Full Length Article

MOLECULAR CHARACTERIZATION, BIOFILM FORMATION AND ANTIBIOGRAM ANALYSIS OF ENTEROTOXIGENIC *BACILUS CEREUS* FROM MILK AND MILK PRODUCTS IN PUDUCHERRY

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ABSTRACT

The present investigation was undertaken to study the occurrence of enterotoxigenic B. cereus from 200 samples including 100 raw milk, 50 pasteurized milk and 50 ice cream samples sold in Puducherry. The results revealed that 68 samples (34 %) belonged to B. cereus group which included 36 from raw milk (36%), 9 from pasteurized milk (18%) and 23 from ice cream samples (46 %). On PCR analysis, 64 out of 68 isolates showed the presence of gyrB gene which differentiates B. cereus from B. cereus group isolates. Multiplex PCR revealed the presence of four enterotoxigenic genes hblA, nheA, cytK and entF Min 32.35 %, 77.94 %, 54.41 % and 94.12 % of the 64 isolates. On biofilm production assay, out of 64 enterotoxigenic B. cereus isolates, 60 (93.75 %) isolates showed the ability to form biofilm in which 13 (20.31 %) were strong, 21 (32.81 %) were moderate and 26 (40.63 %) were weak biofilm producers. All the isolates were resistant to ampicillin and penicillin G and sensitive to ciprofloxacin, gentamicin, vancomycin, streptomycin and norfloxacin. The current study directs the need for proper hygienic measures during collection, production, processing and storage of milk to reduce the contamination level. It also indicates the need for surveillance and action to reduce the contamination of enterotoxigenic B. cereus in milk and milk products in Puducherry.

Key words: *Bacillus cereus*, milk, enterotoxin, biofilm, antibiogram

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INTRODUCTION

Bacillus cereus is a Gram-positive, rod-shaped, motile, spore-forming opportunistic pathogen. They are commonly found in soil, air, grains, rice (row and cooked), vegetables,

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meat and milk. It can grow at temperature from 4° to 50°C and withstand heat and chemicals (Vidic *et al.,* 2020). As this microorganism is ubiquitous in nature, it can enter the dairy processing chain at any stage during milking, production, processing, storage and ripening. They resist the pasteurization process and endospores may revive by germination and out growth and produce spoilage enzymes (proteases, lipases and phospholipases) in the pasteurized milk leading to off-flavours (Tirloni *et al.,* 2022). *B. cereus* causes two distinct types of food poisoning *i.e.,* diarrhoea and emesis caused by two different types of toxins. The emetic type is characterized by the occurrence of nausea and vomiting within 6 hrs after ingestion, caused by small cyclic heat-stable peptide, cereulide (Kumari and Sarkar, 2014) and the diarrhoeal type characterized by the occurrence of abdominal pain and watery diarrhoea within 8 to 16 hrs after ingestion, caused by variety of extracellular toxins including four main type of enterotoxins namely hemolysin BL (*hbl*), non-hemolytic enterotoxin (*nhe*), cytotoxin K (*cyt*K) and enterotoxin FM (*ent*FM). *B. cereus* efficiently form biofilms, although preferentially at air-liquid interfaces under the tested conditions (Wijman *et al*., 2007). Presence of biofilm may lead to recurrent contamination and spoilage of dairy products. *B. cereus* is typically resistant only to *β*–lactams, whereas usually susceptible to aminoglycosides, chloramphenicol, clindamycin, erythromycin, tetracycline and vancomycin (Etikala *et al.,* 2022). Considering the above facts, the present study was undertaken to detect the presence of enterotoxigenic *B. cereus* from milk and milk products in Puducherry.

MATERIALS AND METHODS

Sample collection and processing

A total of 200 samples comprising of Raw milk (100), pasteurized milk (50) and ice cream (50) were collected from different parts of Puducherry. Samples were collected aseptically and transported under refrigerated condition to the laboratory. The collected samples were processed in the Biosafety level - II laboratory in the Department of Veterinary Public Health and Epidemiology, RIVER, Puducherry.

Isolation and identification of *B. cereus*

The procedure for isolation of *B. cereus* group was carried out as described by Shinagawa (1990) with suitable modifications. A homogeneous sample was prepared in 0.85 per cent saline to give an initial 1:10 dilution by thoroughly mixing 10 ml of the sample with 90 ml of diluent. Aliquots of 0.1 ml direct sample and sample homogenates were inoculated on Polymyxin Pyruvate Egg yolk Mannitol Bromothymolblue Agar (PEMBA) by spread plate method and incubated at 37°C for 24 hrs. The typical crenate to fimbriate peacock blue coloured colonies (3-5 mm) surrounded by blue zone of egg yolk hydrolysison PEMBA were presumptively designated as *B. cereus* group. The suspected colonies of *B. cereus* group were subjected to various morphological and biochemical characterization described by Barrow and Feltham (1993).

Molecular characterization of enterotoxigenic *B. cereus*

Molecular confirmation of *B. cereus* was done through detection of *gyr*B gene for differentiation from *B. cereus* group by Polymerase Chain Reaction (PCR) as described by Park *et al.* (2007). The DNA was extracted by using boiling lysis method (Sowmya *et al.,* 2012). The PCR assay was performed in 25 µl reaction volume containing 12.5 µl of master mix, 1 µl of forward and reverse primers each (10 pmol), 5 µl of DNA template and 5.5 µl of nuclease free water. The cyclic condition of the PCR included primary denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 5 min.

Simultaneously all the isolates were screened for four enterotoxigenic genes (*hbl*A, *nhe*A, *cyt*K and *ent*FM) by Multiplex PCR as per Ngamwongsatit *et al.* (2008). The PCR assay was performed in 25 µl reaction volume containing 12.5 µl of master mix, 1 µl each of 4 forward and reverse primers (10pmol), 3 µl of DNA template and 1.5 µl of nuclease free water. The cyclic condition of the mPCR included primary denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 60 sec and extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min. The amplified PCR products were run in 1.5% agarose gel and analyzed using gel documentation system (Bio- rad). The primers used in this study are given in the table 1.

Biofilm production assay

Determining of adherence and biofilm forming ability of the isolates was analysed by qualitative test tube methodas described by Christensen *et al*. (1982) with slight modification. Briefly, a loop full of the bacterial culture from the agar plate was inoculated into a sterile glass test tube containing five ml of BHI broth and incubated at 37°C for 48 hrs. Each tube was decanted and stained with 0.25 % safranin, gently rotated to ensure uniform staining and then the contents were decanted. The tubes were then placed upside-down to ensure draining. The tube's inner surface colour was observed and used for interpretation. An adherent film on the surface of the glass tube was taken as an evidence of slime formation. Based on slime production, the positive results were recorded as strong (+++), moderate (++), weak (+) and the absence of film was represented as negative (-) (Murugan *et al*., 2010).

Antibiogram

Antibiotic susceptibility tests were performed for *B. cereus* according to the Clinical and Laboratory Standards Institute (CLSI, 2013) using commonly used antibiotics. Briefly the isolates were grown in nutrient broth at 37°C for 16 hrs. Individual broth cultures were smeared on the Mueller-Hinton (MH) agar plates with the help of sterile cotton swabs. The plates were allowed to dry for few minutes. The antibiotic discs were placed on the agar surface within 15 min of inoculation of plates and incubated overnight at 37°C. The isolates were tested against ampicillin (10 mcg), amoxyclav (30 mcg), chloramphenicol (30 mcg), co-trimaxazole (10 mcg), ciprofloxacin (30 mcg), cefotaxime (30 mcg), gentamicin (30 mcg), penicillin G (10 units), tetracycline (30 mcg), erythromycin (15 mcg), vancomycin (5 mcg), streptomycin (10 mcg), nitrofurantoin (300 mg), norfloxacin (10 mcg), doxycycline hydrochloride (30 mcg) and ofloxacin (5 mcg) antibiotic discs. Based on the zone of inhibition, the isolates were graded as sensitive, intermediate and resistant as per the interpretative chart provided by the manufacturer (HiMedia Laboratories, Mumbai).

RESULTS AND DISCUSSION

In the present study, out of the 200 samples screened, thirty six (36 %) samples from raw milk, nine (18 %) from pasteurized milk and twenty three samples (46 %) from ice cream produced typical crenate to fimbriate peacock blue coloured colonies (3-5 mm) surrounded by blue zone of egg yolk hydrolysis on PEMBA with an overall occurrence of 34%. When subjected to biochemical tests, it was found that all the isolates were positive for catalase, motility, Voges-Proskauer test, citrate utilization, growth in 7 per cent NaCl and nitrate reduction tests. The isolates were negative for oxidase test, indole production and methyl red tests. Also, 64 out of 68 isolates produced β hemolysis on 5 % sheep blood agar. The aerobic spore forming bacilli with lecithinase positive and mannitol non fermenting character clearly indicates that they belong to *B. cereus* group. (Table 2).

The occurrence of *B. cereus* in the raw milk in this study was in line with the findings of Adamski *et al.* (2023) who reported 38.9 % of raw milk samples were contaminated with *B. cereus* in Poland whereas Tewari *et al.* (2013) and Ashraf *et al*. (2023) reported 11.3 % and 16.7 % of raw milk samples were contaminated with *B. cereus* in Uttarakhand and Egypt respectively. These findings may be attributable to the variations in environmental factors such as temperature, humidity and geographical location which significantly affect the growth and survival of *B. cereus* along with poor milking, handling and storage practices.

Earlier studies (Yamada *et al*., 1999; Park *et al*., 2007) suggested the use of *gyr*B genes that encode the subunit B protein of DNA gyrase (topoisomerase type II) for the differentiation of *B. cereus* from other species. The *gyr*B gene was present in 64 out of 68 isolates (94.1 %) in the present study with a desired product size of 475 bp (Fig. 1). Murugan and Villi (2014) observed *gyr*B gene in all the 28 *B. cereus* isolated from milk and dairy products in Namakkal, India. Also, Meena *et al.* (2019) obtained *gyr*B gene in 10 out of 10 isolates from milk and milk products in Rajasthan, India. These results show that the detection of *gyr*B gene based PCR can be used as an effective tool for the confirmation of *B. cereus* isolates.

The pathogenicity of *B. cereus* group, whether intestinal or non-intestinal, is intimately associated with tissue-destructive/ reactive exo-enzyme production. In the present study, all 68 isolates when screened by mPCR, revealed 64 isolates (94.12 %) carried enterotoxigenic genes of *B. cereus* and none of the enterotoxigenic genes were present in 4 isolates (5.88 %) which were also negative for *gyr*B gene. Among these *B. cereus* group isolates, *hbl*A, *nhe*A, *cyt*K and *ent*FM genes were present in 32.35 %, 77.94 %, 54.41 % and 94.12 % with a desired product size of 884 bp, 759 bp, 565 bp and 486 bp respectively (Fig. 2).

Concordant report was given by Tewari *et al*. (2015), who reported that *ent*FM, *nhe* complex, *cyt*K and *hbl* complex genes were found in 93%, 89.72 %, 41.4 % and 55.2 % of *B. cereus* isolates respectively in Uttarakhand, India. Also, Meena *et al.* (2019) reported *cyt*K and *hbl*A genes in 60% and 40% of the isolates obtained from milk and milk products in Rajasthan, India. This study shows a high occurrence of enterotoxigenic genes *viz. hbl*A*, nhe*A*, cyt*K and *ent*F Min ferring that *B. cereus* isolated from milk and milk products could be regarded as potential pathogenic strains indicating possible risk of food borne infection.

B. cereus biofilms may develop particularly in industrial storage and piping systems and these biofilms can act as a nidus for spore formation and subsequently can release their spores into food production environments. Germination and subsequent outgrowth of spores may result in the development of biofilms (Wijman *et al.,* 2007). In the present study, out of 64 enterotoxigenic *B. cereus* isolates, 60 (93.75 %) isolates showed the ability to form biofilm in which 13 (20.31 %) were strong, 21 (32.81 %) were moderate and 26 (40.63 %) were weak biofilm producers whereas remaining 4 (6.25 %) isolates were non biofilm producers (Table 3).

Similar results were observed by Kumari and Sarkar (2014) who analysed biofilm forming potential of 73 *B. cereus* group isolates from milk and milk products in India reported most of the isolates (54 %) were weak biofilm formers, 9 % were moderate and 8 %were strong biofilm formers. Didouh *et al.* (2023) studied the biofilm forming ability of 31 *B. cereus* isolates from milk samples in Algeria who observed 3.1 %, 12.1 % and 9.1% were strong, moderate and weak biofilm formers respectively.

Since majority of the isolates in the present study were biofilm formers with a good number of moderate and strong biofilm producers, the biofilms formed by them in dairy processing lines can be responsible for recurrent contamination and spoilage of dairy products or facilitate transmission of diseases.

The extensive use of antibiotics in the livestock production systems could lead to the presence of antimicrobial resistance genes in bacteria that contaminate or naturally occur in milk and dairy products, thereby introducing them into the food chain. All enterotoxigenic *B. cereus* isolates obtained in this study were resistant to ampicillin and penicillin G, whereas sensitive to ciprofloxacin, gentamicin, vancomycin, streptomycin and norfloxacin (Table 4).

Table 1: Details of the primers used for *gyr*B gene and enterotoxigenic genes of *B. cereus* group

Sl.No.	Target gene	Primer	Primer sequence (5'→3')	Size (bp)
1	*gyr*B	Forward Reverse	TCATGAAGAGCCTGTGTACG CGACGTGTCAATTCACGCGC	475
2	*hbl*A	Forward Reverse	GCAAAATCTATGAATGCCTA GCATCTGTTCGTAATGTTTT	884
3	*nhe*A	Forward Reverse	TAAGGAGGGGCAAACAGAAG TGAATGCGAAGAGCTGCTTC	759
4	*cyt*K	Forward Reverse	CGACGTCACAAGTTGTAACA CGTGTGTAAATACCCCAGTT	565
5	*ent*FM	Forward Reverse	GTTCGTTCAGGTGCTGGTAC AGCTGGGCCTGTACGTACTT	486

Table 2: Identification tests for enterotoxigenic *B. cereus* group

Sl. No.	Identification tests	Reaction
1	Gram' staining	Gram + rods
2	Spore staining	Positive
3	Catalase	Positive
4	Oxidase	Differential
5	Motility	Variable
6	Indole production	Negative
7	Methyl red	Negative
8	Voges-Proskauer	Positive
9	Citrate utilization	Differential
10	Growth at 7 per cent NaCl	Differential
11	Gelatin hydrolysis	Negative
12	Nitrate reduction	Positive
13	Haemolysis on 5 per cent sheep blood agar	Differential

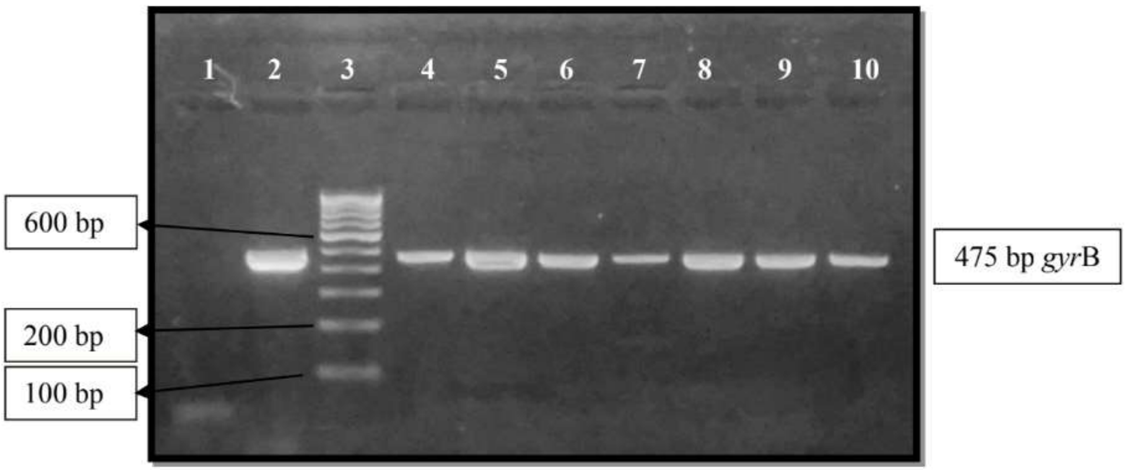

Fig. 1: Detection of *gyr*B gene of *B. cereus*

Lane 1- Negative template control
Lane 2 - Positive control
Lane 3 - 100 bp ladder
Lane 4 to Lane 10 - Positive field isolates

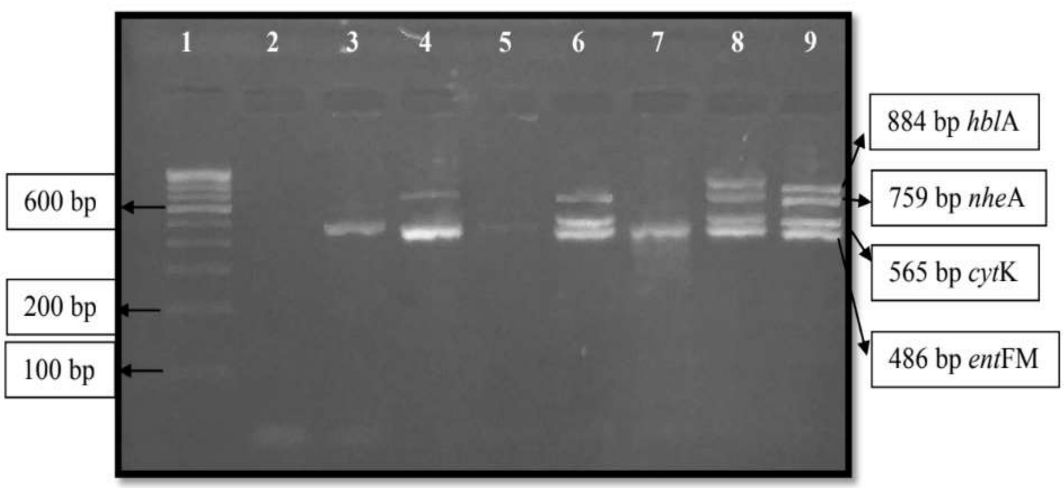

Fig. 2: mPCR profile of *B. cereus* group isolates for enterotoxigenic genes

Lane 1 - 100 bp DNA ladder
Lane 2 - Negative control
Lane 3 - Positive for *ent*FM
Lane 4 - Positive for *nhe*A and *ent*FM
Lane 5 - Negative for all enterotoxigenic genes
Lane 6 - Positive for *cyt*K
Lane 7 - Positive for *ent*FM
Lane 8 and Lane 9 - Positive for *hbl*A, *nhe*A, *cyt*K and *ent*FM

Table 3: Biofilm forming potential of enterotoxigenic *B. cereus* isolates

Biofilm forming potential	Raw milk isolates n=32 (%)	Pasteurized milk n=9 (%)	Ice cream n=23 (%)	Total n=64 (%)
Strong	6 (18.75)	2 (22.22)	5 (17.39)	13 (20.31)
Moderate	9 (28.13)	5 (55.56)	7 (30.43)	21 (32.81)
Weak	15 (46.87)	2 (22.22)	9 (39.13)	26 (40.63)
Negative	2 (6.25)	0 (0)	2 (8.69)	4 (6.25)

Table 4: Antibiogram of all enterotoxigenic *B. cereus* isolates

Sl. No.	Antibiotics	Total % (64)		
		R	I	S
1	Amoxyclav	98.4 (63)	-	1.6 (1)
2	Ampicillin	100 (64)	-	-
3	Cefotaxime	79.7 (51)	15.6 (10)	4.7 (3)
4	Chloramphenicol	4.7 (3)	9.4 (6)	85.9 (55)
5	Ciprofloxacin	-	-	100 (64)
6	Co- trimoxazole	56.3 (36)	32.8 (21)	10.9 (7)
7	Doxycycline hydrochloride	-	6.3 (4)	93.8 (60)
8	Erythromycin	31.3 (20)	12.5 (8)	56.3 (36)
9	Gentamycin	-	-	100 (64)
10	Nitrofurantoin	35.9 (23)	29.7 (19)	34.4 (22)
11	Norfloxacin	-	-	100 (64)
12	Ofloxacin	1.6 (1)	1.6 (1)	96.9 (62)
13	Penicillin G	100 (64)	-	-
14	Streptomycin	-	-	100 (64)
15	Tetracycline	3.1 (2)	15.6 (10)	81.3 (52)
16	Vancomycin	-	-	100 (64)

R – Resistant, I – Intermediate and S - Sensitive

The findings of the present study regarding susceptibilities to these agents are in concordance with Abraha *et al.* (2017) who investigated 103 milk samples in Ethiopia and reported that, all isolates of *B. cereus* showed resistance to penicillin, ampicillin whereas 100 % susceptible to vancomycin and enrofloxacin and majority of isolates (80 %) showed susceptibility towards doxycycline. Ashraf *et al.* (2023) studied the antibiogram of *B. cereus* isolates obtained from milk and dairy products in Egypt who observed 100 % resistance to ampicillin and 100 % susceptible to vancomycin followed by 93.3 % susceptible to tetracycline.

In the present investigation the antibiotic resistance pattern was almost similar among the *B. cereus* isolates from different sources. It was also found that ciprofloxacin, gentamicin, vancomycin, streptomycin and norfloxacin were the most effective antibiotics against *B. cereus*. Many of the *B. cereus* isolates obtained in the study were multidrug resistant which could be of major health concern.

CONCLUSION

The presence of *B. cereus* in raw milk and milk products in Puducherry is a matter of public health concern. Its contamination could cause adverse health effects and produce economic loss due to spoilage of milk and milk products by heat stable enzymes. Implementation of hazard analysis and critical control points (HACCP) and good manufacturing practices (GMP) in dairy processing lines could reduce the contamination level.

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