within a short period of time.

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