Indian Journal of Animal Sciences **96** (5): 346–351, May 2026/Article
https://doi.org/10.56093/ijans.v96i5.171894

Molecular characterization and genotyping of *Bacillus cereus* isolated from buffalo raw milk and milk products

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Received: 19 September 2025; Accepted: 03 July 2026

ABSTRACT

Bacillus cereus, a ubiquitous organism, is mainly responsible for foodborne illness. The present study was conducted in order to determine the incidence, virulence gene and antibiogram profiles along with genetic diversity of *B. cereus* isolated and confirmed through PCR assay from different milk and milk products (raw milk, basundi, burfi, junnu (colostrum milk pudding), kalakhand and khoa). *B. cereus* was isolated from 73 (22.81%. 73/320) different samples by cultural method (20 raw milk, 9 basundi, 11 burfi, 3 flavoured milk, 10 junnu, 10 kalakand and 10 khoa samples). Of the 73 cultural positive isolates, 31 (9.68%, 31/320) were confirmed to be *B. cereus* by species specific PCR (9.68%) with 8% (12/150), 15% (3/20), 12.1% (4/33), 11.8% (2/17), 10.8% (4/37) and 21.4% (6/28) occurrence in raw milk, basundi, burfi, colostrum milk pudding (junnu), kalakhand and khoa samples, respectively and none of the flavoured milk and ice cream samples were positive for *B. cereus* by PCR The virulence genes, *nheA*, *nheB, entFM* and *nheC* were found. None of the isolates were positive for the *hbl* complex and *cytK* genes. The three component Nhe complex (all *nheA*, *nhe B* and *nheC genes*) was detected in some isolates, indicating the possibility of non-haemolytic diarrheal food poisoning syndrome. All the isolates were resistant to beta-lactam antibiotics but highly sensitive to gentamicin and tetracycline. ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction) found to be superior to REP- PCR (Repetitive Extragenic Palindromic PCR) in differentiating the *B. cereus* isolates in this study.

Key words: *Bacillus cereus,* Enterotoxin, Genetic diversity, mPCR

B. cereus is a Gram-positive, motile, facultatively aerobic spore former. Due to the ubiquity of the organism and ability to form highly resistant spores, it poses a great threat to food safety, particularly of milk and milk products (Tallent *et al.* 2012). The high resistance of spores to pasteurization temperature, its adhesion to various metallic surfaces and milking equipment and ability to form biofilm make *B. cereus* a high and persistent contaminant of dairy products. Soiling of the animal udder acts as main source of contamination for the raw milk. Some of the strains of *B. cereus* are psychrotrophic, so can be easily grown in refrigerated dairy products (Montanhini *et al.* 2014) and responsible for two types of food poisoning, one is early "emetic syndrome" responsible for nausea and vomiting and the other one is late "diarrheal syndrome" characterized by diarrhoea and abdominal pain (Kramer and Gilbert 1989). *B. cereus* produces three different enterotoxins of which two are three component enterotoxins i.e. Hbl (*hblA, hblC* and *hblD*) and Nhe (*nheA, nheB* and *nheC*). All the three components are required for maximum biological activity and the other enterotoxin is *cytK* (Beattie and Williams 2002). Emergence of multi drug resistant *B. cereus* strains is of public health importance and is mainly due to long term use or abuse of antibiotics and horizontal transfer among the strains of *B. cereus* (Agerso *et al.* 2002). In recent years, there has been a growing concern over its ability to cause non gastro intestinal infections mainly in immune compromised or debilitated patients. In this regard, the present study was conducted to determine occurrence, virulence profile, antibiogram and genotyping of *B. cereus* in various raw buffalo milk and milk products collected from different areas of Andhra Pradesh and provide an insight about the common contamination

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sources, public health risks and helps to take measures towards outbreak containment.

MATERIALS AND METHODS

Isolation and identification of Bacillus cereus: In the present study, a total of 320 samples comprising of 150 raw buffalo milk samples and 170 milk product samples were collected (20 basundi samples, 33 burfi samples, 20 flavoured milk samples, 15 ice cream samples, 17 junnu samples, 37 kalakhand and 28 khoa samples) from different locations in Krishna, Eluru, East Godavari and Vizianagaram districts of Andhra Pradesh, India under aseptic conditions (Supplementary Figure-1). Briefly, ten grams of each of milk product samples collected from various sources were mixed with 90 mL of Phosphate Buffer Saline (PBS) and homogenized. Following homogenization, one mL of suspension was inoculated in nine mL of Brain Heart Infusion (BHI) broth for enrichment and incubated at 37°C for 24 h aerobically. Then the cultures were streaked on selective agar i.e MYP (Mannitol Egg Yolk Polymyxin) agar supplemented with polymyxin B and incubated at 37°C for 18-24 h (Fuchs *et al.* 2022). Two to five typical eosin pink colonies with a surrounding zone of precipitation were selected as presumptive *B. cereus* colonies (Supplementary Figure-2) and inoculated into BHI broth and incubate at 37°C for 24 h. The enriched broth was used for further DNA extraction and antibiogram studies.

Genomic DNA extraction and PCR assay: Genomic DNA was extracted by boiling and chilling method (Bindu *et al.* 2021). About 1-1.5 mL of enriched BHI broth was centrifuged at 8000 rpm for 10 min and kept in water bath at 100°C for 10 min, then the tubes were immediately placed on ice for 15 min and again centrifuged at 10,000 rpm for 5 min to obtain the genomic DNA. The extracted DNA was stored at -20 °C until further use. The concentration of DNA was determined with spectrophotometer and adjusted to 50 ng/µL. Standard DNA of *B. cereus* (ATCC 10876) procured from Himedia was used as positive control for PCR assays. All *B. cereus* isolates characterized biochemically were further confirmed by species specific *gyrB* gene primers BCI: ATTGGTGACACCGATCAAACA and BC 2r: TCATACGTATGGATGTTATTC (Rather *et al.* 2011). Standard PCR mixture (25 µL) comprising of 12.5 µl. of 2x master mix (Go Taq Green Master Mix, Promega) 1.25 µL forward primer (10 pmol/µL), 1.25 µL reverse primer (10 pmol/µL), 2 µL template DNA and 8 µL nuclease free water was used for amplification of DNA under standardized PCR conditions: initial denaturation at 95°C for 5 min followed by 30 cycles of amplification at 95°C for 1 min (denaturation), 57°C for 1.5 min (annealing), 72°C for 2.5 min (extension) and final extension at 72°C for 7 min (Rather *et al.* 2011).

Detection of virulence genes: This study targeted detection of eight virulence/enterotoxin genes (Supplementary Table-1): haemolytic BL (*hblA*, *hblC* and *hblD*), Non Hemolytic Enterotoxins (*nheA*, *nheB* and *nheC*), cytotoxin K (*cytK*) and enterotoxin FM (*entFM*). Using m-PCR optimized in 25 µL reaction mixture containing 12.5 µl. of 2x master mix (Go Taq Green Master Mix, Promega), 4 µL forward primer (10 pmol/ µL), 4 µL reverse primer (10 pmol/ µL), 2 µL template DNA and 2.5 µL nuclease free water under standardized amplification conditions: initial denaturation at 95°C for 5 min followed by 30 cycles of amplification for final denaturation at 94°C for 45 sec, annealing at 54°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min (Rather *et al.* 2011). PCR amplicons for species specific *gyrB* gene and toxin genes were subjected to gel electrophoresis using 1.5% agarose gel stained with ethidium bromide and the bands were visualised using gel documentation unit (BIORAD, USA).

Antimicrobial susceptibility testing: All *B. cereus* isolates were tested for antimicrobial susceptibility using twelve different commonly used antibiotics by Kirby-Bauer disc diffusion method on Muller Hinton agar according to performance standards of Clinical and Laboratory Standards Institute (CLSI 2015). The twelve antibiotics tested included amoxicillin + clavulanic acid (AMC, 30 µg), cefepime (CPM, 50 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), clindamycin (CD, 10 µg), colistin (CL, 10 µg), erythromycin (E, 10 µg), gentamicin (GEN, 10 µg), penicillin-G (P, 10 U), tetracycline (TE, 30 µg), rifampicin (RIF, 5 µg) and vancomycin (VA, 30 µg). Direct-colony suspension of each isolate was made in PBS (P^H 7.4) and the turbidity was adjusted to 0.5 McFarland units (equivalent to approximate cell density of $1.5*10^8$ CFU/ mL) using PBS (pH 7.4). Since there was no data regarding *B. cereus* susceptibility patterns, the inhibition zones of *Staphylococcus aureus* were used as interpretative zone criteria for *B. cereus* as given in Clinical and Laboratory Standards Institute guidelines (Fraccalvieri *et al.* 2022)

Genetic diversity of B. cereus isolates: Genetic diversity among *B. cereus* isolates was analysed by ERIC PCR and REP PCR as described by Versalovic *et al.* (1991) and Lee *et al.* (2012), respectively. The ERIC-PCR reaction mixture (25 µL) contained 12.5 µL of 2x master mix (Go Taq Green Master Mix, Promega), 1.00 µL (10 pmol/ µL) each of forward and reverse primer, 2 µL of template DNA and 8.5 µL of nuclease free water with amplification conditions: initial denaturation at 95°C for 7 min, followed by 30 cycles of amplification for final denaturation at 94°C for 30 sec, primer annealing at 50°C for 1 min, extension at 65°C for 8 min and final extension at 65°C for 16 min whereas REP-PCR was done by using single oligonucleotide primer (GTG)5 (5'GTGGTGGTGGTGGTG 3) with PCR reaction mixture (25 µL) containing 12.5 µL master mix, 2 µL of $(GTG)_5$ primer, 2 µL DNA template and 8.5 µL nuclease free water under standardized conditions: initial denaturation at 95°C for 7 min followed by 30 cycles of amplification at 94°C for 30 sec (final denaturation), 40°C for 1 min (primer annealing), 65°C for 8 min (extension) and a final extension at 65°C for 16 min.

Table 1. Distribution of virulence genes in *B. cereus* isolates

Type of sample	No. of samples analyzed	No. of *B. cereus* isolated by cultural method	No. of *B. cereus* isolated by PCR	No. of *B. cereus* isolates showing atleast one virulence gene	nheA	nheB	nheC	entFM
Raw milk	150	20	12	9	2	4	8	5
Basundi	20	9	3	2	1	1	1	2
Burfi	33	11	4	3	2	2	2	2
Flavoured milk	20	3	-	-	-	-	-	-
Junnu	17	10	2	2	1	2	1	1
Ice cream	15	-	-	-	-	-	-	-
Kalakhand	37	10	4	3	1	1	1	2
Khoa	28	10	6	4	3	2	3	2
Total	320	73	31	23	10	12	16	14

* None of the isolates showed presence of *hblA, hblC, hblD* and *cytK* genes

RESULTS AND DISCUSSION

In the current study, *B. cereus* was isolated from 73 (22.81%. 73/320) different samples by cultural method (20 raw milk, 9 basundi, 11 burfi, 3 flavoured milk, 10 junnu, 10 kalakand and 10 khoa samples). Of the 73 cultural positive isolates, 31 (9.68%, 31/320) were confirmed to be *B. cereus* by species specific PCR (9.68%) with 8% (12/150), 15% (3/20), 12.1% (4/33), 11.8% (2/17), 10.8% (4/37) and 21.4% (6/28) occurrence in raw milk, basundi, burfi, colostrum milk pudding (junnu), kalakhand and khoa samples, respectively (Table 1) and none of the flavoured milk and ice cream samples were positive for *B. cereus* by PCR (Fig-1). The existence of *B. cereus* in dairy products might be due the hydrophobicity of the spore which could have resulted in their stronger adhesion to different surfaces and its survival during processing of milk and milk products (Iurlina *et al.* 2006). In our study, the higher isolation rates were observed in khoa, which might be due to improper storage conditions, packaging and poor hygiene level of the place from where the samples were collected. The findings were similar to that of Murugan and Villi (2014) who reported 10.4% prevalence (28/269) in milk and different milk products.

Among 31 *B. cereus* isolates, 23 (74.2%) isolates were positive for atleast one of the targeted virulence genes. Of the 31 *B. cereus* isolates, *nheA*, *nheB* and *nheC* genes were found in 10 (32.3%), 12 (38.7%) and 16 (51.6%) isolates, respectively whereas *entFM* was detected in 14 (45.2%) of the isolates (Fig 2). None of the isolates were positive for the *hbl* complex and *cytK* genes (Table 1). One isolate showed presence of four genes, *nheA*, *nheB*, *nheC* and *entFM*. In contrast, Osama *et al.* (2020) reported presence

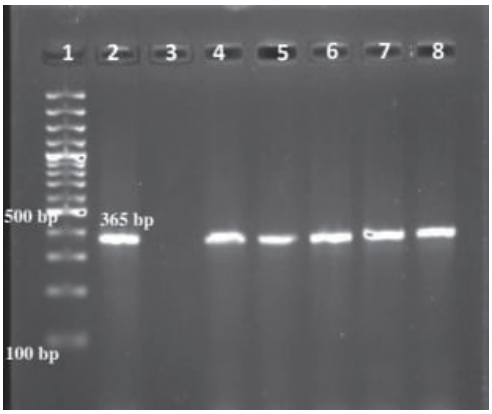

Fig.1. Species specific *gyrB* gene of *Bacillus cereus*
Lane 1: 100 bp DNA ladder;
Lane 2: Positive control of *B. cereus* ATCC10876
Lane 3: Negative control (nuclease free water)
Lane 4-8: *B. cereus* isolated from different samples

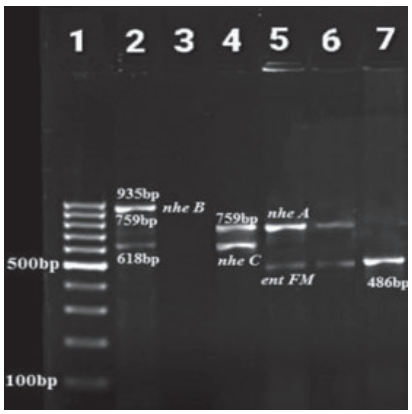

Fig. 2. Virulence/enterotoxin genes of *Bacillus cereus* isolates
Lane 1: 100 bp DNA ladder
Lane 2: Positive control *B. cereus* ATCC10876 showing *nheC* (618bp) and *nheB* (935bp)
Lane 3: Negative control (nuclease free water)
Lane 4: *B. cereus* isolate showing *nheC* (618 bp) and *nheA* genes (759 bp)
Lane 5-6: *B. cereus* isolates showing *nheA (*759) and *entFM* (486 bp)
Lane 7: *B. cereus* isolate showing *entFM* (486 bp)
Lane 8: *nheA* at 759 bp

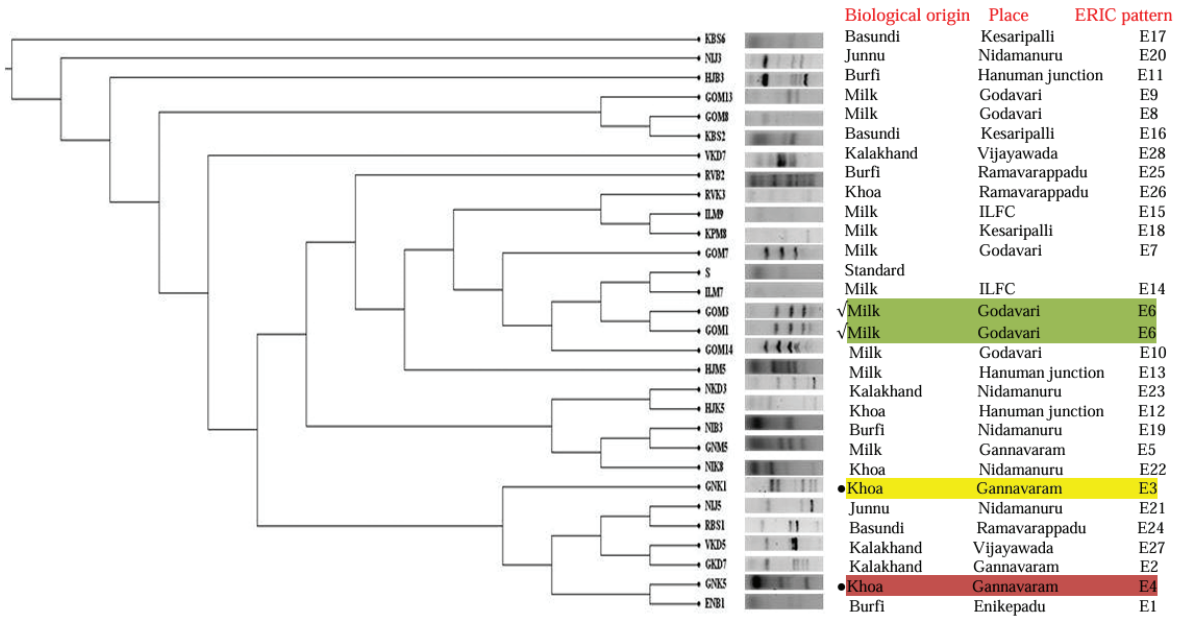

Fig. 3. Dendrogram analysis of ERIC-PCR fingerprints of *B. cereus* isolates from different sources. The dendrogram was generated using " branch and bound method" using dollop program of phylip version 3.6.

of *hblA*, *hblC* and *hblD* genes in 58.6% of *B. cereus* isolates and 86.9%, 93.4% and 89.1% for *nheA*, *nheB* and *nheC*, respectively. The three component Nhe complex (all *nheA*, *nhe B* and *nheC genes*) was detected in 12.9% (4/31) isolates indicating the possibility of non-haemolytic diarrheal food poisoning syndrome (Granum and Lund 1997).

All the 31 *B. cereus* isolates were found highly resistant to penicillin and amoxicillin- clavulanic acid (100%) followed by cefepime (87.1%) whereas all the isolates were highly sensitive to gentamicin and tetracycline (100%) followed by erythromycin (87.1%), chloramphenicol (83.8%) and ciprofloxacin (80.6%) (Supplementary Table-2). The organisms which exhibited resistance to more than three classes of antibiotics were classified as multi drug resistant organisms (Magiorakos *et al.* 2012). In this study, 51.6% (16/31) of the isolates were multi drug resistant showing resistance to more than three antibiotic classes which was in concurrence to the findings of Zhang *et al.* (2017) who reported 50% isolates to be multidrug resistant. All the 16 MDR *B. cereus* isolates were also positive for atleast one of the virulence genes which adds to the public health significance of the isolates.

Molecular typing is best for tracing the sources and understanding the epidemiology of an organism (Gao *et al.* 2018). Genetic diversity analysis could improve the understanding of population characteristics of *B. cereus,* thereby, establishing accurate detection methods for definitive tracking of *B. cereus* and employing targeted prevention and control measures (Park *et al.* 2009). The positions of ERIC and REP elements vary between different species and have been used as some as genetic marker considering one of the best techniques to characterize isolates within bacterial species (Versalovic *et al.* 1991). REP patterns are generally more complex than ERIC patterns but application of both ERIC and REP PCR to the samples to be typed increases the discriminatory power over that of either technique used alone (Wolska and Szweda, 2012). The discriminatory power is determined by Simpson diversity of index (D) as the quantitative measure of the probability of two unrelated strains being distinguished as different types. Discriminatory powers above 0.90 are generally considered highly significant (Hunter and Gaston, 1988).

In the current study, ERIC-PCR fingerprints consist of 2-8 bands ranging from 50 to 900bp whereas REP-PCR revealed 2-8 fragments per isolate ranging from 150 to 850 bp. Out of the 31 *B. cereus* isolates, ERIC-PCR was able to type only 29 isolates whereas REP-PCR demonstrated banding pattern in all the isolates. Two isolates (1 milk and 1 from burfi) did not show any ERIC patterns. Out of 29 *B. cereus* ERIC typable isolates, only 28 distinguishable ERIC patterns were obtained whereas out of 31 *B. cereus* REP typable isolates, only 29 were distinguishable REP patterns. Two milk isolates (GOM1 and GOM3) revealed indistinguishable ERIC and REP PCR genotypes whereas another pair of isolates obtained from khoa samples (GNK1 and GNK5) revealed indistinguishable REP-PCR pattern which were distinguished by ERIC-PCR into two different genotypes (Fig-3 and Fig-4). Cluster analysis of ERIC-PCR profiles differentiated *B. cereus* isolates into five clusters in which the isolates with 70% similarity. The clusters I, II and III were again divided into sub clusters having 90% similarity in ERIC band pattern indicating the possible common source for those isolates. The standard 'S' is sub clustered with milk isolate (ILM7) whereas six other isolates were unclustered separately, indicating the genetic diversity (Supplementary Figure-3). In REP-PCR, cluster

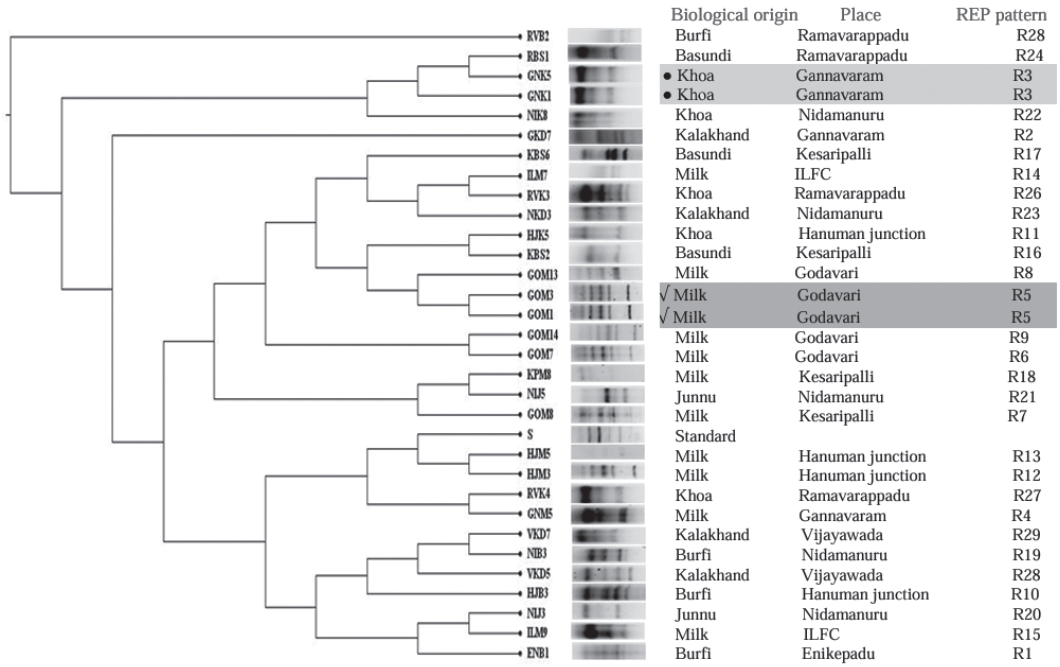

Fig.4. Dendrogram analysis of REP-PCR fingerprints of *B. cereus* isolates from different sources. The dendrogram was generated using " branch and bound method" using dollop program of phylip version 3.6

analysis differentiated *B. cereus* isolates into seven clusters where standard 'S' was clustered with HJM3 and HJM5 (milk samples) with 90% similarity whereas two other isolates (GKD7 and RVB2) were unclustered separately (Supplementary Figure-4). The discriminatory power (D) of two typing methods i.e ERIC-PCR and REP-PCR was found to be 0. 997 and 0.995, respectively which was in correlation with that of Gao *et al.* (2018) who reported a D value of 0.996 for ERIC-PCR, thereby concluding both ERIC and REP-PCR as the best fingerprinting methods for typing of *B. cereus* isolates since the discriminatory powers above 0.90 are considered highly significant.

Bacillus cereus is a common cause of food poisoning related to milk and milk products since it is a common contaminant of raw milk and dairy products and often causes emetic or diarrheal type of food poisoning when dairy items are improperly refrigerated. It is also one of the important opportunistic pathogens and can cause severe potentially fatal local or systemic infections in immune compromised individuals, infants or people with indwelling medical devices. In this study, the high occurrence of *B. cereus* in milk and different milk products indicates the maintenance of poor hygiene at farm level. The isolation and molecular characterization of *Bacillus cereus* from food samples, particularly milk and milk products, helped in assessing public health risks, tracing the contamination sources and guiding food safety protocols. Molecular profiling targeted the specific virulence genes, identifying toxigenic strains which can further help in validating pasteurization efficiency and prevent foodborne illnesses. High occurrence of multidrug resistant *B. cereus* organisms in combination with virulence genes is highly threatening in terms of public health and food safety and is likely leading to hassles in antibiotic therapy. So, a constant vigilance over MDR food microbes is needed as the antibiotic therapy is the main empirical treatment in the treatment of *B. cereus* gastro and non-gastro intestinal infections.

ACKNOWLEDGEMENT

The authors are thankful to Sri Venkateswara Veterinary University, Tirupati for providing the facilities to carry out the current study.

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