Journal of Animal Environment

(E-ISSN 2717-1396, ISSN 2717-1388)

Original Research Paper

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF B. CEREUS CONTAMINATION IN MILK, MEAT, AND STREET FOODS: A STUDY ON PREVALENCE AND FOOD SAFETY.

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ABSTRACT:

Bacillus cereus is a ubiquitous bacterium that poses a significant concern in food safety due to its ability to produce toxins that cause foodborne illnesses. The study assesses the prevalence of B. cereus in meat, milk, and street foods collected from various regions. Perform genotypic characterization to identify key toxin-producing genes [hbl, nhe, cytK], and conduct phenotypic characterization to evaluate toxin production, antibiotic resistance, and environmental adaptability. Analyze the correlation between genotypic and phenotypic traits to understand the pathogenic potential of B. cereus better. A total of 300 samples were collected from various sources, and isolates were subjected to genotypic analysis targeting toxin-producing genes and phenotypic tests for toxin production and antibiotic resistance. The findings revealed a contamination rate of 65%, with a high prevalence of key toxin-producing genes [hbl, nhe, cytK] and notable phenotypic diversity in toxin production. This study highlights the critical need for enhanced food safety measures and a stringent monitoring protocol to mitigate the risks associated with B. cereus contamination in the food supply chain.

KEYWORDS: B cereus, Food safety, Genotypic characterization, Phenotypic characterization, Toxin production, Antibiotic resistance, Meat, Milk, Street foods.

1: INTRODUCTION

Bacillus cereus is a Gram-positive, spore-forming bacterium that is widely present in natural environments such as soil, dust, and raw food materials [1]. It is recognized for its dual pathogenic potential, being responsible for both emetic and diarrheal syndromes in humans. The emetic form arises from cereulide toxin production, whereas the diarrheal form is linked to enterotoxins including hemolysin BL (hbl), non-hemolytic enterotoxin (nhe), and cytotoxin K (cytK) [2]. These virulence factors establish *B. cereus* as a major public health concern, particularly in foodborne outbreaks. Its ability to form spores further complicates control measures, as spores withstand heat, radiation, and desiccation, enabling persistence during food processing and storage [3]. Contamination typically results from inadequate handling, storage, or cooking practices, contributing to widespread foodborne illness globally.

RELEVANCE OF STUDYING CONTAMINATION IN MEAT, MILK, AND STREET FOODS:

Milk, meat, and street foods represent some of the most susceptible categories to *Bacillus cereus* contamination, owing to their rich nutrient content and frequent exposure to handling. Under inadequate storage conditions, these products provide an optimal environment for bacterial proliferation and toxin production [4]. In developing regions, street foods are particularly vulnerable due to poor hygiene practices and limited regulatory enforcement [5]. Likewise, raw and processed milk can serve as reservoirs for *B. cereus* spores, posing risks of foodborne outbreaks [6]. Focusing on these food groups is essential for understanding contamination dynamics and for shaping effective food safety interventions and public health policies.

GAPS IN CURRENT RESEARCH ON GENOTYPIC AND PHENOTYPIC CHARACTERIZATION:

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Received: 25th May, 2026; Revised: 6th June, 2026; Accepted: 8th June, 2026; Available Online: 04rd August, 2026
(DOI): 10.70102/AEJ.2026.18.2s.71

Although numerous studies have examined the prevalence of *Bacillus cereus* in food products, investigations integrating both genotypic and phenotypic characterization of isolates remain scarce. Genotypic analyses generally emphasize the detection of toxin-associated genes, yet they often fail to align with phenotypic attributes such as actual toxin expression or antibiotic resistance [7]. Moreover, data on regional differences in contamination and the impact of diverse food preparation practices on *B. cereus* prevalence are limited. Addressing these research gaps is essential for elucidating the pathogenic potential of *B. cereus* strains and for establishing robust, comprehensive risk assessment frameworks.

GENOTYPIC AND PHENOTYPIC METHODS FOR BACTERIAL CHARACTERIZATION:

Genotypic characterization of *Bacillus cereus* commonly employs molecular approaches such as polymerase chain reaction (PCR) to identify toxin-encoding genes, including *hbl*, *nhe*, and *cytK* [2]. More advanced techniques, such as whole-genome sequencing (WGS), have been utilized to investigate genetic diversity and evolutionary features of *B. cereus* strains [4]. In contrast, phenotypic characterization emphasizes toxin expression, antibiotic resistance profiles, and environmental adaptability. Pathogenic potential is typically assessed using toxin production assays, such as ELISA and cytotoxicity tests [7]. Antibiotic susceptibility studies have further highlighted the growing resistance of *B. cereus* to clinically important drugs, raising concerns regarding therapeutic options for foodborne infections [3].

PREVALENCE IN DIFFERENT REGIONS AND TYPES OF FOOD:

The prevalence of *Bacillus cereus* contamination varies considerably across regions and food categories. In developing countries, street foods often show elevated contamination rates due to weak regulatory oversight and inadequate hygiene practices [5]. Conversely, processed products such as dairy and meat are more frequently contaminated in these settings due to lapses in post-processing sanitation [6]. For instance, a study in Belgium detected *B. cereus* in 47% of food samples, with the highest occurrence in meat and dairy products [7]. Comparable investigations in Asia reported contamination levels ranging from 70% to 80%, underscoring the global scale of the issue [1].

FOODBORNE ILLNESS ASSOCIATED WITH BACILLUS CEREUS TOXINS:

Bacillus cereus is primarily linked to two forms of foodborne illness: emetic and diarrheal syndromes. The emetic form, driven by cereulide toxin, is most often associated with contaminated rice and pasta dishes [2]. In contrast, the diarrheal form results from enterotoxins such as *hbl* and *nhe*, with symptoms typically following the consumption of contaminated meat, milk, or vegetables [4]. Both syndromes represent notable public health concerns, particularly in communities with limited healthcare access. While most infections are self-limiting, severe cases may arise, especially among immunocompromised individuals.

2: MATERIALS & METHODS:

2.1 Sample collection:

Samples were obtained from multiple sources, including retail markets, dairy farms, and street vendors in Hyderabad. The collection encompassed raw meat, both pasteurized and unpasteurized milk, as well as a variety of ready-to-eat street foods such as fried snacks and rice-based dishes.

2.2 Sampling Technique:

Samples were aseptically collected in sterile containers and promptly transported to the laboratory under chilled conditions (4–6 °C) to maintain microbial integrity. In total, 300 samples were obtained over a period of two to three months to ensure representative coverage.

2.3 Isolation and identification:

For the isolation of *Bacillus cereus*, samples were homogenized in sterile saline and cultured on selective media, specifically Mannitol Egg Yolk Polymyxin (MYP) agar. Plates were incubated at 30 °C for 24 hours, after which colonies were examined for characteristic traits. Suspected *B. cereus* colonies—mannitol-negative with a blue appearance and lecithinase-positive, indicated by a precipitation zone—were selected and subcultured on nutrient agar (Oxoid). Identification was confirmed through biochemical assays, including catalase, oxidase, urease, lecithinase activity, nitrate reduction, and carbohydrate fermentation (D-glucose, maltose, D-xylose, lactose, and D-mannitol), as well as growth at elevated temperatures (100 °C). Reference strains *B. cereus* ATCC 11778 and ATCC 14579 were employed for phenotypic validation. Morphological identification was performed using Gram staining to confirm rod-shaped, spore-forming bacteria.

2.4 GENOTYPIC CHARACTERIZATION:

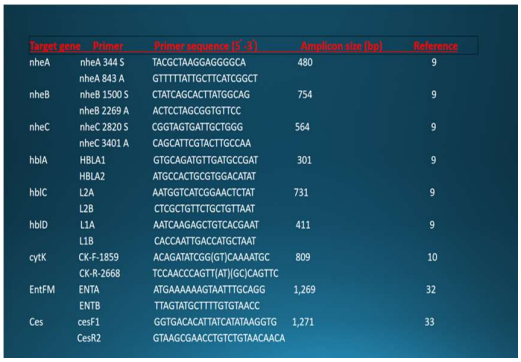
DNA Extraction:

Before DNA extraction, bacterial cultures were prepared by streaking onto nutrient agar and incubating at 36 °C for 24 hours to generate template DNA for PCR screening. Genomic DNA was subsequently extracted using the Instagene Matrix DNA Extraction Kit, following the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

= PCR Screening:

PCR screening was performed to detect toxin-encoding genes such as *hbl*, *nhe*, and *cytK*, along with their variants [2]. The primer sequences used for amplification of *B. cereus* virulence genes are presented in Table 1. PCR reactions were conducted following the protocol described by Kim et al. [8]

Sequence of PCR primers targeting various virulent factor genes shown in Table 1.



Target gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
nheA	nheA 344 S	TACGCTAAGGAGGGGCA	480	9
	nheA 843 A	GTTTTTATTGCTTCATCGGCT		
nheB	nheB 1500 S	CTATCAGCACTTATGGCAG	754	9
	nheB 2269 A	ACTCCTAGCGGTGTTCC		
nheC	nheC 2820 S	CGGTAGTGATTGCTGGG	564	9
	nheC 3401 A	CAGCATTCGTACTTGCCAA		
hblA	HBLA1	GTGCAGATGTTGATGCCGAT	301	9
	HBLA2	ATGCCACTGCGTGGACATAT		
hblC	L2A	AATGGTCATCGGAACTCTAT	731	9
	L2B	CTCGCTGTTCTGCTGTTAAT		
hblD	L1A	AATCAAGAGCTGTCACGAAT	411	9
	L1B	CACCAATTGACCATGCTAAT		
cytK	CK-F-1859	ACAGATATCGG(GT)CAAAATGC	809	10
	CK-R-2668	TCCAACCCAGTT(AT)(GC)CAGTTC		
EntFM	ENTA	ATGAAAAAAGTAATTTGCAGG	1,269	32
	ENTB	TTAGTATGCTTTTGTGTAACC		
Ces	cesF1	GGTGACACATTATCATATAAGGTG	1,271	33
	CesR2	GTAAGCGAACCTGTCTGTAACAACA		

PCR amplification was carried out under the following conditions: an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at gene-specific temperatures for 30 seconds, and extension at 72 °C for 1 minute.

2.5 Phenotypic Characterization:

Antimicrobial susceptibility of *Bacillus cereus* isolates was assessed in Mueller–Hinton (MH) broth using the broth dilution method, following Clinical and Laboratory Standards Institute (CLSI) guidelines [11,12]. Fifteen antibiotics—Amoxicillin, Ampicillin, Cefepime, Chloramphenicol, Ciprofloxacin, Clindamycin, Erythromycin, Gentamicin, Oxacillin, Penicillin, Quinupristin/Dalfopristin, Rifampin, Tetracycline, Trimethoprim/Sulfamethoxazole, and Vancomycin—were tested in two-fold serial dilutions ranging from 64 to 0.015 mg/L in MH broth. The final inoculum was adjusted to approximately 1×10^3 CFU/ml. Cultures were incubated at 35 ± 2 °C for 18–20 hours, and growth was measured using a microplate reader at 610 nm. Breakpoints for *B. cereus* were interpreted according to CLSI guidelines M45-A2E (2010) and M45-P (2005), except for Oxacillin and Quinupristin/Dalfopristin, where breakpoints for *Staphylococcus* spp. from CLSI M100-S22 (2012) and S24 (2014) were applied [12].

Toxin Production Assay:

Toxin production was evaluated using a commercial ELISA kit specific for *Bacillus cereus* toxins, including the BDE-VIA™ (Bacillus Diarrheal Enterotoxin Visual Immunoassay). In addition, cytotoxicity assays were performed on Vero cells to assess pathogenic potential. Quantitative measurements of toxin concentrations were recorded.

Growth Conditions and Environmental Adaptability:

The growth behaviour of *Bacillus cereus* isolates was examined under varying environmental conditions, including different temperatures (10 °C, 30 °C, and 45 °C), pH levels (4.0–9.0), and salt concentrations (0.5%–10% NaCl). These experiments were designed to evaluate the adaptability of isolates to adverse environments

3: RESULTS:

The prevalence of *Bacillus cereus* contamination across food categories is summarized in Table 2. Street foods exhibited the highest contamination rate (65%), followed by meat (45%) and milk (30%). The mean counts (log CFU/g) were 3.8 ± 2.0, 1.8 ± 1.4, and 4.8 ± 1.8, respectively, based on analysis of 300 samples. The elevated prevalence in street foods is likely attributable to poor hygiene practices, inadequate storage, and frequent exposure to environmental contaminants [5]. Seasonal variation was also observed, with higher contamination levels during summer months, presumably due to elevated temperatures that favour bacterial growth.

Table 2: Prevalence and Mean Counts of *Bacillus cereus* in Meat, Milk, and Street Foods

Sample type	Total samples	Positive for B cereus	prevalence	Mean count [log CFU/gm]
Meat	100	45	45%	3.8± 2.0
Milk	100	30	30%	1.8± 1.4
Street foods	100	65	65%	4.8± 1.8

Bacillus cereus is ubiquitous in the environment and exhibits considerable ecological diversity, which enhances its capacity to contaminate a wide range of raw and processed food products, including milk, meat, and street foods [13–18]. In general, ingestion of food containing *B. cereus* cells and/or spores at levels between 10^5 and 10^8 is sufficient to cause illness [19,20].

3.1 Genotypic Characterization Findings:

Virulence factors such as HBL, NHE, and Cytotoxin K are the primary contributors to *Bacillus cereus* enterotoxin production [21–23]. PCR-based detection targeted genes encoding both enterotoxins and emetic toxins, including the hemolytic (*hblA*, *hblC*, *hblD*) and non-hemolytic (*nheA*, *nheB*, *nheC*) complexes, as well as Cytotoxin K (*cytK*). Genotypic analysis revealed the presence of *hbl* (78%), *nhe* (62%), and *cytK* (50%) genes among the positive isolates, with street food isolates showing the highest frequency [2]. Genetic diversity was further examined through PCR sequencing, which identified variations within toxin gene clusters, suggesting potential evolutionary adaptations. Phylogenetic analysis grouped isolates into distinct clades based on gene sequences, with notable differences observed between those derived from processed foods and raw materials [7].

3.2 Phenotypic Characterization Findings:

All isolates exhibited phenotypic and biochemical traits consistent with *Bacillus cereus* identification. They were motile rods with peritrichous flagella and demonstrated hemolytic activity, producing β-hemolysis or a lavender-green coloration under heavy growth, indicative of proteolytic activity. The isolates produced catalase, lecithinase, and reduced nitrate. Fermentation tests showed consistent utilization of D-glucose and maltose, while sucrose and lactose fermentation were variable. None of the isolates fermented D-xylose or D-mannitol. Oxidase and urease production were variable, whereas indole was not produced. All isolates were capable of growth at 100 °C [24].

Resistance of B cereus to Antibiotics:

Antimicrobial resistance and susceptibility patterns of *Bacillus cereus* isolates are summarized in Table 3. Regardless of source, isolates exhibited high resistance to Ampicillin (98%), Oxacillin (92%), Penicillin (100%), Amoxicillin (100%), Cefepime (100%), and Trimethoprim/Sulfamethoxazole (80%, with 20% showing intermediate resistance). In contrast, they remained susceptible to several other agents, including Chloramphenicol (99%), Ciprofloxacin (100%), Clindamycin (100%), Erythromycin (92%), Gentamicin (100%), Quinupristin/Dalfopristin (100%), Rifampin (100%), Tetracycline (97%), and Vancomycin (100%) [24].

Table 3: Antimicrobial Resistance and Susceptibility Patterns of *Bacillus cereus*

Antibiotics	Breakpoints		Interpretation n (%)		
	S (≤)	R (≥)	S	I	R
Amoxycillin	4	8	0	0	96(100)
Ampicillin	0.25	0.5	0	2(0.02)	94(98)
Cefepime	0	0	0	0	96(100)
Chloramphenicol	8	32	95(99)	1(0.01)	0
Ciprofloxacin	1	4	96(100)	0	0
Clindamycin	0.5	4	96(100)	0	0
Erythromycin	0.5	4	88(92)	8(8)	0
Gentamicin	4	16	96(100)	0	0
Oxacillin	2	4	3(3)	5(5)	88(92)
Penicillin	0.12	0.25	0	0	96(100)
Quinupristin/dalfopristin	1	4	96(100)	0	0
Rifampin	1	4	96(100)	0	0
Tetracycline	4	16	93(97)	3(3)	0
Trimethoprim/sulfamethoxazole	2	4	0	19(20)	77(80)
Vancomycin	4	>4	96(100)	0	0

a. Antimicrobial breakpoints for *Bacillus cereus* were interpreted according to CLSI guidelines M45-A2E (2010) and M45-P (2005). Exceptions were made for Oxacillin and Quinupristin/Dalfopristin, for which breakpoints established for *Staphylococcus* spp. in CLSI guidelines M100-S22 (2012) and S24 (2014) were applied, as described by Luna et al. [12].

b. S susceptibility, I intermediate, R resistant.

In this study, *Bacillus cereus* isolates recovered from meat, milk, and street foods demonstrated predominant resistance to β-lactam antibiotics. The widespread production of β-lactamases by bacteria, including *Bacillus* species, is recognized as a major mechanism underlying antibiotic resistance [14,26].

Environmental Adaptability:

Environmental adaptability assessments revealed that *Bacillus cereus* exhibited robust growth across diverse conditions, including temperatures from 10°C to 45°C, pH values between 5 and 9, and salt concentrations up to 6% NaCl. Strains isolated from street foods proved especially versatile, showing resilience even at the limits of these environmental ranges [5].

4: Discussion:

The detection of *Bacillus cereus* in street foods (65%) highlights the significant risk posed by informal food markets, where hygiene practices and regulatory controls are often lacking [5]. Contamination was also evident in meat (45%) and milk (30%), pointing to weaknesses in the handling, storage, and processing of perishable products. Regional and seasonal differences observed in the study further illustrate how environmental factors and infrastructure shape contamination rates. The increased prevalence during warmer periods underscores the role of temperature in bacterial growth, consistent with earlier reports that *B. cereus* proliferates more readily under elevated thermal conditions [1].

By combining genotypic and phenotypic analyses, this study emphasizes the value of connecting genetic traits with functional outcomes to better understand the pathogenic potential of *Bacillus cereus*. The high frequency of toxin genes (hbl: 78%, nhe: 62%, cytK: 50%), together with strong toxin production profiles, demonstrates a close link between genetic composition and virulence [2]. Phenotypic testing also revealed differences in toxin expression, indicating that environmental influences or regulatory pathways may affect gene activation. These results align with earlier research underscoring the importance of genetic characterization for reliable risk evaluation and effective management strategies [4].

5: Conclusion:

This study underscores the widespread contamination of *Bacillus cereus* in food sources, with street foods showing the highest prevalence (65%) due to poor hygiene practices and limited regulatory oversight. Meat (45%) and milk (30%) also exhibited notable contamination, reflecting vulnerabilities in the handling and preservation of perishable items. Genotypic screening revealed a high occurrence of toxin genes (hbl, nhe, cytK), particularly among street food isolates, pointing to their elevated pathogenic potential. Phenotypic analyses confirmed toxin production, though expression varied depending on environmental and regulatory factors. The detection of substantial antibiotic resistance further raises pressing public health concerns, highlighting the urgent need for antimicrobial stewardship within food production systems.

These results emphasize the value of integrating both genotypic and phenotypic analyses to gain a complete understanding of *Bacillus cereus* pathogenicity, which is essential for effective risk evaluation and management. The study also demonstrates the organism's remarkable ability to withstand diverse environmental conditions, highlighting its persistence within food supply chains and the difficulties associated with controlling contamination.

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