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Molecular detection and toxigenic profiling of *Bacillus cereus* in milk-based sweets of Bikaner, Rajasthan: A public health perspective

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Abstract

Milk-based sweets are extensively consumed across India and are often produced under artisanal or small-scale conditions where adherence to strict hygienic practices may be limited, thereby posing risks to microbiological safety. *Bacillus cereus*, a spore-forming foodborne pathogen, is of particular public health relevance owing to its ability to withstand heat treatment and its capacity to produce a range of enterotoxins responsible for gastrointestinal disorders. In this study, the prevalence and toxigenic potential of *B. cereus* in milk-based sweets marketed in Bikaner, Rajasthan, were assessed using conventional culture techniques in combination with polymerase chain reaction (PCR). A total of 300 milk-based sweet samples were analysed, comprising 150 mawa-based and 150 chhana-based products. Presumptive *B. cereus* isolates were molecularly confirmed by PCR amplification of the *gyrB* gene, followed by screening for virulence-associated genes (*nheA*, *hblA*, *cytK*, and *cesB*). Overall, *B. cereus* was detected in 13% of the samples, with a higher prevalence in mawa-based sweets (16.66%; 25 isolates) than in chhana-based sweets (9.33%; 14 isolates). Among isolates from mawa-based sweets, the occurrence of virulence genes was 36% for *nheA*, 40% for *hblA*, 16% for *cesB*, and 48% for *cytK*. In contrast, isolates recovered from chhana-based sweets showed lower prevalence rates, with 14.28% each for *nheA*, *hblA*, and *cytK*, while *cesB* was not detected. Notably, several isolates carried more than one toxin gene, suggesting enhanced pathogenic potential. The presence of toxigenic *B. cereus* in widely consumed traditional milk sweets represents a considerable food safety risk. These findings emphasize the need for regular molecular monitoring and the implementation of improved hygiene and handling practices during the preparation and marketing of traditional dairy products.

Keywords: *Bacillus cereus*, Bikaner, milk-based sweets, PCR, virulence genes, food safety

Introduction

Traditional milk-based sweets constitute a vital part of the Indian diet and hold significant cultural importance. In Rajasthan, particularly in the city of Bikaner, these products are largely prepared from concentrated milk solids and distributed through small-scale, informal production and retail systems. Although highly popular, the manufacture of these sweets often lacks standardized processing and quality control measures and relies on traditional, open-air methods with extensive manual handling, rendering them susceptible to microbial contamination. Among the many pathogens, especially those causing foodborne illnesses, *Bacillus cereus* is one of the 22 foodborne pathogens identified by the World Health Organization as a key organism for evaluating the global burden of foodborne diseases (Kirk *et al.*, 2015) [8]. *Bacillus cereus* is a widely distributed, Gram-positive, aerobic, spore-forming bacterium commonly present in soil, dust, water, and raw milk. The spores of this organism display exceptional resistance to heat and drying, enabling them to survive pasteurization and cooking. When conditions during storage become favourable, the spores may germinate and multiply, resulting in toxin production and the potential onset of foodborne disease. The pathogenicity of *B. cereus* is linked to its ability to produce a range of toxins responsible for two clinically distinct forms of food poisoning. The diarrheal form arises following ingestion of viable cells or spores that synthesize enterotoxins in the small intestine, including non-hemolytic enterotoxin (NHE), hemolysin BL (HBL), and cytotoxin K (CytK), encoded by the *nheA*, *hblA*, and *cytK* genes, respectively (Beecher and Macmillan, 1991) [3] (Lund and Granum, 1996) [12] (Lund *et al.*, 2000) [13].

Conversely, the emetic form is caused by cereulide, a heat-stable cyclic dodecadepsipeptide encoded by the *ces* gene cluster, which is pre-formed in food and remains biologically active despite exposure to high temperatures and resistance to gastric proteolysis (Ehling-Schulz and Scherer, 2004) [4]. Molecular identification targeting the *gyrB* gene offers greater discriminatory accuracy than conventional 16S rRNA-based methods, which lack sufficient resolution to reliably differentiate *B. cereus* from closely related species such as *B. thuringiensis* (Yamada *et al.*, 1999) [18]. Accordingly, the present study employed *gyrB* (DNA gyrase subunit B) for precise species-level identification, while simultaneous detection of virulence-associated genes enabled a more accurate evaluation of public health risk. Nevertheless, information on the molecular prevalence and toxigenic profiles of *B. cereus* in traditional milk-based sweets from Rajasthan is scarce. This study was therefore undertaken to address this gap by molecularly detecting *B. cereus* and characterizing its virulence gene distribution in milk-based sweets marketed in Bikaner.

Materials and Methods

Sample Collection

A total of 300 milk-based sweet samples were obtained from various sweet shops across Bikaner district, Rajasthan, comprising 150 mawa-based sweets and 150 chhana-based sweets. Samples were collected aseptically in sterile containers and subsequently transported and stored under refrigerated conditions until further analysis. Sweet shops were selected using a random sampling approach to encompass a broad geographic distribution across the city, thereby ensuring that the samples were representative (Acharya *et al.*, 2013) [2].

Isolation and Phenotypic Identification

One gram of each sample was aseptically homogenized in 9 mL of brain heart infusion broth and incubated at 37 °C for 24 h for enrichment. Following incubation, a loopful of the enriched culture was streaked onto Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) and incubated at 37 °C for a further 24 h. After incubation, the plates were inspected for the presence of characteristic peacock blue colonies, which were selected for subsequent analyses. The presumptive isolates were further examined by Gram staining and a series of biochemical tests to achieve preliminary identification and confirmation. (Wong *et al.*, 1988) [17]

Preparation of Bacterial Cell Culture

Pure cultures of *Bacillus cereus* were grown overnight in 10 mL of Brain Heart Infusion (BHI) broth at 37 °C in a shaker incubator. Following incubation, the cultures were centrifuged at 13,000 rpm for 10 minutes to pellet the bacterial cells, after which the supernatant was carefully discarded.

Genomic DNA Extraction

Genomic DNA from the confirmed *Bacillus cereus* isolates was extracted using a commercial DNA extraction kit following the manufacturer's protocol with minor modifications. Briefly, 1.5 mL of an overnight-grown Brain Heart Infusion (BHI) broth culture, prepared from a single colony and exhibiting visible turbidity, was transferred into

a capped microcentrifuge tube and centrifuged at 12,000 rpm for 2 minutes. The resulting cell pellet was retained, and the supernatant was carefully discarded. The pellet was resuspended in 180 µL of resuspension buffer, mixed thoroughly, and subsequently treated with 20 µL of Proteinase K. The mixture was incubated at 55 °C for 30 minutes in a thermomixer to facilitate enzymatic digestion. Following this, 200 µL of lysis buffer was added, and the tube was further incubated at 55 °C for 10 minutes. Subsequently, 180 µL of absolute ethanol was added, and the contents were mixed gently. The entire lysate was then transferred to a spin column and centrifuged at 8,000 rpm for 4 minutes at 4 °C. The flow-through was discarded, after which the column was washed with 500 µL of pre-wash buffer and centrifuged at 8,000 rpm for 1 minute. This step was followed by the addition of 500 µL of wash buffer and centrifugation at 8,000 rpm for 1-2 minutes. To remove residual ethanol, the column was subjected to a dry spin at 9,000 rpm for 4 minutes. The spin column was then placed into a fresh sterile microcentrifuge tube, and 100 µL of elution buffer was added directly onto the membrane. DNA was eluted by centrifugation at 8,000 rpm for 5 minutes. The eluted buffer containing genomic DNA was used as the template for PCR amplification. DNA purity was assessed by measuring the OD260/OD280 ratio using a spectrophotometer, and the extracted DNA samples were stored at -20 °C until further analysis.

PCR-Based Detection of Species-specific and Virulence Genes

PCR assays were employed for molecular confirmation of *Bacillus cereus* through amplification of the *gyrB* gene and for screening of virulence-related genes (*nheA*, *hblA*, *cytK*, and *cesB*). The primer sequences used for detecting the *gyrB*, *nheA*, *hblA*, *cesB*, and *cytK* genes in *B. cereus* isolates are presented in Table 1. Each PCR reaction was performed in a total volume of 15 µL, comprising 10 µL of 2× DreamTaq Green Master Mix, 0.5 µL each of forward and reverse primers (25 pM/µL), 2.0 µL of template DNA, and 2.0 µL of nuclease-free water, under the optimized cycling conditions detailed in Table 2. The amplified products were separated by electrophoresis on 1.5% agarose gels and visualized using a gel documentation system.

Electrophoresis of PCR products

A 1% agarose gel was prepared by dissolving 1.25 g of agarose in 100 ml of diluted 1× TBE buffer, followed by heating in a microwave oven until a clear and homogeneous solution was obtained. The molten agarose was then allowed to cool to approximately 50-60 °C before adding 4 µl of ethidium bromide to achieve the desired final concentration, and the solution was mixed thoroughly. The gel was poured into a suitable casting tray fitted with an acrylic comb and allowed to solidify. Once the gel had set, the comb was carefully removed. For electrophoresis, the gel was placed in a horizontal electrophoresis chamber filled with 1× TBE buffer, ensuring that the buffer level remained 2-3 mm above the gel surface and that the wells were oriented toward the cathode. Amplified PCR products (10 µl) were gently loaded into the wells using disposable micropipette tips. A 100 bp or 1 kb DNA ladder was loaded in a separate well to estimate the size of the amplified fragments, while a negative control was included in the final well. Electrophoresis was performed at 70 V for 60 minutes, and

the PCR amplicons were subsequently visualized using a UV transilluminator.

Results and Discussion

Among the 300 milk-based sweet samples examined, all presumptive isolates recovered from mawa-based sweets (25; 100%) and chhana-based sweets (14; 100%) produced the specific 475 bp amplicon and were conclusively identified as *Bacillus cereus* through PCR targeting the *gyrB* gene, as detailed in Table 3 and Figure 1. The agarose gel electrophoresis profiles demonstrating *gyrB* gene amplification in *B. cereus* isolates are additionally illustrated in Plate 1. Overall, mawa-based sweets showed a higher prevalence of *B. cereus* contamination compared to chhana-based sweet products.

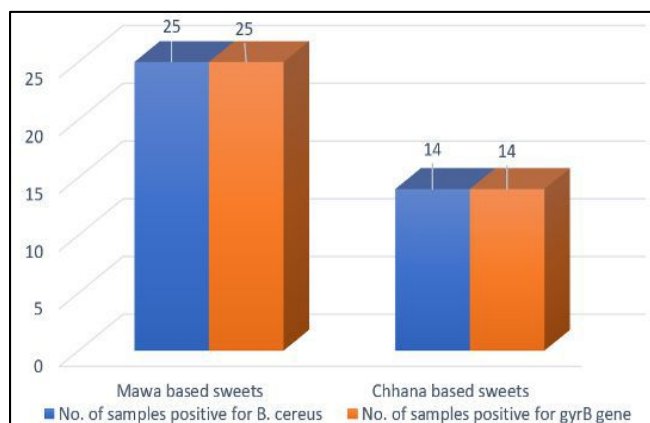


Fig 1: Bar chart of samples positive for *gyrB* gene

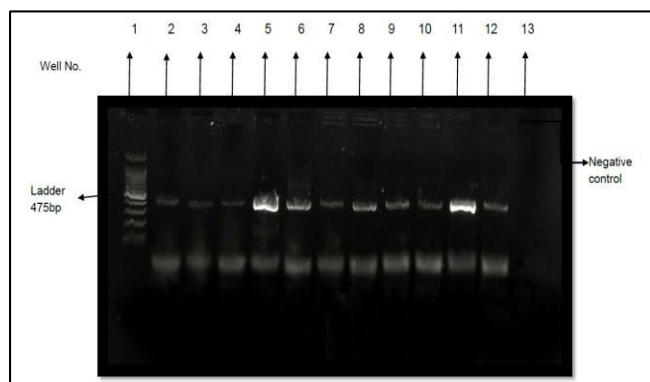


Plate 1: Agarose gel showing the amplification product of *gyrB* gene of *B. cereus* (475 bp)

These results are consistent with earlier studies, including that of Tewari *et al.* (2013) [15], who reported amplification of the *gyrB* gene in all of their isolates (100%, 29/29), and Rather *et al.* (2011) [14], who confirmed 98.3% (59/60) of isolates as *Bacillus cereus* using *gyrB*-specific primers. In addition, reliance on *gyrB*-based molecular assays improves the precision and reliability of pathogen detection and serves as an important tool for surveillance of foodborne bacteria, facilitating early identification and effective public health interventions.

Distribution of Virulence Genes

Detection of *nheA* gene

Among the 39 isolates examined, 11 were found to be positive for the target virulence gene. Of these, 9 isolates (36%) originated from mawa-based sweets, while 2 isolates

(14.28%) were recovered from chhana-based sweets, resulting in an overall prevalence of 28.20%. These findings are summarized in Table 4 and illustrated in Figure 2. Additionally, the PCR agarose gel electrophoresis results demonstrating amplification of the *gyrB* gene specific to *Bacillus cereus* are depicted in Plate 2.

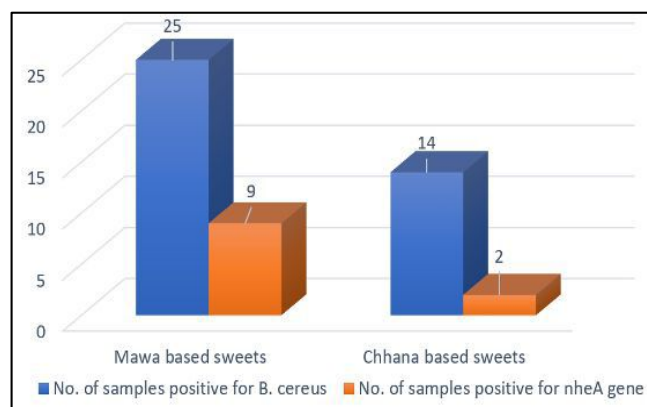


Fig 2: Bar chart of samples positive for *nheA* gene

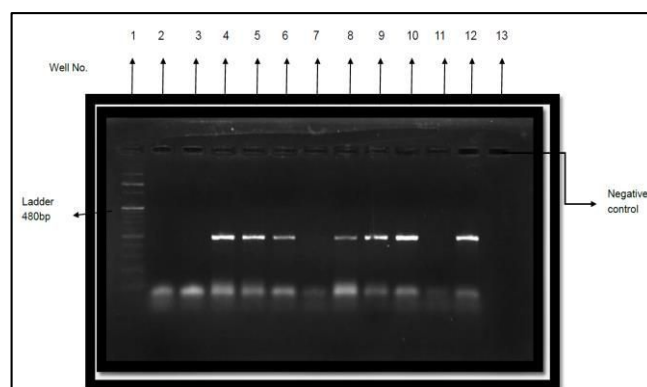


Plate 2: Agarose gel showing the amplification product of *nheA* gene of *B. cereus* (480 bp)

Abdelaziz *et al.* (2024) [1] analysed a total of 179 samples comprising milk-derived products and raw chicken meat and reported that 22.34% were positive for *Bacillus cereus* sensu stricto. Of the recovered isolates, 17.5% harboured the *nheABC* enterotoxin gene cluster, with the *nheA* gene detected in 55% of isolates—a prevalence notably higher than that observed in the present investigation. In a related study, Elgushi *et al.* (2023) [16] evaluated 180 dairy samples, including raw cow milk, milk powder, and Ras cheese (60 samples from each category). Using PCR assays targeting the *nheA* gene, which encodes the non-hemolytic enterotoxin, they identified enterotoxigenic *B. cereus* in 10.5% of the samples. This prevalence closely aligns with the results reported in the current study.

Detection of *hblA* gene

Of the 39 isolates examined, 12 were found to be positive, comprising 10 isolates (40%) from mawa-based sweets and 2 isolates (14.28%) from chhana-based sweets. This corresponds to an overall prevalence of 30.76% for the targeted virulence gene, as detailed in Table 5 and illustrated in Figure 3. In addition, the PCR agarose gel electrophoresis results demonstrating amplification of the *gyrB* gene specific to *Bacillus cereus* are shown in Plate 3.

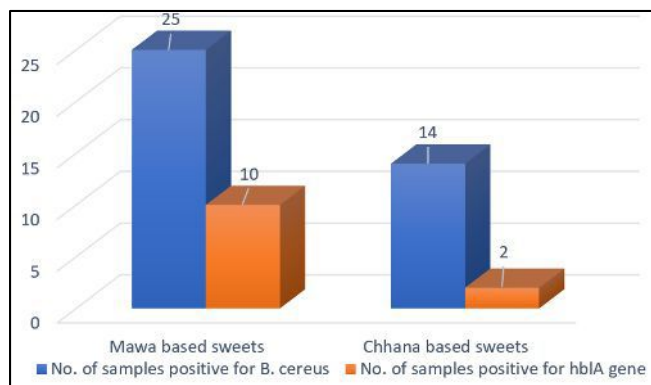


Fig 3: Bar chart of samples positive for *hblA* gene

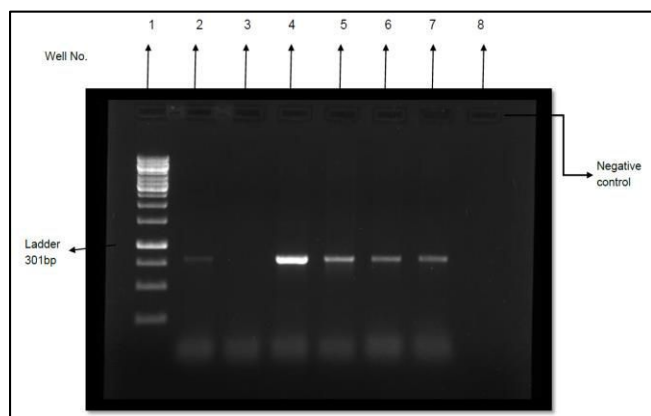


Plate 3: Agarose gel showing the amplification product of *hblA* gene of *B. cereus* (301 bp)

Abdelaziz *et al.* (2024) ^[1] analysed 179 samples and reported that 22.34% were positive for *Bacillus cereus* sensu stricto. Notably, milk products and raw chicken meat exhibited a 50% prevalence of the *hblA* gene, which is substantially higher than that observed in the present

investigation. Similarly, Thabet *et al.* (2023) ^[16] evaluated 100 samples comprising raw milk, pasteurized milk, Damietta cheese, and yoghurt, and detected the *hblA* gene—associated with emetic syndrome—in 60% of the samples, a markedly greater proportion than that recorded in the current study. In another study, Kowalska *et al.* (2022) ^[9] assessed 267 *B. cereus* isolates recovered from various food products and identified the *hblA* gene in 53.56% of the strains, which also considerably exceeds the prevalence reported in the present findings.

Detection of *cesB* gene

Of the 39 isolates analysed, only four were found to be positive for the *cesB* gene. All four positive isolates (16%) originated from mawa-based sweets, while none of the isolates recovered from chhana-based sweets harboured this gene, indicating that this virulence determinant was present in a limited proportion of the total isolates (10.25%), as detailed in Table 6 and Figure 4. Additionally, the PCR agarose gel electrophoresis results demonstrating the amplification of the *gyrB* gene specific to *Bacillus cereus* are illustrated in Plate 4.

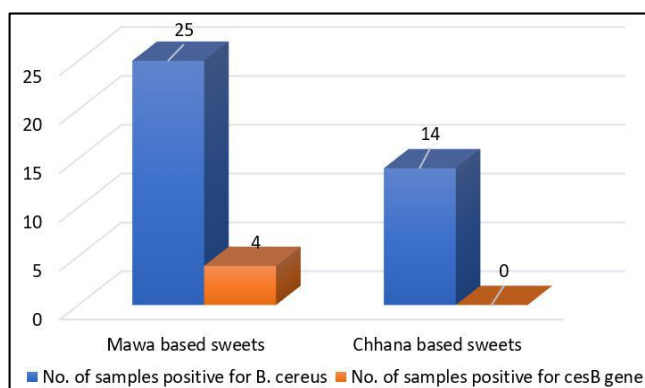


Fig 4: Bar chart of samples positive for *cesB* gene

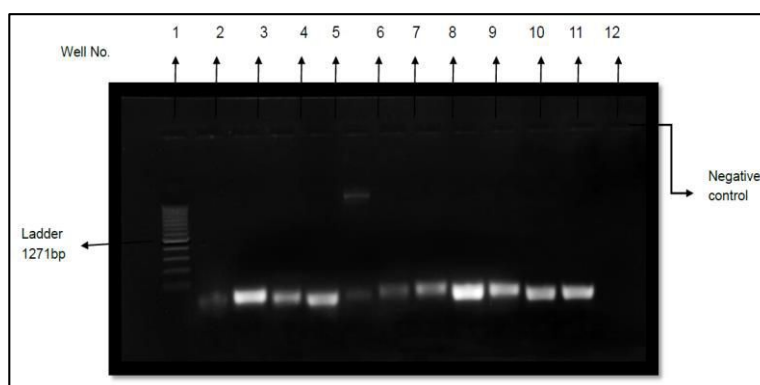


Plate 4: Agarose gel showing the amplification product of *cesB* gene of *B. cereus* (1271 bp)

Abdelaziz *et al.* (2024) ^[1] analysed 179 samples and reported that 22.34% were positive for *Bacillus cereus* sensu stricto. Among the isolates recovered from milk products and raw chicken meat, only seven strains (17.5%) harboured the emetic toxin-encoding *ces* gene, a finding that is comparable with the results observed in the present investigation. Similarly, Thabet *et al.* (2023) ^[16] evaluated 100 samples comprising raw milk, pasteurized milk, Damietta cheese, and yogurt, with 25 samples from each category. The *ces* gene associated with emetic toxin production was detected in 5 of the 34 confirmed *B. cereus* isolates, corresponding to a prevalence of 14.7%. This

detection rate is also consistent with the observations of the current study.

Detection of *cytK* gene

Among the 39 isolates analysed, 14 were found to be positive for the virulence gene, including 12 isolates (48%) from mawa-based sweets and 2 isolates (14.28%) from chhana-based sweets, reflecting that this gene was present in a notable proportion of the isolates (35.89%), as shown in Table 7 and Figure 5. The PCR agarose gel results demonstrating the amplification of the *gyrB* gene in *Bacillus cereus* are presented in Plate 5.

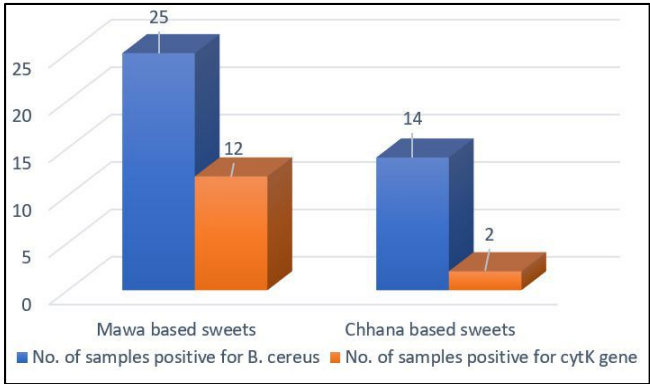


Fig 5: Bar chart of samples positive for *cytK* gene

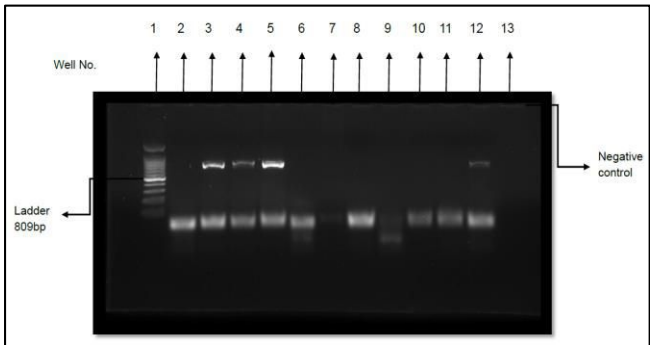


Plate 5: Agarose gel showing the amplification product of *cytK* gene of *B. cereus* (809 bp)

Thabet *et al.* (2023) ^[16] examined 100 samples comprising raw milk, pasteurized milk, Damietta cheese, and yogurt (25 samples of each type) and reported the presence of the *cytK* virulence gene in 80% of the samples, a detection rate

considerably higher than that observed in the present study. Similarly, Mostafa *et al.* (2022) ^[10] investigated multiple virulence genes, including *cytK*, in 18 different *Bacillus cereus* strains and found a prevalence of 63.64%, which also surpasses the results obtained in the current research. These results reveal considerable variability in the distribution of virulence genes among the *Bacillus cereus* isolates. The *nheA* gene was the most frequently detected, suggesting a higher potential for causing diarrheal illness. The predominance of *nheA* and *hblA* supports previous findings that strains producing diarrheal toxins are commonly associated with dairy products. The detection of *cytK* further heightens public health concerns, as this gene is linked to severe gastrointestinal effects. Although the proportion of *cesB*-positive isolates was relatively low, the remarkable heat resistance of cereulide makes even minimal prevalence epidemiologically important. Several isolates carried combinations of multiple virulence genes, indicating an increased pathogenic versatility. Overall, this study demonstrates that traditional milk-based sweets sold in Bikaner may act as reservoirs for toxigenic *B. cereus*. The prevalence observed is consistent with reports from other warm-climate regions, where insufficient temperature control and post-processing contamination facilitate bacterial survival. The co-occurrence of multiple toxin genes in single isolates highlights the importance of molecular surveillance, as reliance solely on conventional culture methods may underestimate the risk. From a veterinary public health perspective, these findings underscore the urgent need to enhance hygiene practices, strengthen cold chain management, and incorporate molecular diagnostic approaches into routine food safety monitoring programs.

Table 1: The primers used for the detection of *gyrB*, *nheA*, *hblA*, *cesB* and *cytK* gene

S. No.	Oligo name	Sequence (5'-3')	Size of Amplified product (bp)	References
1.	<i>gyrB</i>	F-TCATGAAGAGCCTGTGTACG R-CGACGTGTCAATTCACGCGC	475 bp	Tewari <i>et al.</i> (2013) ^[15]
2.	<i>nheA</i>	F-TACGCTAAGGAGGGGCA R-GTTTTATTGCTTCATCGGCT	480 bp	Fagerlund <i>et al.</i> (2004) ^[7]
3.	<i>hblA</i>	F-GTGCAGATGTTGATGCCGAT R-ATGCCACTGCGTGGACATAT	301 bp	Fagerlund <i>et al.</i> (2004) ^[7]
4.	<i>cesB</i>	F-GGTGACACATTATCATATAAGGTG R-GTAAGCGAACCTGTCTGTAACAACA	1271 bp	Ehling-Schulz <i>et al.</i> (2006) ^[5]
5.	<i>cytK</i>	F-ACAGATATCGGGTCAAAATGC R-TCCAACCCAGTTATGCCAGTTC	809 bp	Kwarteng <i>et al.</i> (2017) ^[11]

Table 2: Steps and Conditions of Thermal Cycling for PCR of *B. cereus* isolates:

Primers	Cycling conditions				
(Forward and Reverse)	Initial denaturation	Denaturation	Annealing	Extension	Final extension
<i>gyrB</i> (F) <i>gyrB</i> (R)	94 °C 10 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 10 minutes
Repeated for 40 cycles					
<i>nheA</i> (F) <i>nheA</i> (R)	94 °C 10 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 10 minutes
Repeated for 40 cycles					
<i>hblA</i> (F) <i>hblA</i> (R)	94 °C 10 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 10 minutes
Repeated for 40 cycles					
<i>cesB</i> (F) <i>cesB</i> (R)	94 °C 10 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 10 minutes
Repeated for 40 cycles					
<i>cytK</i> (F) <i>cytK</i> (R)	94 °C 10 minutes	94 °C 1 minute	55 °C 1 minute	72 °C 1 minute	72 °C 10 minutes
Repeated for 40 cycles					

Table 3: Detection of *gyrB* gene in the test isolates

S. No.	Sample type	No. of samples examined	No. of samples positive for <i>B. cereus</i>	No. of samples positive for <i>gyrB</i> gene	Prevalence of <i>gyrB</i> gene (%)
1.	Mawa based sweets	150	25	25	25/25 (100%)
2.	Chhana based sweets	150	14	14	14/14 (100%)
	Total	300	39	39	39/39 (100%)

Table 4: Detection of *nheA* gene in the test isolates

S. No.	Sample type	No. of samples examined	No. of samples positive for <i>B. cereus</i>	No. of samples positive for <i>nheA</i> gene	Prevalence of <i>nheA</i> gene (%)
1.	Mawa based sweets	150	25	9	9/25 (36%)
2.	Chhana based sweets	150	14	2	2/14 (14.28%)
	Total	300	39	11	11/39 (28.20%)

Table 5: Detection of *hblA* gene in the test isolates

S. No.	Sample type	No. of samples examined	No. of samples positive for <i>B. cereus</i>	No. of samples positive for <i>hblA</i> gene	Prevalence of <i>hblA</i> gene (%)
1.	Mawa based sweets	150	25	10	10/25 (40%)
2.	Chhana based sweets	150	14	2	2/14 (14.28%)
	Total	300	39	12	12/39 (30.76%)

Table 6: Detection of *cesB* gene in the test isolates

S. No.	Sample type	No. of samples examined	No. of samples positive for <i>B. cereus</i>	No. of samples positive for <i>cesB</i> gene	Prevalence of <i>cesB</i> gene (%)
1.	Mawa based sweets	150	25	4	4/25 (16%)
2.	Chhana based sweets	150	14	0	0/14 (0%)
	Total	300	39	4	4/39 (10.25%)

Table 7: Detection of *cytK* gene in the test isolates

S. No.	Sample type	No. of samples examined	No. of samples positive for <i>B. cereus</i>	No. of samples positive for <i>cytK</i> gene	Prevalence of <i>cytK</i> gene (%)
1.	Mawa based sweets	150	25	12	12/25 (48%)
2.	Chhana based sweets	150	14	2	2/14 (14.28%)
	Total	300	39	14	14/39 (35.89%)

Conclusion

The present study effectively employed a molecular approach to characterize the toxigenic profile of *Bacillus cereus* in Bikaner's popular milk sweets, confirming the presence of toxigenic strains in these widely consumed dairy products. PCR-based detection of the *gyrB* gene along with virulence-associated genes offers critical insights into the potential public health risks linked to these sweets. The observed high prevalence of enterotoxin genes and detection of emetic markers underscore the need for strengthened monitoring. Implementation of Good Manufacturing Practices (GMP), Hazard Analysis and Critical Control Point (HACCP) systems, and regular molecular screening is strongly recommended to safeguard consumer health.

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