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Mohamed A. Abdelhak, Mohamed A. Abdelmonem, Sahar T. Tolba

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Prevalence of Virulent *Bacillus cereus* isolated from various food samples in the Egyptian food markets

Mohamed A. Abdelhak[1], Mohamed A. Abdelmonem[1], Sahar T.M. Tolba[2]
[1]*Central laboratory of Residue Analysis of Pesticides and Heavy Metals in Food (QCAP), Egypt*
[2]*Microbiology Department, Faculty of Science, Ain Shams University, Egypt*

Bacillus cereus is a common, widely distributed contaminant mostly found in soil. It is known as a food-borne pathogen that can be detected in various food types and causes emetic and diarrheal illnesses in humans. This research examined 200 food samples of various food products including rice, starch, flavors, milk and dairy products, fruits, herbs, and ready-to-eat foods. Examination revealed 50 samples (25%) of the total sample were contaminated with *B. cereus*. Antibiotic susceptibility, detection of hemolytic (*hbl*), emetic (*ces*), and nonhemolytic (*nhe*) genes were conducted for each isolate. The findings indicated that all *B. cereus* isolates were resistant to β-lactam antibiotics (ampicillin, penicillin-G), with 38% of the isolates being resistant to vancomycin, which raises serious concerns about the spread of antibiotic resistance in the environment. Analysis revealed that non-hemolytic genes were detected as 94% for *nhe*A, 100% for *nhe*B and 86% for *nhe*C. Hemolytic genes were detected in lower percent with 66% for *hbl*A, 64% for *nbl*B and 48% for *nbl*D, while the emetic *ces* gene was present in 6% of all isolates. This study demonstrates the frequency of virulent *B. cereus* in food samples found in markets and emphasizes the need of utilizing both conventional and molecular approaches to identify virulent *B. cereus* in food.

Keywords: *B. cereus, nonhemolytic genes, hemolytic, emetic genes, food*

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CORRESPONDANCE TO
Sahar T. M. Tolba,
Microbiology Department, Faculty of Science,
Ain Shams University, Egypt
Email: s.tolba@sci.asu.edu.eg
ORCID ID: 0000-0002-7779-2873
DOI: 10.21608/ejbo.2024.287475.2836

EDITOR
Prof. Khaled Ghanem,
Botany and Microbiology Department, Faculty of Science, Alexandria University, Egypt
Email: kmghanem@yahoo.com

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INTRODUCTION

Bacillus cereus is a Gram-positive, rod-shaped, facultative, spore-forming anaerobic bacteria, and strongly beta-hemolytic. It is widely common in the environment, as it can be isolated from soil, water and various food matrices, as cereals, rice, milk, vegetables, fruits, poultry and drinks (Felis *et al.,* 2009; Berthold-Pluta *et al.,* 2019; Abou El Nour & Sakr, 2020). *B. cereus* has shown tolerance to harsh environmental conditions due to its production of spores, as it can grow at a wide range of temperature ranging 4 to 50 °C, with those spores highly tolerant to heat, freezing, drying and UV radiation (Luo *et al.,* 2007; Felis *et al.,* 2009; Bottone *et al.,* 2010). It is one of the pathogens responsible for numerous foodborne illnesses outbreaks around the world (Yang *et al.,* 2023).

Bacillus cereus is a serious foodborne pathogen causing gastroenteritis-like symptoms as it causes two forms of food poisoning named diarrheal and emetic, in rare cases, fatal poisonings may occur to humans (Castiaux *et al.,* 2014). The culprit behind emetic food poisoning is a substance called cerulide, which is released into food and remains active throughout the digestion process as it is highly resistant to heat, a variety of pH levels, and protease enzymes. Therefore, emetic toxin-induced sickness does not require the consumption of living *B. cereus*. (Agata *et al.,* 2002; Stenfors *et al.,* 2008).

On the other hand, diarrheal food poisoning is caused by the effect of heat-labile enterotoxins in the small intestines during bacterial growth. The emetic form causes vomiting within 1-5 h after consumption of the contaminated foods. Diarrheal food poisoning is not caused by the toxins excreted in food, it is caused by the vegetative *B. cereus* cells (not spores) that produce enterotoxins in the small intestines (Ceuppens *et al.,* 2012). The diarrheal form causes abdominal pain, and it occurs 8-16 h after consumption of the contaminated food, then disappears generally within 12 - 24 h (Ehling- Schulz *et al.,* 2005; Senesi & Ghelardi, 2010).

Because *B. cereus* can damage food products, it also has an impact on agriculture and food processing (Arslan *et al.,* 2014). Antibiotic treatment is the first line of treatment for *B. cereus* infections, even though antibiotic misuse has led to a rise in antibiotic-resistant *B. cereus* strains (Barbosa & Levy, 2000). Acquisition of resistance genes through horizontal gene transfer has long been reported (Brown 2003; Tolba & Wellington 2004). It is well known that *Bacillus cereus* has a wide variety of plasmids, including conjugative plasmids. Plasmid transfer across the *Bacillus* group has been documented in numerous investigations, particularly for plasmids containing important toxins (Hinnekens *et al*., 2022). The gene cluster *ces*ABCD of *B. cereus* emetic toxin is located on a large plasmid (Stenfors *et al*., 2008). The whole genome sequence of *B. cereus* strains isolated

from ice cream samples revealed the presence of 27 sequence type strains (STs), including one that causes food poisoning (Yu *et al*., 2019). Additionally, toxin genes detection revealed the presence of the genes encoding nonhemolytic enterotoxin (NHE), hemolysin BL (HBL), cytotoxin K (cytK) and cereulide (ces) in these isolates (Fraccalvieri *et al*., 2022). The objective of this research was to determine the prevalence of *B. cereus* in various food products and detect their toxigenic genes that may cause poisoning.

MATERIALS AND METHODS

Food Samples Collection

A total of 200 samples: rice 30, starch 20, peanut 20, flavors 20, herbs 20, milk 20, milk with sugar and full cream 10, yoghurt 20, strawberry 20, frozen vegetables 10, and processed meat 10 were collected from various markets and examined for the presence of *Bacillus cereus.*

Detection of *B. cereus* in food samples

B. cereus was detected in food samples using the international standard protocol (ISO 7932:2014), horizontal method for the enumeration of *B. cereus* using the colony-count technique at 30˚C. Ten grams of each food sample were added to 90 mL Maximum Recovery diluent (MRD) (oxoid), in a sterile stomacher bag, then 10-fold serial dilutions of each sample was made. 0.1 mL of each dilution sample was inoculated on the surface of the mannitol egg yolk polymyxin B (MYP) (oxoid) agar medium. Plates were incubated for 24 h at 30 °C. The target *B. cereus* does not ferment mannitol, so colonies appear pink in color. In addition, *B. cereus* splits lecithin in egg yolk by the phospholipase C into an insoluble diglyceride which precipitate around the colonies. Polymyxin inhibits Gram negative bacteria. *B. cereus* colonies appear large, pink, and generally surrounded by a zone of precipitation.

Confirmation of *B. cereus* colony by detection of hemolysin

Four to five colonies from MYP medium were picked and inoculated in Muller Hinton broth (MHB) (Oxoid), incubated overnight at 30 °C for enrichment. Turbidity of the culture was adjusted approximately to 1.5 x 10^8 CFU/ml, according to McFarland standard turbidity scale. Five microliters of each suspension were spotted and stabbed on blood agar base supplemented with 5% defibrinated sheep blood then plates were incubated at 37°C. The plates were examined after 24h. The hemolytic activity was expressed as clear halo zone surrounding the colony. The potency of hemolysis was then classified according to diameter of zone as strong, intermediate, and weak hemolysis.

Confirmation of presumptive colonies using MALDI-TOF (Vitek®MS)

Single pure colony was picked from TSA agar medium to be analysed by MALDI-TOF (Vitek®MS, BioMérieux, France. The picked colony placed on the target slide and was added from matrix CHCA (BioMérieux, France) and left to dry, the target slide with all sample were loaded into the instrument and the instrument was set to run (Manzulli *et al.,* 2021).

Antimicrobial susceptibility testing

The antibiotic sensitivity test was determined using Kirby-Bauer disc susceptibility test (Bauer *et al.,* 1966) on Mueller-Hinton agar (oxoid) by sterile inoculating loop, four or five isolated colonies was selected and inoculated in Mueller Hinton broth and was incubated for 24h. Then, turbidity was adjusted to 0.5 McFarland. Mueller Hinton agar was inoculated from incubated broth and plates were then allowed to sit at room temperature for 3-5 min for the surface of the agar plate to eliminate moisture from the agar surface. Fifteen antibiotic disks (oxoid) were used for susceptibility testing: gentamicin (GM200), norfloxacin (NOR10), clindamycin (CD10), streptomycin (S10), tetracycline (T30), imipenem (IMI10), penicillinG (PG10), ciprofloxacin (CIP5), chloramphenicol (C30), trimethoprim/ sulphamethoxazole (TS25), ampicillin (SAM20), erythromycin (E15), vancomycin (VA 5). Plates were placed in a 37°C incubator for 24h. The susceptibility of the bacterial species was detected by a clear zone around the discs expressed in mm. As no criteria was mentioned in CLSI (2018) for judging the results of *Bacillus cereus,* the results were interpreted according to the zone diameter of *Staphylococcus aureus* in CLSI (2018).

Extraction of genomic DNA

DNA extraction was carried out using mericon DNA Bacterial Plus Kit from QIAGEN according to manufacturer's instruction, one ml of overnight enriched sample was transferred in micro centrifuge tube and then centrifuged at (15000rpm/5min). The supernatant was removed, and the pellet was re-suspended in 400µl of Fast Lysis Buffer. The contents were mixed using the vortex mixer. The mixture was transferred into Pathogen Lysis Tube and vigorous vortex at maximum speed for 10 min, then centrifuged at (15000 rpm/3 min). 100 µl of the

supernatant were transferred to new micro-centrifuge tube, the supernatant was used as DNA template for PCR.

Detection of virulence genes by PCR

Using designed primers by eurofins Genomics to detect hemolytic enterotoxin HBL complex genes (*hbl*A, *hbl*C and *hbl*D), non-hemolytic enterotoxin (*nhe*A, *nhe*B and *nhe*C). The detection rates of *cyt*k gene according to (Owusu-Kwarteng *et al.,* 2017) as shown in Table 1, the following mixture was prepared, 12.5µl of Master Mix (EmeraldAmp® GT-PCR) , 1 µl of forward primer and 1 µl of reverse primer , and 5.5 µl of molecular free DNA water, 20 µl from this mixture was added to each well in the T100 Bio-Rad (Thermal Cycler) 96 well and 5 µl of the prepared DNA was added. PCR protocol was carried out in compliance with (Owusu-Kwarteng *et al.,* 2017) As one cycle of denaturation at 95 °C, 3 min followed by 35 cycles of (denaturation at 94°C, 30s, annealing at 58°C for 45 s, extension 72°C, 1.5 min and a final step 5 min for the final extension at 72 °C. PCR products were investigated by electrophoresis on 1% agarose gel stained by 5 µl ethidium bromide submerged in 1X TBE buffer at 80 V for 50 min. Ten µl of PCR products were loaded in the wells and 5 µl of DNA ladder (genedirex 100bp) was loaded in the first well. UV Transilluminator was used to visualize amplified product and gel documentation system (Bio-Rad XR+) was used to record the bands.

Statistical analysis

The data were presented in mean ±SD. IBM SPSS Statistics 21 (IBM, Chicago, IL, USA) as Windows was used for analysis. Pearson's Chi-square test was performed to evaluate antibiotic resistance patterns. The significance level was set at $P<0.05$.

RESULTS

Isolation and purification of bacterial isolates

Results indicated that 50 out of the 200 samples (25%) were positive for the presence of *Bacillus cereus* (2 rice, 4 starch, 3 peanuts, 10 flavors, 8 herbs, 11 milk, 3 milk with sugar and full cream, 5 yoghurt, 2 strawberry,1 frozen vegetable, and 1 processed meat) as shown in Table 2. The highest percentage was detected in milk (55%) and food flavors (50%).

Characterization of bacterial isolates

Bacterial isolates were cultured on MYP agar medium for the detection of mannitol fermentation and lecithinase production. The bacterial appearance was large pink colonies indicating that *Bacillus cereus* does not ferment mannitol which indicated by the presence of phenol red supplement in the medium. Lecithinase production is indicated by a white precipitate around the colonies as shown in Photo 1. Hemolytic activity showed that *B. cereus* isolates were strongly hemolytic and produce 2-4 mm zone of complete (β) hemolysis surrounding the colony Photo 2. The bacterial isolates were identified by culture technique and by Mass spectrometry device MALDI-TOF (Vitek®MS). The results showed that the bacterial isolates were *Bacillus cereus* with percentage of identification 99.9% as shown in Photo 3.

Antimicrobial susceptibility of *Bacillus cereus* isolates

All the tested isolates were sensitive to ciprofloxacin (5µg), norfloxacin (10µg), imipenem (10µg) by 100% and gentamicin (200µg) by 90%, while all tested samples showed intermediate resistance to clindamycin (2µg) by 56%, erythromycin (15µg) by 50%, vancomycin (5µg) by 62%, streptomycin (10µg) by 26% and trimethoprim/ sulphamethoxazole (25µg) by 26%. All tested strains were totally resistant to ampicillin (20µg) and penicillin G (10µg) (Table 3, photo 4). Statistical analysis of the antibiotic's resistance test in terms of intermediate and sensitivity showed high significance with P-value < 0.05, Chi-Square Value: 749.2517 and degrees of Freedom: 24.

Detection of virulence genes in *Bacillus cereus* isolates: Conventional PCR screening was performed to detect hemolytic (*hbl*A, *hbl*C and *hbl*D), non-hemolytic (*nhe*A, *nhe*B and *nhe*C) genes and emetic *ces*-genes (Table 1) which are responsible for production of endotoxin in food products causing harmful symptoms to human consumption.

Detection of non-hemolytic genes: DNA of 50 bacterial isolates were tested for detection of non-hemolytic genes A, B and C. A band with the right size (450 bp) was detected in 47 (94%) isolates representative (nheA), while (nheB) was detected in all the isolates (100%) with band size (754 bp). The non-hemolytic gene (nheC) were detected in 43 isolates (86%) with band size (564 bp) (Photo 5).

Detection of hemolytic genes: DNA of 50 bacterial isolates were tested for detection of hemolytic genes (*hblA, hblC, hblD*). The results showed 33 isolates (66%) were positive for the gene (*hblA*) with band size (301 bp). While (*hblC*) was detected in 32 isolates (64%) of the isolates with band size (731 bp) and (*hblD*) gene was detected in 24 isolates (48%) with band size (411 bp) as shown in Photo 6.

Table 1. Primers used in conventional-PCR assays for virulence genes (Owusu-Kwarteng *et al.,* 2017)

Target gene	Name	Type	Sequence (5'--> 3')
nheA	nheA 344 S	Forward	TACGCTAAGGAGGGGCA
	nheA 843 A	Reverse	GTTTTTATTGCTTCATCGGCT
nheB	nheB 1500 S	Forward	CTATCAGCACTTATGGCAG
	nheB 2269 A	Reverse	ACTCCTAGCGGTGTTCC
nheC	nheC 2820 S	Forward	CGGTAGTGATTGCTGGG
	nheC 3401 A	Reverse	CAGCATTCGTACTTGCCAA
hblA	HBLA1	Forward	GTGCAGATGTTGATGCCGAT
	HBLA2	Reverse	ATGCCACTGCGTGGACATAT
hblC	L2A	Forward	AATGGTCATCGGAACTCTAT
	L2B	Reverse	CTCGCTGTTCTGCTGTTAAT
hblD	L1A	Forward	AATCAAGAGCTGTCACGAAT
	L1B	Reverse	CACCAATTGACCATGCTAAT
ces	cesF1	Forward	GGTGACACATTATCATATAAGGTG
	cesR2	Reverse	GTAAGCGAACCTGTCTGTAACAACA

Table 2. Prevalence of *Bacillus cereus* in food samples.

Type of samples	No. of samples	No. of positive samples	Percentage of positive
Rice	30	2	6.7%
Starch	20	4	20%
Peanut	20	3	15%
Flavors	20	10	50%
Herbs	20	8	40%
Milk	20	11	55%
Milk with sugar &Full Cream	10	3	30%
Yoghurt	20	5	25%
Strawberry	20	2	10%
Frozen vegetables	10	1	10%
Processed meat	10	1	10%

Table 3. Antibiotics percentage of sensitivity and resistance against Bacillus *cereus* isolates

Antibiotics	Symbol	Resistance		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Gentamicin	GM200 (200µg)	5	10	-	0	45	90
Norfloxacin	NOR10 (10µg)	-	0	-	0	50	100
Clindamycin	CD2 (2µg)	1	2	28	56	21	42
Streptomycin	S10 (10µg)	1	2	13	26	36	72
Tetracycline	T30 (30µg)	2	4	2	4	46	92
Imipenem	IMI10 (10µg)	-	0	-	0	50	100
Penicillin G	PG10 (10µg)	50	100	-	0	-	0
Ciprofloxacin	CIP5 (5µg)	-	0	-	0	50	100
Chloramphenicol	C30 (30µg)	1	2	7	14	42	84
Trimethoprim/ sulphamethoxazole	TS25 (25µg)	-	0	13	26	37	73
Ampicillin	SAM20 (20µg)	50	100	-	0	-	0
Erythromycin	E15 (15µg)	-	0	25	50	25	50
Vancomycin	VA 5 (5µg)	19	38	31	62	-	0

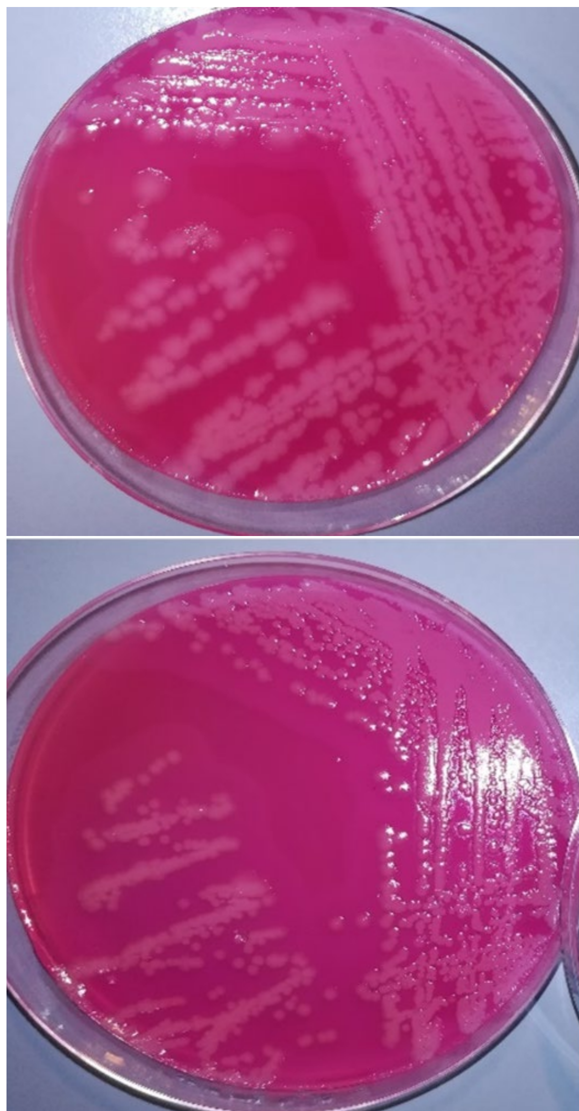

Photo 1. The typical colony of *Bacillus cereus* on MYP agar. Pink colonies with white precipitation around the colonies.

Detection of *ces* gene: Out of 50 bacterial isolates investigated for the presence of hemolytic-A gene *Ces*, only 3 isolates (6%) were found to carry the gene (Photo 7, Figure 1).

DISCUSSION

One of the most common bacteria causing foodborne outbreaks worldwide is *Bacillus cereus* (Yang et al., 2023). Due to the production of four different types of diarrheal toxins, it causes symptoms like gastroenteritis, including vomiting and diarrhea (Mandappa et al., 2016). According to Yang et al. (2023), the Health and Safety Executive (HSE) has classified B. cereus as a human pathogen that falls within risk group 2 (RG2).

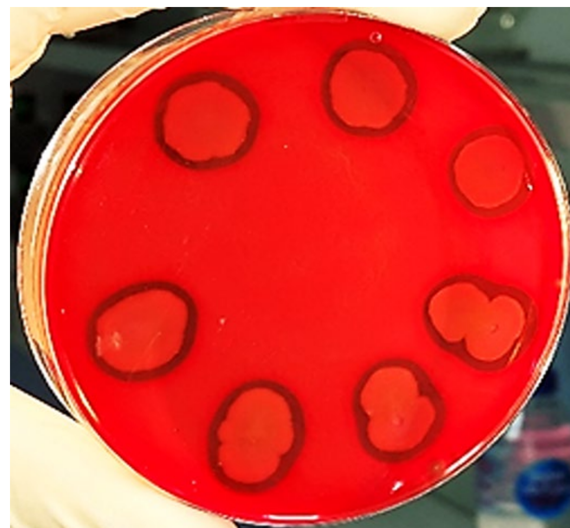

Photo 2. Beta-hemolysis (β) of representative *B. cereus* isolates indicated by the lysis of the RBCs and the presence of halo transparent zone around the colonies.

In this study, 50 of the 200 samples of various food matrices that were collected from several Egyptian marketplaces were contamination with *Bacillus cereus* Bonerba et al. (2010) found *B. cereus* in 250 food samples; their ratio of detection was between 10.5-45.9% in various food samples. Owusu-Kwarteng et al. (2017) gathered 114 samples from farmland soil where cattle were grazing, and they found that *B. cereus* was prevalent in soft cheese, yoghurt-like products, and raw milk. Samapundo et al. (2011) isolated *B. cereus* from food products in Belgium markets, they found that *B. cereus* group spp. was detected in 56.3% of the samples. In the current study, a high percent *of B. cereus* was detected in milk and flavors by 55, 50%, respectively. This indicated that the presence of this bacterium does not depend on the humidity of the source. These results provide evidence of the prevalence of *B. cereus* in food. In this study, all the isolates showed (β) hemolysis which were indicated by the lysis of the RBCs and the media looked completely transparent around the colonies. The pathogenicity of *B. cereus* is closely associated with the production of tissue-destructive exoenzymes. Among these secreted toxins are four hemolysins, three distinct phospholipases, and three pore-forming enterotoxins (Nguyen & Tallent, 2019). The enterotoxins which activate the nod-like receptor protein-3 (NLRP3) are hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K. Results suggested that *Bacillus cereus* that lack HBL can cause inflammation and disease in humans, suggesting that other virulence

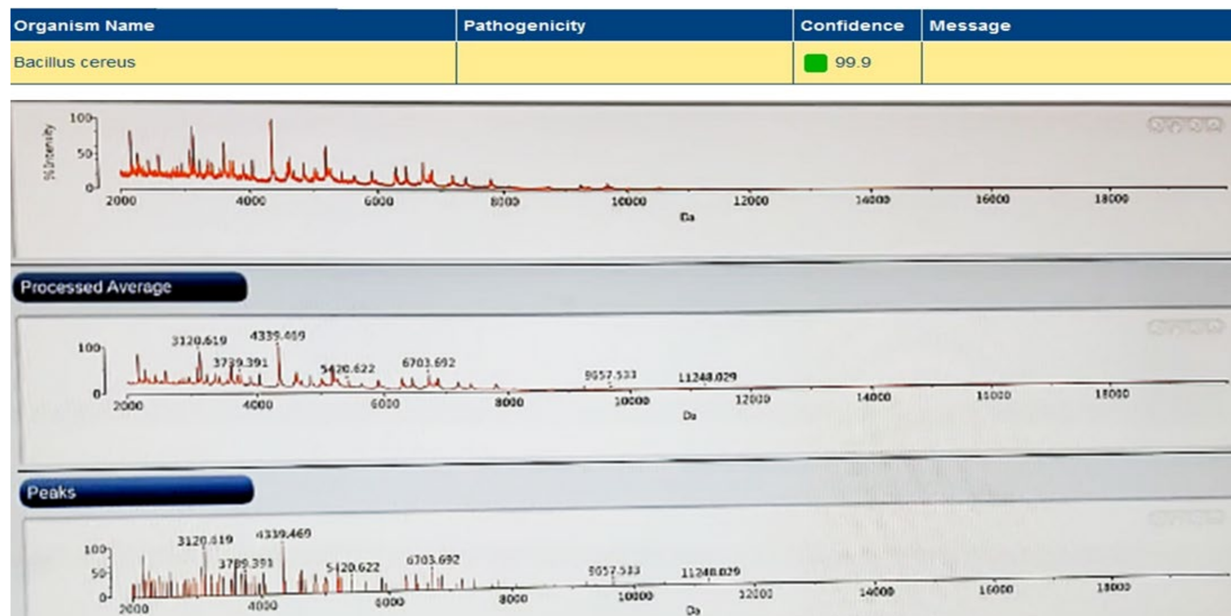

Photo 3. Representative identification of Bacillus *cereus* isolate using mass spectrometry device MALDI-TOF showing 99.9% confidence identification.

factors are critical in the pathogenesis. Fox et al. (2020) demonstrated that non-haemolytic enterotoxin (NHE) of *B. cereus* can induce activation of the NLRP3 inflammasome and pyroptosis via a mechanism targeting the plasma membrane of host cells.

MALDI-TOF MS analysis is a fast and efficient way to identify environmental isolates. The results are generally expressed with log (score) values between 0 and 3.0, demonstrating the correspondence between the MSPs in the reference database and the sample spectrum. A log (score) <1.7 shows that it was unable to determine the strain's genus or species; a log (score) between 1.7 and 2.0 suggests that only the genus level of identification could be reliably determined, while a log (score) 2.0 indicates that identification could be reliable at the species level of the organism. In the current study all the bacterial isolates were identified by culture technique. The results showed that the bacterial isolates were Bacillus cereus with percentage of identification 99.9% as it gives score more than 2.0 and these results were almost like (Muigg et al., 2022) who used routine analysis using the MALDI-TOF MS sDB to diagnose samples of *B. cereus*.

The excessive use of antibiotics had led to resistance to most antibiotics. In the present study, *B. cereus* isolated from different kinds of food was tested against 13 antibiotics from different groups.

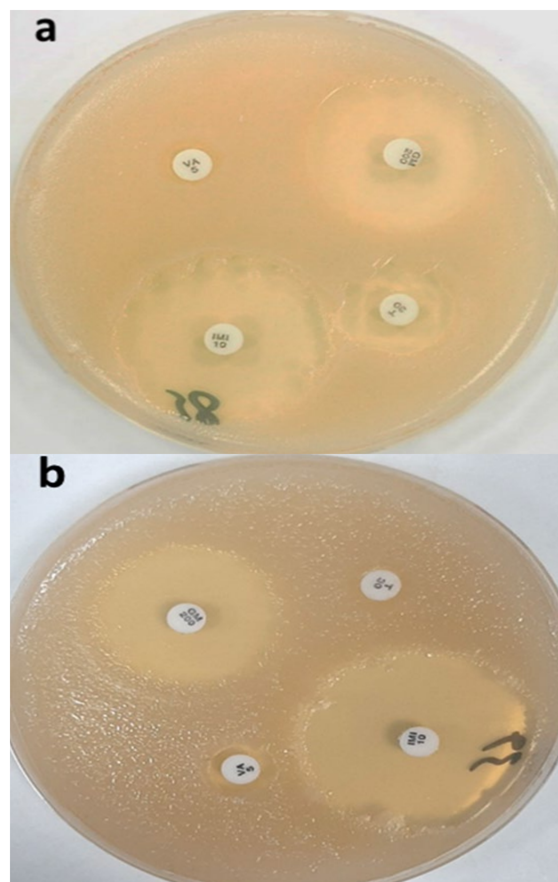

Photo 4. Susceptibility of representative *Bacillus cereus* isolates to antibiotics (vancomycin, gentamycin, imipenem tetracycline). a) isolate resistant to vancomycin b) isolate resistant to tetracycline.

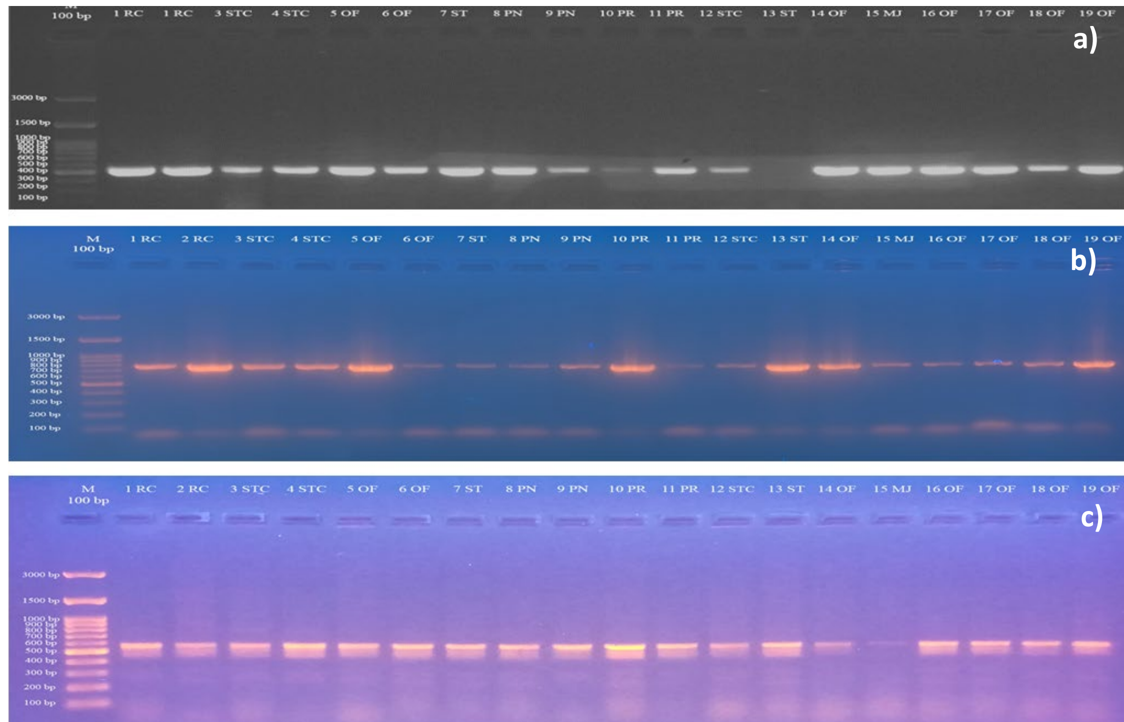

Photo 5. PCR amplification of (a) *nhe*A gene, (b) *nhe*B gene, (c) *nhe*C gene in representative *B. cereus* isolates. Lane M, 100bp DNA ladder.

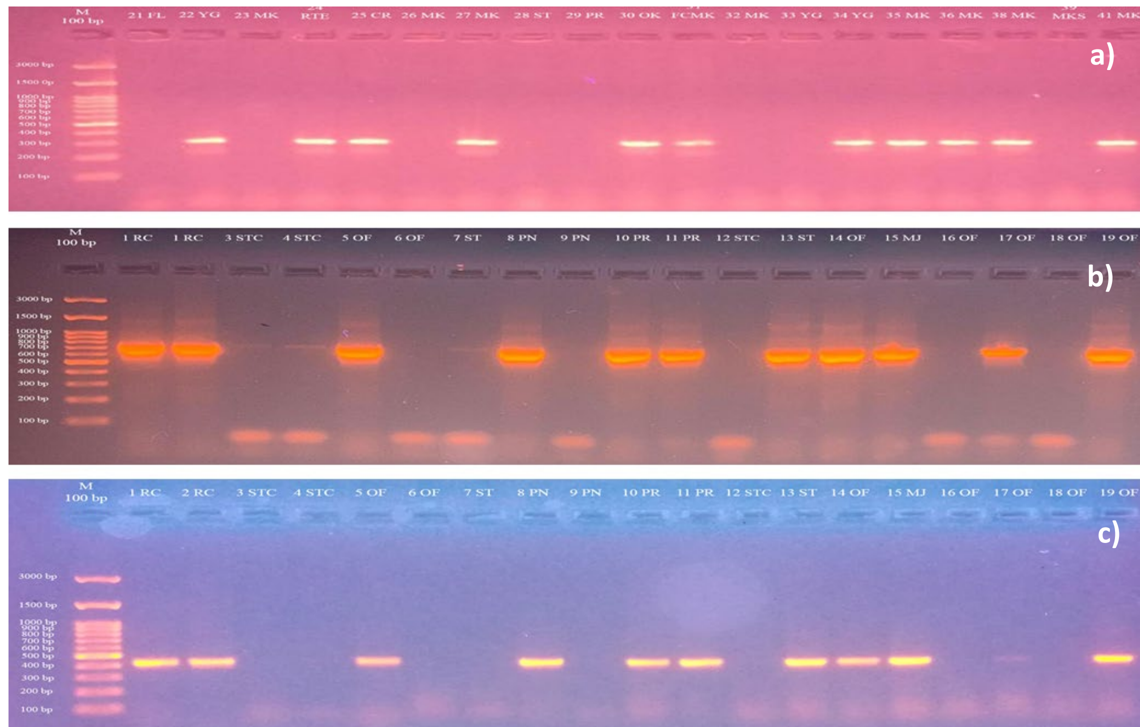

Photo 6. PCR amplification of (a). *hbl*A gene, (b) *hbl*C gene, (c*). hbl*D gene in representative *B. cereus* isolates. Lane M, 100bp DNA ladder.

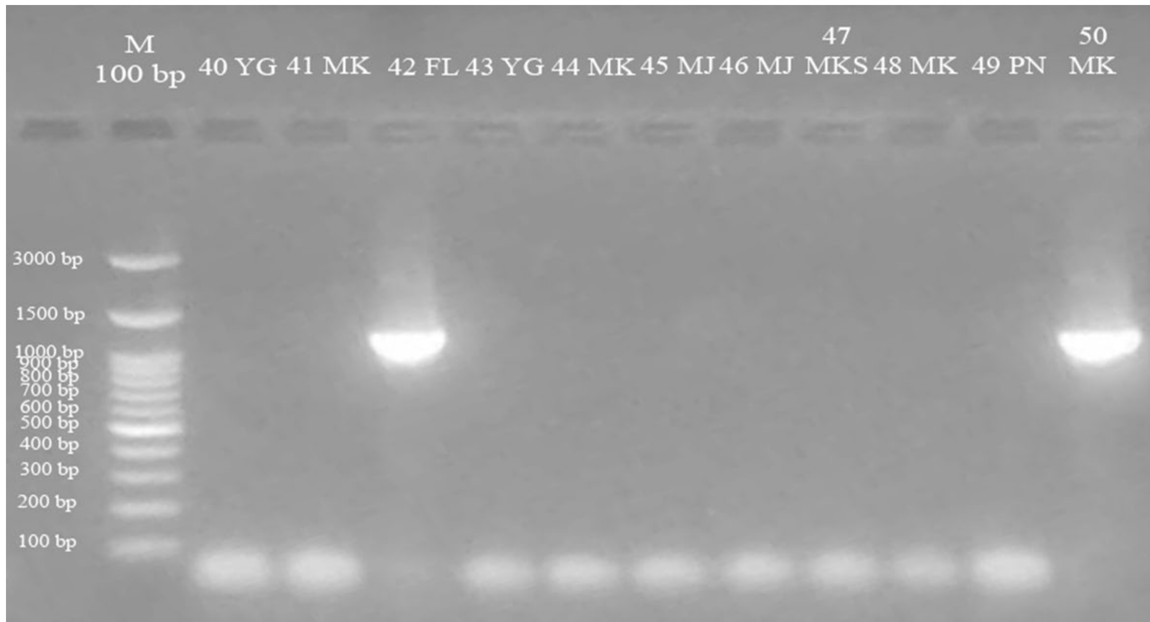

Photo 7. PCR amplification of *ces* gene in representative *Bacillus cereus* isolates.

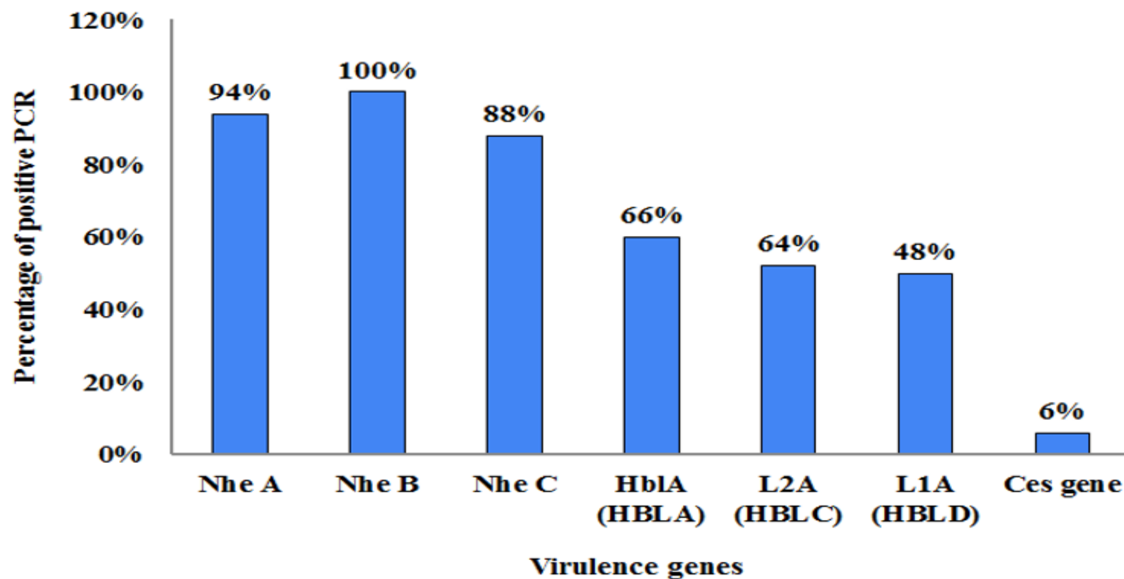

Figure 1. Prevalence of virulence gene in *Bacillus cereus* isolates. Lane M, 100bp DNA ladder.

The results showed that all tested strains were 100% sensitive to ciprofloxacin, norfloxacin, Imipenem and 90-92% to gentamicin and tetracycline. However, high resistance was detected against penicillin, ampicillin, and vancomycin. These data are in congruence with data of Gdoura-Ben Amor et al. (2019) who tested 11 antibiotics against their samples and found that most of the isolates were sensitive to streptomycin, kanamycin, erythromycin, and tetracycline but showed resistance to ampicillin and novobiocin. However, in our study, the resistance to vancomycin is considered a record as in many reports *B. cereus* was sensitive to vancomycin (Gdoura-Ben Amor et al., 2019).

Vancomycin is regarded as one of the best treatments for *B. cereus* infections (Tatara et al., 2013; Godič Torkar et al., 2016). However, the emergence of vancomycin resistance strains will make the treatment of *B. cereus* harder. The European Food Safety Authority (EFSA) reports that 16–20% of food poisoning outbreaks are caused by *B. cereus* bacterial

toxins, which are the primary cause of endotoxins in food products that cause detrimental effects when consumed by humans. In the present study, non-hemolytic (nheA, nheB, nheC) genes were detected in higher percentage than hemolytic (hblA, hblC, hblD) genes, while the emetic ces genes were found only in 3 isolates. These findings align with Gdoura-Ben Amor et al. (2019) who detected the enterotoxin genes among *B. cereus* isolates in descending sequence, nheA (98.9%), nheC (97.7%) and nheB (86.8%) versus hblC (54.6%), hblD (54.6%), hblA (29.9%) and hblB (14.9%). In addition, Zeighami et al. (2020) demonstrated high frequency of nonhemolytic genes upon hemolytic in *B. cereus* isolates from meat samples. The study was also consistent with Berthold-Pluta et al. (2019) who investigated the occurrence of toxigenic B. cereus strains in all tested market products and found that food of both plant and animal origin had nhe genes more presentable than hbl genes and only 0.9% ces genes were detected.

CONCLUSION

The emergence of antibiotic resistant strains of *B. cereus* in food is a strong alarm for the dissemination of the resistance to antibiotics in the environment. Routine methods for identification of *B. cereus* in food products are very important to monitor the virulent strains of *B. cereus*. Molecular methods are very important for monitoring the pathogenic and toxigenic *B. cereus* in food.

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