Current Scenario in Biotechnology

Editors

P. PONMURUGAN
PUNIETHAA PRABHU
G. AYYAPPADASAN
R. GANESH BABU

Molecular Characterization of Enterotoxigenic *Bacillus cereus* in Tropical Fisheries Environment of Cochin, India

Sanjoy Das* and K.V. Lalitha
Central Institute of Fisheries Technology, Matsyapuri, Cochin, Kerala
E-mail: sanjoy125@yahoo.co.in

INTRODUCTION

Bacillus cereus is a Gram positive spore-forming organism, which causes two different types food poisoning viz. diarrhoeal type and emetic type. Apart from these, *B. cereus* has also been reported to be associated with opthalmitis, respiratory tract infection and central nervous system disorder (Bekemeyer and Zimmerman, 1985; Beer *et al.*, 1990; Barrie *et al.*, 1992). In *B. cereus*, *hbla*, *bceT* and *entFM* are the important genes, which are responsible for diarrhoeal enterotoxin production (Heinrichs *et al.*, 1993; Agata *et al.*, 1995; Asano *et al.*, 1997). Being a spore-former organism, it can survive heat-treated foods. Some strains of *B. cereus* are psychrotrophic in nature and thus can grow even in refrigerated food items (TeGiffel *et al.*, 1997). This organism is also capable of producing biofilm on different food-contact surfaces (Oosthuizen *et al.*, 2002) and the possibility of transmission from those surfaces to different food products can't be ruled out. According to Centers for Disease Control, United States of America, approximately 2% of the total food-borne illnesses in USA are due to enterotoxigenic *B. cereus* (Centers for Disease Control and Prevention, 1994). In European countries, food poisoning caused by enterotoxigenic *B. cereus* is very common and attributes to 47, 33 and 22% of the total food poisoning cases reported in Iceland, Norway and Finland, respectively (Granum and Lund, 1997). Although in India, no major outbreak due to this

*Corresponding author.

pathogen has been reported so far, enterotoxigenic *B. cereus* has been frequently isolated from different types of food items (Kamat *et al.*, 1989; Agarwal *et al.*, 1997; Das *et al.*, 2009). In one study carried out among diarrhoea patients in and around Kolkata, India, *B. cereus* was isolated from 3.5% of the stool samples of the patients (Banerjee *et al.*, 2011). As per the tropical seafood is concerned, it was observed that 24.91% of the samples collected from Cochin and surrounding area were positive to enterotoxigenic *B. cereus* (Das *et al.*, 2009). So, this is very much required to study the occurrence of this pathogen in the tropical fisheries environment. Keeping this in view, the present study was designed to assess the occurrence and molecular characterization of *B. cereus* from tropical fisheries environmental samples of Cochin and surrounding areas.

MATERIALS AND METHODS

Samples and Isolation of the Organism

A total of 51 samples collected during 2009–10 from fish markets and landing centres of Cochin and surrounding areas comprising of 30 ice, 18 mud and sand and 3 sea water were screened for the presence of *B. cereus*. Selective plating technique on Polymixin-pyruvate-egg yolk-mannitol-bromocresol purple agar (PEMPA) was done for Isolation of *B. cereus* (Szabo *et al.*, 1984). Twenty five grams of sample was blended in 225 ml of sterile normal saline in a stomacher blender (Seward Ltd, U.K.) and serial 10 fold dilutions were made on normal saline. From each dilution, 0.5 ml was spread-plated on PEMPA plates. After incubating at 37°C for 24 h, suspected *B. cereus* colonies showing lecithinase reaction were subcultured and subjected to identification using Gram's staining and standard biochemical reactions as mentioned in Bacteriological analytical manual of United States Food and Drug Administration (Rhodehamel and Harmon, 1998). The starch hydrolytic ability of the organisms was checked on nutrient agar containing 1% starch.

Screening of *B. cereus* Isolates for Haemolytic Activity and Production of Enterotoxin

The haemolytic activity of the suspected isolates was checked on spot inoculation on blood agar viz. Columbia agar base with 5% human blood. The blood agar plates were incubated at 37°C for 24 h. The ability to produce diarrhoeal enterotoxin was detected by Reverse passive latex agglutination (RPLA) test using BCET-RPLA kit (Oxoid) as per manufacturer's instruction.

Preparation of Crude PCR Template

Crude PCR template from the isolates was prepared by boiling method. Overnight grown culture of the isolate was centrifuged at 7000 × g for 10 minutes at 4°C. The pellet was washed once with normal saline and suspended in autoclaved MilliQ distilled water. The suspended bacterial cells were lysed on boiling water bath for 10 min. Then it was immediately frozen at –20°C. After freezing, the lysate was thawed and centrifuged at 4000 × g to pellet the debris. 5 µl of the supernatant was used as template in the PCR reaction.

Polymerase Chain Reaction

Total 4 sets of primers were used for this study. For *B. cereus* group specific PCR, BalF and BalR primers were used. All the isolates were screened for the presence of three virulent genes viz. *hbla*, *entFM* and *bceT* genes, which are generally responsible for enterotoxin production. The primer pairs HblA1/HblA2, BceT1/BceT2 and ENTA/ENTB were used to detect the presence of *hbla*, *bceT* and *entFM* genes, respectively. The details of primers including sequences have been depicted in Table 1. PCRs were carried out in 25 µl of reaction mixture, which consisted of 5 µl of template, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas), 1 U *Taq* DNA polymerase (Fermentas) and 10 p mol of respective primers. For each PCR assay at the beginning of the reaction, the initial

Table 1: PCR Primers and Cycling Condition Details

Primer	*Sequence*	*Target and Reference*	*PCR Cycling Condition*
BalF BalR	5'- TGCAACTGTATTAGCACAAGC T -3' 5'- TACCACGAAGTTTGTTCACTACT -3'	*Bacillus cereus* Group (Chang *et al.*, 2003)	94°C for 45 seconds (denaturation), 55°C for 45 seconds (annealing) and 72°C for 45 seconds (extension).
HblA1 HblA2	5'- CTAATGTAGTTTCACCTGTAGCAAC- 3' 5'- AATCATGCCACTGCGTGGACATATAA- 3'	*hbla* gene (Mantynen and Lindstrom, 1998)	94°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 minute.
ENTA ENTB	5'- ATGAAAAAAGTAATTTGCAGG- 3' 5'- TTAGTATGCTTTTGTGTAACC- 3'	*entFM* gene (Asano *et al.*, 1997)	94°C for 45 seconds, 52°C for 45 seconds and 72°C for 1 minute.
BceT1 BceT2	5'- GAATTCCTAAACTTGCACCATCTC G- 3' 5'- CTGCGTAATCGTGAATGTAGTCAAT- 3'	*bceT* gene (Mantynen and Lindstrom, 1998)	94°C for 45 seconds, 55/65°C for 45 seconds and 72°C for 45 second

denaturation was done at 95°C for 5 min and at the end of the cycle, the final extension was carried out at 72°C for 5 min. The other details of PCR cycling condition for each PCR have been mentioned in Table 1. *bceT* gene specific PCR was run at two different annealing temperatures viz. 55 and 65°C. Following PCR, the amplified products were resolved in 1.5% agarose gel prepared in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid and 2 mM EDTA, pH 8.0) containing ethidium bromide (0.3 µg/ml). Electrophoresis was done at 7-volts/cm for 1 hour and the gel was photographed by an UV gel documentation system (Alpha Innotech Corporation, USA).

RESULTS AND DISCUSSION

Occurrence of *B. cereus* and Enterotoxin Production

Among the 51 samples screened, the presence of *B. cereus* was detected in 16 samples (4 ice and 12 mud and sand). Total 41 isolates have been recovered from the positive samples. All the isolates were haemolytic on blood agar and 38 isolates showed starch hydrolytic property. By Reverse passive latex agglutination (RPLA) test, 36 isolates were found to produce diarrhoeal enterotoxin. Those 36 isolates were sourced from 4 ice and 11 mud and sand samples. All the enterotoxin producing isolates were found to hydrolyze starch (Table 2).

Detection by *B. cereus* Group Specific PCR

In PCR using *B. cereus* group specific primers BalF/BalR, all the 41 isolates yielded 533 bp amplified product. Standard strains of *Bacillus subtilis* NCIM 2545 and *Staphylococcus aureus* NCIM 2079 were also employed in this PCR as negative controls, but none of them yielded any amplified product in this PCR.

Screening for Virulence Genes

The isolates were screened for the presence of three enterotoxin producing genes viz. *hbla*, *entFM* and *bceT* genes. *hbla* gene specific PCR using primers HblA1/HblA2 yielded 834 bp amplified product in case of 36 enterotoxigenic isolates. The isolates, which were negative to enterotoxin production by RPLA method, didn't yield any amplified product in this PCR. In case of *entFM* gene specific PCR using ENTA/ENTB primers, 1300 bp amplified product was observed in case of 35 isolates. On the other hand, *bceT* gene specific PCR didn't yield any amplified product in any of the isolates tested. The results of each PCR, enterotoxin production by RPLA test and starch hydrolysis test for each isolate has been depicted in Table 2.

Table 2: Details of *B. cereus* Isolates

Isolates	*Starch Hydrolysis*	*RPLA**	*BCG***	*Hemolysis*	*hbla PCR*	*entFM PCR*	*bceT PCR*
BC-env-1	+	+	+	+	+	+	–
BC-env-2	+	+	+	+	+	+	-
BC-env-3	+	+	+	+	+	+	–
BC-env-4	+	+	+	+	+	–	–
BC-env-5	+	+	+	+	+	+	–
BC-env-6	+	+	+	+	+	+	–
BC-env-7	+	+	+	+	+	+	–
BC-env-8	+	+	+	+	+	+	–
BC-env-9	+	+	+	+	+	+	–
BC-env-10	+	–	+	+	–	+	–
BC-env-11	+	+	+	+	+	+	–
BC-env-12	+	+	+	+	+	+	–
BC-env-13	+	+	+	+	+	+	–
BC-env-14	+	+	+	+	+	+	–
BC-env-15	+	+	+	+	+	+	–
BC-env-16	+	+	+	+	+	+	–
BC-env-17	–	–	+	+	–	–	–
BC-env-18	+	+	+	+	+	+	–
BC-env-19	+	+	+	+	+	+	–
BC-env-20	+	+	+	+	+	+	–
BC-env-21	+	+	+	+	+	+	–
BC-env-22	+	+	+	+	+	+	–
BC-env-23	+	+	+	+	+	+	–
BC-env-24	+	+	+	+	+	+	–
BC-env-25	+	+	+	+	+	+	–
BC-env-26	+	+	+	+	+	–	–
BC-env-27	+	+	+	+	+	–	–
BC-env-28	+	+	+	+	+	+	–
BC-env-29	+	+	+	+	+	+	–
BC-env-30	–	–	+	+	–	+	–
BC-env-31	–	–	+	+	–	+	–
BC-env-32	+	+	+	+	+	+	–
BC-env-33	+	+	+	+	+	+	–
BC-env-34	+	+	+	+	+	+	–
BC-env-35	+	+	+	+	+	+	–
BC-env-36	+	+	+	+	+	+	–
BC-env-37	+	+	+	+	+	+	–
BC-env-38	+	+	+	+	+	+	–
BC-env-39	+	+	+	+	+	+	–
BC-env-40	+	–	+	+	–	–	–
BC-env-41	+	+	+	+	+	–	–

* BCG = Bacillus cereus group specific PCR
** RPLA = Enterotoxin production by RPLA test

The aim of the this study was to study the occurrence of enterotoxigenic *B. cereus* in fishery environmental samples collected from fish markets and landing centers and also to study the presence of different virulence genes in the isolates of *B. cereus*. In this study, 31.37% (16 out of 51) of the samples were found to possess *B. cereus* and enterotoxigenic *B. cereus* was isolated from 15 samples (Occurrence rate 29.41%). The occurrence of enterotoxigenic *B. cereus* was found 13.33% (4 out of 30) and 61.11% (11 out of 18) in case of ice and mud/sand, respectively. On other hand, all the three sea water samples were found negative for this organism. The comparatively high occurrence was found in case of mud and sand collected from the fishery environment. In previous study, it was observed that 29.41% of fishes of tropical area of Cochin possessed enterotoxigenic *B. cereus* (Das *et al.*, 2009). As per this study, it can be stated that occurrence of enterotoxigenic *B. cereus* is moderately high in tropical fisheries environment and the fisheries environment may be possible source of transmission of *B. cereus* to fish.

On screening for virulence genes, it was observed that all the enterotoxigenic isolates possessed *hbla* gene while this gene was absent in all non-enterotoxigenic isolates (Table 2). Similar type of finding was reported by Das *et al.* (2009) in case of *B. cereus* isolates from fish. *entFM* gene was detected in three non-enterotoxigenic isolates, while this gene was not detected in four enterotoxigenic isolates (Table 2). So, it can be stated that no relationship can be established between presence of *entFM* genes and enterotoxin production. *bceT* gene was not detected in any of the isolates. So, this gene can be considered rare in case of tropical fishery environmental isolates of Cochin. So based on this study, it can be concluded that *hbla* gene can be considered as marker for diarrhoeal enterotoxin production in case of *B. cereus* isolates from tropical fishery environment.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Central Institute of Fisheries Technology for providing necessary facilities for conducting this research work.

REFERENCES

Agarwal, R.K., Kapoor, K.N., Bachhil, V.N., Singh, B.R., Kumar, A. and Bhilegaonkar, K.N. (1997). Studies of toxigenicity of *Bacillus cereus* strains isolated from food. *Ind J Comp Microbiol Immunol Infect Dis*, 18: 162–165.

Agata, N., Ohta, M., Arakawa, Y. and Mori, M. (1995). The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology*, 141: 983–988.

Asano, S.I., Nukumizu, Y., Bando, H., Hzuka, T. and Yamamoto, T. (1997). Cloning of novel enterotoxin gene of *Bacillus cereus* and *Bacillus thuringiensis. Appl Environ Microbiol,* 63: 1054–1057.

Banerjee, M., Nair, G.B. and Ramamurthy, T. (2011). Phenotypic and genotypic characterization of *Bacillus cereus* isolated from acute diarrheal patients. *Ind J Med Res,* 133: 88–95.

Barrie, D., Wilson, J.A., Hoffman, P.N. and Kramer, J.M. (1992). *Bacillus cereus* meningitis in two neurosurgical patients: an investigation into the source of the organism. *J Infect,* 25: 291–297.

Beer, P.M., Ludwig, I.H. and Packer, A.J. (1990). Complete visual recovery after *Bacillus cereus* endophthalmitis in a child. *Am J Ophthalmol,* 110: 212–213.

Bekemeyer, W.B. and Zimmerman, G.A. (1985). Life-threatening complications associated with *Bacillus cereus* pneumonia. *Am Rev Respir Dis,* 131: 466–469.

Centers for Disease Control and Prevetion (1994). *Bacillus cereus* food poisoning associated with fried rice at two day care centers-Virginia, 1993. *Morbidity and Mortality weekly report,* 43: 177–178.

Das, S., Surendran, P.K. and Thampuran, N. (2009). PCR-based identification of enterotoxigenic isolates of *Bacillus cereus* from tropical seafood. *Ind J Med Res,* 129: 316–320.

Granum, P.E. and Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol Lett,* 157: 223–228.

Heinrichs, J.H., Beecher, D.J., Macmillan, J.D. and Zilinskas, B.A. (1993). Molecular cloning and characterization of *hbla* gene encoding the B component of hemolysin BL from *Bacillus cereus. J Bacteriol,* 175: 6760–6766.

Kamat, A.S, Nerkar, D.P. and Nair, P.M. (1989). *Bacillus cereus* in some Indian foods: incidence and antibiotic, heat and radiation resistance. *J Food Safety,* 10: 31–41.

Mantynen, V. and Lindstrom, K. (1998). A rapid PCR-based DNA lest for enterotoxigenic *Bacillus cereus. Appl Environ Microbiol,* 64: 1634–1631.

Oosthuizen, M.C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Holy, A.V. and Brozel, V.S. (2002). Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. *Appl Environ Microbiol,* 68: 2770–2780.

Rhodehamel, E.J. and Harmon, S.M. (1998). *Bacillus cereus.* In Bacteriological Analytical Manual, 8th Ed., Revision A, Chap. 14, U.S. Food and Drug Administration.

Szabo, R.A., Todd, E.C.D. and Rayman, M. (1984). Twenty-four hour isolation and confirmation of *Bacillus cereus* in foods. *J Food Prot,* 47: 856–862.

Te Giffel, M.C., Beumer, R.R., Granum, P.E. and Rombouts, F.M. (1997). Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in Netherlands. *Int J Food Microbiol,* 34: 307–318.

History of the Society

The Society for Biotechnologists (India) (SBTI) is a nonprofit organization formed in 1995. SBTI is an association of eminent biotechnologists, students, researchers, academicians, doctors, industrialists and professionals working in biotechnology. SBTI serves as a liaison between the various specialized fields of theoretical and applied biotechnology, promotes exchange of advanced scientific information through workshops, meetings and publications, in such areas as recombinant DNA technology, neuroscience, cancer biology, industrial biotechnology, molecular biology, medical biotechnology, plant and animal biotechnology, aquatic with marine biotechnology, environmental science, bioprocess technology, stem cell research and nanotechnology.

The society maintains its national headquarters in the Department of Biotechnology, Cochin University of Science and Technology, Cochin-682 022, Kerala, India.

The Institution

K.S. Rangasamy College of Technology was established during the academic year 1994-95 at Tiruchengode, Namakkal district, Tamil Nadu. The campus of K.S. Rangasamy College of Technology is functioning in an expansive area of about 350 acres in K.S.R. Kalvi Nagar located between Tiruchengode and Erode. The institution is run by the K.S. Rangasamy Educational and Charitable Trust. Within a short span of its existence, it has developed into a pioneer institution in providing quality technical education in 12 U.G. and 12 P.G. programmes. The salient features of our college includes accreditation of U.G/P.G courses by the AICTE, well established infrastructure, telecommunication facilities, digital libraries and Centres of Research Excellences etc. The college has been certified with ISO 9001-2000 for its performance followed by NAAC (A Grade).

The Department

The Department of Biotechnology offers B.Tech. Biotechnology at the U.G. level, M.Tech. Biotechnology and M.Sc. Biotechnology at the P.G. level. The department has well equipped laboratories, which permits the students to work and use the facilities at any time. The department has 24 qualified faculty members for imparting quality education. The department conducts seminars, refresher courses and workshops frequently to help the students of biotechnology to acquire knowledge in the latest technologies. It has seven funded projects from DST, DBT, ICMR & DRDO, New Delhi and NTRF, Kolkata which is under Ministry of Commerce. An in-house magazine called BIOZOOM is being released by the department every semester. The faculty members have filed three Indian patents and five US patents so far and published 130 research papers and 12 nucleotide sequences have been deposited in the NCBI.

COVER DESIGN: KAM STUDIO

₹ 2400

ISBN 978-93-82563-27-3

9 789382 563273

BLOOMSBURY PUBLISHING INDIA PVT. LIMITED