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Prevalence of *Bacillus cereus* in some dairy desserts in Egypt

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KEYWORDS:

Raw milk, Buffalo, Microbial quality, Chemical quality.

Abstract

Pudding and rice pudding are very popular dairy desserts consumed in Egypt that are processed and stored in conditions suitable for many microorganisms to flourish. *Bacillus cereus* is a Gram-positive, spore-forming pathogen that was incriminated in several foodborne outbreaks of two distinct types; the diarrheal and the emetic Both types have serious effect on human health.. Therefore, this study aim to detect the prevalence of *B. cereus* in some locally manufactured dairy desserts that sold in Mansoura city, Moreover, the enterotoxigenicity of recovered isolates was detected through scanning the isolates for some virulence genes. In this study, *B. cereus* could be detected in 58 , 74% of pudding and rice pudding samples with mean values of $8.38\times 10^5 \pm 1.8\times 10^5$ and $4.4 \times 10^6 \pm 1.4 \times 10^6$CFU/g, respectively. It was found that 100, 77.8, 100 and 22.2 % of *B. cereus* isolates obtained from the examined samples have *nhe*, *hbl*, *cytK* and *ces* genes, receptively.

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Introduction

Bacillus cereus is a Gram-positive, rod-shaped, and is an important cause of food poisoning (15). It is a facultative anaerobic, ubiquitous, endospore-forming bacteria with high frequency of its isolation from various kinds of contaminated raw and processed food products, such as rice, spices, milk, dairy products, vegetables, desserts and cakes (22). After cooking if food is not adequately refrigerated and in the absence of competitive flora, B. cereus grows well (22). B. cereus causes negative effects on dairy products quality and safety (23). B. cereus may be able to produce its harmful pathogenic substances like toxins which make it afamous foodborne pathogen implicated in many types of food poisoning (14). Bacillus cereus is the causative agent of two different foodborne diseases one emetic (intoxication) due to a preformed small heat-stable cyclic peptide; cereulide (6) encoded by ces gene (29) and one diarrhoeal (infection) due to enterotoxins production in intestines (10,19) which are hemolysin BL (HBL), nonhemolytic enterotoxin (NHE) and cytotoxin K (CytK) and are heat labile, acid sensitive and proteolysis (26). Haemolysin (Hbl) and nonhaemolytic enterotoxin (Nhe) are two different three-component enterotoxins (25). B. cereus as a food borne pathogen still underestimated because its illness not a reportable illness (21). Among dairy desserts mahallbia (pudding) and rice milk (rice pudding) are very popular desserts that are palatable, rich with dairy nutrients, healthy and cheap in Egypt. These products are made from different ingredients like milk as abasic constituents, rice, corn starch, sugar, vanilla, nuts, raisins and coconut to enhance its nutritive value and flavor (12).

Contamination by Bacillus cereus in food may occur after heat treatment or food production, during food processing, preparation, transport, storage and distribution (7). Also the source of contamination may be from the various ingredients such as rice which is the most ingredient implicated in B. cereus emetic intoxication (18,14). This study aims to i) Enumeration of B. cereus in some locally manufactured dairy desserts ; mahallbia (pudding) and rice milk (rice pudding) that sold and consumed in Mansoura city, Egypt . ii) Detection of B. cereus isolates enterotoxigenicity through scaning the isolates for some of B.cereus virulence genes.

Methodology :

1. Collection and preparationSamples: One hundred samples of dairy desserts (mahallbia and rice milk, 50 of each) were collected from different localities in Mansoura city, Egypt. These products were sampled in a hygienic manner in clean, dry and sterile containers and transferred to the laboratory as soon as possible in a condition microbiologically unchanged from that existing at the time of sampling for the examination of prevalence of Bacillus cereus (31).

2. Enumeration and isolation of bacillus cereus : According to (31) every sample of mahallbia and rice milk was prepared . According to (28) aseptically inoculate 0.1 ml from each of previously prepared dilutions directly onto the surface of *Bacillus cereus* selective agar base CM0617 plates (duplicate plates for each dilution) and surface spreading of the

inoculum with a sterile bent glass rod. Icubate the plates at 37°C for 24 hours. Examine for typical colonies of *Bacillus cereus* after 24 hours if no colonies appear, further 24 hours incubation at 37°C is done. Examine the incubated plates for typical and characteristic *Bacillus cereus* colonies; the colonial appearance, precipitation of hydrolysed lecithin and the failure to utilisemannitol are the specific characters in diagnosis of *Bacillus cereus*. Count the typical *Bacillus cereus* colonies which are large about 5mm in diameter, crenated, dull and have the characteristic turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same colour because of production of lecithinase (28) .

3. Identification of bacillus cereus : All *Bacillus cereus* isolates were biochemically confirmed by anaerobic growth and hemolytic activity, acid production from sugars (glucose, mannitol, arabinose, xylose) , nitrate reduction test (31).

4. Molecular examination of *Bacillus cereus* isolates: Through scaning the isolates for some of *Bacillus cereus* virulence genes for the presence of the Nhe, Hbl, cytK and ces genes after detecting the family gene groEL in the scanned isolates.

4.1. Extraction of DNA : according to QIAamp DNA mini kit (Qiagen, USA).

4.2. Preparation of PCR Master Mix : By using Emerald Amp GT PCR mastermix (Takara) kit and Oligonucleotide primers sequences Metabion (Germany) Table (1) . DNA amplificarion, cycling conditions shown in Table (2).

4.3. Agarose gel electrophoreses (33) with modification :

According to (37) Using electrophoresis grade agarose (ABgene), DNA ladder Gel Pilot 100 bp plus ladder supplied from QIAGEN (USA) and gel documentation system (Alpha Innotech).

Results

One hundred samples of dairy desserts (mahallbia and rice milk, 50 of each) were examined for presence and enumeration of *Bacillus cereus*. Table (3) represented that the organism could be detected in 66 % of the total samples. From the results revealed in table (3), The incidence of *Bacillus cereus* was 58% of mahallbia (pudding) samples with mean count of $8.38\times 10^5 \pm 1.8\times10^5$ CFU/g . While 74% of rice milk (rice pudding) samples were contaminated with *Bacillus cereus* in amean count of $4.4 \times10^6 \pm 1.4\times10^6$ CFU/g . It is clear from data in (Table 4) that F.D was 34.4% of pudding and 40.63 % of rice pudding (rice milk) samples B. cereus in the range of $10^5 \geq 10^6$ CFU/g .

Ten *Bacillus cereus* isolates obtained from the examined dairy desserts were molecularly scanned for groEL gene which is the specific *Bacillus cereus* group gene , 9 (90%) of examined isolates found to harbor it. These nine isolates which confirmed to be *Bacillus cereus* were examined for the presence of the potential toxin genes known to be responsible for virulence *Bacillus cereus.* Emetic toxin ces gene could be detected in 22.2 % of the isolates. Diarrheal toxin genes nhe, hbl and cytK could be detected in 100% , 77.8 % ,

100% , respectively of isolates obtained from dairy desserts (Fig. 1 , photo 1:5) .

Discussion

In this study *Bacillus cereus* has been isolated from 58% of pudding (mahallbia) samples with mean count of 8.38× 10^5 CFU/g and approximately similar results were reported by (13,17). Lower incidences have been reported 44% and 48% by (4,16) respectively but with lower mean value of 3.83×10^5 CFU/g (16). Much higher result 80% has been detected from examined mahallbia samples by (1). From the data in Table (4) it is obvious that the highest frequency distribution (34.4%) of pudding (mahallbia) samples was with the count range of ($10^5 \geq 10^6$ CFU/g) lower than that recorded by (13) with the highest score (44.4%) of mahallbia samples in the same count range. Any of constituents may introduce *Bacillus cereus* contamination to the final products and many studies reported that *Bacillus cereus* could be isolated from raw milk (27) , from food containing corn starch (14). Starchy and rice based food mostly identified as typical vehicles of B. cereus emetic outbreaks (22,35).

The results registered in Table (3) it can be revealed that B. cereus could be isolated from 74% of rice pudding (rice milk) samples with a mean value of 4.4 $\times10^6 \pm 1.4 \times10$. CFU/g. Higher incidence has been recorded 76.6% with ahigher mean value of $1.4 \times10^7 \pm 6.6 \times 10^6$ CFU/g by (13).lower results were recorded 64% with ahigher mean value of 2.4×107± 1.5× 107 CFU/g by (4). Lower incidences have been reported 60% ,55% , 48% ,35% and 15% from rice pudding samples by (32,27,16, 30,17) respectively. lower mean value than our has been recorded $2.38\times10^5\pm1.4\times10^5$ CFU/g by (16) .A much lower incidence 5.8% was revealed by (8).

According to the obtained results in Table (3) it was cleared that rice pudding samples has higher mean value $4.4\times10^6 \pm 1.4 \times 10^6$ CFU/g than pudding samples $8.38\times10^5\pm1.8\times10^5$ CFU/g ; This may be due to rice as it is known to be amain vehicle to B. cereus . Also the product is processed and hold in room tempreture ; ambient for B. cereus growth and not refrigerated at soon, B.cereus geminate rapidly in cooked rice to infective dose and liberate emetic toxin at both 30-35 ºC (3).Even when refrigerated B. cereus group are psychrotrophic organisms ; able to grow at low tempretures especially with long storage of the food.

Results represented in Table (3) shows that, the examined samples positive for B. cereus were able to cause food borne illness as B. cereus levels associated with food poisoning range from10^3 to 10^{10}cfu/g (2).

Based on molecular examination of B. cereus isolates virulence genes of which are responsible for toxin production in rice pudding samples , it is clear that emetic toxin ces gene could be found in 22.2 % of the isolates which is ahigher incidence than obtained by (11) who reported that 1.1% of B. cereus tested were emetic toxin producers. The ces gene encoding cereulide has no incidence in the isolates obtained from food samples examined by (13,5,20).

Diarrheal toxin genes nhe, hbl and cytK could be detected in 100%, 77.8 %, 100%, respectively of isolates obtained from dairy desserts (Fig. 1, photo 1-4) . These results agree with what recorded

before higher incidences of nhe 100% and cytK 91.6% but hbl was with lower incidence 4.2% in cooked rice samples *Bacillus cereus* isolates by (24). Also, it was revealed that in the examined rice milk samples the NHE gene 86.9% has more frequency than the Hbl gene 43.5% agree with our results but the cytK gene has much lower score 17.4% than us by (13).

In previous studies it is found that HBL gene was less in frequency than the NHE gene by (36,38) and both are shown to be the major virulence toxins of *Bacillus cereus* causing food poisoning (34).

With regard to results of molecular examination of some isolates which showed toxigenic activity, *Bacillus cereus* should be considered as a public health hazard to dairy desserts consumers . Being the cause of two types of food poisoning emetic type caused by cereulide toxin produced in food with short incubation period and diarrheal type by enterotoxins . Strict hygyienic measures should be applied in food production , insure clean sources for constituents, sufficient heat treatments when cooking , refrigeration as soon as possible to the products .

Table (1) Oligonucleotide Primers Sequences Source: Metabion (Germany).

Target gene	Primer Sequences	Amplified product	Reference
hbl	GTA AAT TAI GAT GAI CAA TTTC	1091 bp	(11)
	AGA ATA GGC ATT CAT AGA TT		
nhe	AAG CIG CTC TTC GIA TTC	766 bp	
	ITI GTT GAA ATA AGC TGT GG		
cytK	ACA GAT ATC GGI CAA AAT GC	421 bp	
	CAA GTI ACT TGA CCI GTT GC		
ces	GGTGACACATTATCATATAAGGTG	1271 bp	
	GTAAGCGAACCTGTCTGTAACAACA		
groEL	TGCAACTGTATTAGCACAAGC T	533 bp	(9)
	TACCACGAAGTTTGTTCACTACT		

Table (2) : Cycling conditions of the different primers during PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Number of cycles	Final extension
Toxins (hbl, nhe, cytK and ces)	94˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.
groEL gene	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

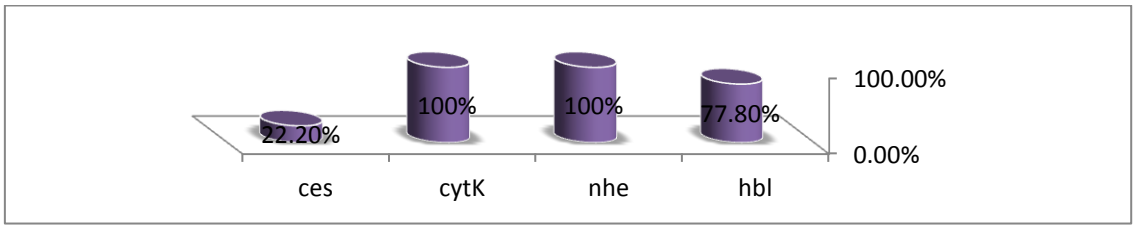
Table (3) Statistical analytical results of *Bacillus cereus* **in the examined dairy desserts (**mahallbia and rice milk**):**

Examined Products	No. of samples	Positive samples		Min.	Max.	M±SE
		No.	%			
Mahalbia (Pudding)	50	29	58 %	2.18×10^3	2.9×10^6	$8.38\times 10^5 \pm 1.8\times 10^5$
Rice milk (rice pudding)	50	37	74 %	1.01×10^4	3.9×10^7	$4.4\times \pm 1.4\times 10^6$ 10^6

Table (4) : Frequency distribution of *Bacillus cereus* **in the examined dairy desserts(** mahallbia and rice milk**)**

Examined samples / Intervals	Pudding (Mahalbia)		Rice pudding (rice milk)	
	No.	%	No.	%
$10^3 \geq 10^4$	3	10.3	0	0
$10^4 \geq 10^5$	7	24.14	4	10.8
$10^5 \geq 10^6$	10	34.4	15	40.6
$10^6 \geq 10^7$	9	31	14	37.8
$10^7 \geq 10^8$	0	0	4	10.8
Total	29	100 %	37	100 %

Figure 1: Prevalence of virulence (toxin) genes in *Bacillus cereus* **isolates obtained from dairy dessert samples.**



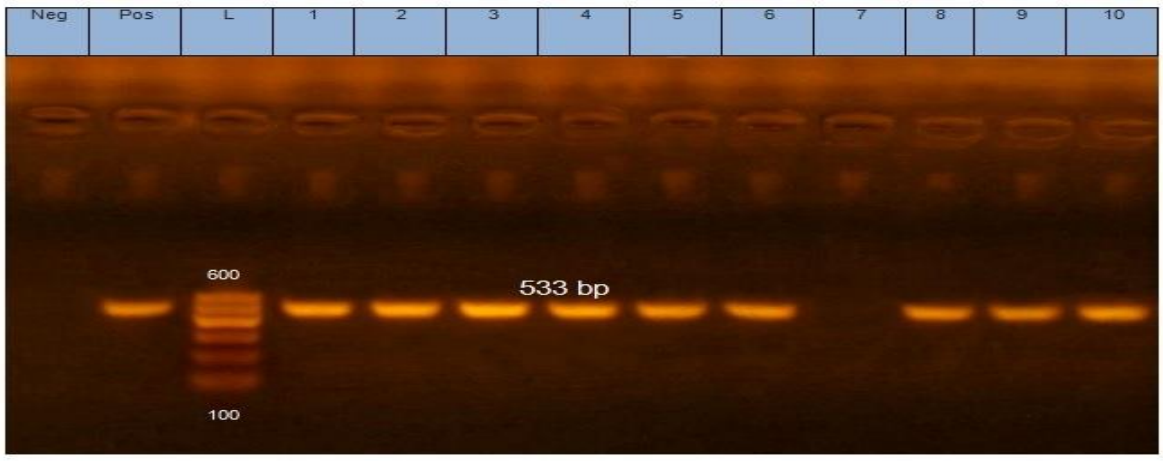


Photo 1. Agarose gel electrophoresis of uniplex PCR of groEl gene 533bp which is a diagnostic gene for B.cereus : L: Ladder, +ve: positive control forgroEl gene, -ve: negative control for groEl gene. Lane 1-10: B. cereus isolates obtained from examined dairy desserts samples .lanes 1,2,3,4,5,6,8,9,10 :positive B. cereus isolates for groEl gene.

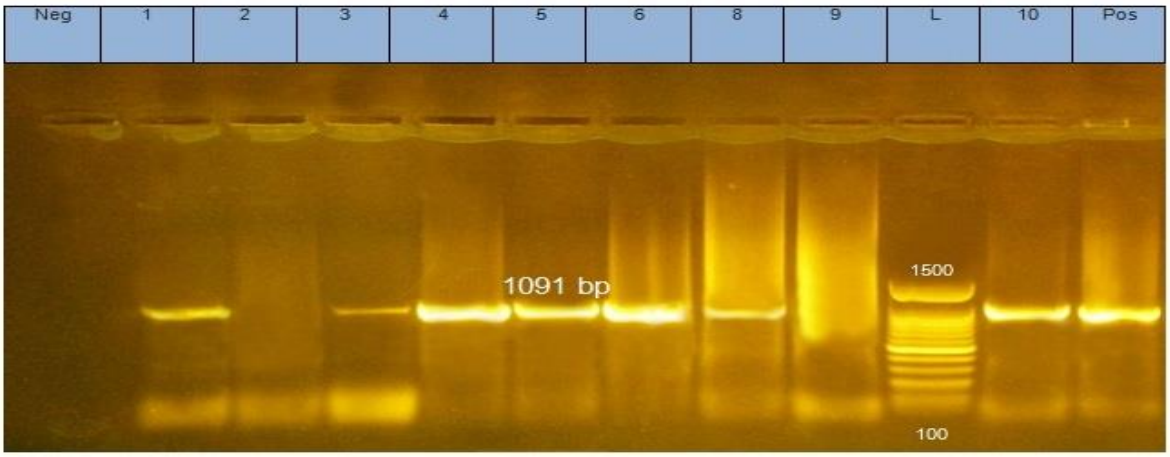


Photo 2. Agarose gel electrophoresis of uniplex PCR ofhbl gene 1091bp which is avirulence gene for b.cereus : L: Ladder, +ve: positive control for hbl gene, -ve: negative control for hbl gene. Lane 1-10: B. cereus isolates obtained from examined dairy desserts samples .lanes 1,3,4,5,6,8,10 :positive b. cereus isolates for hbl gene.

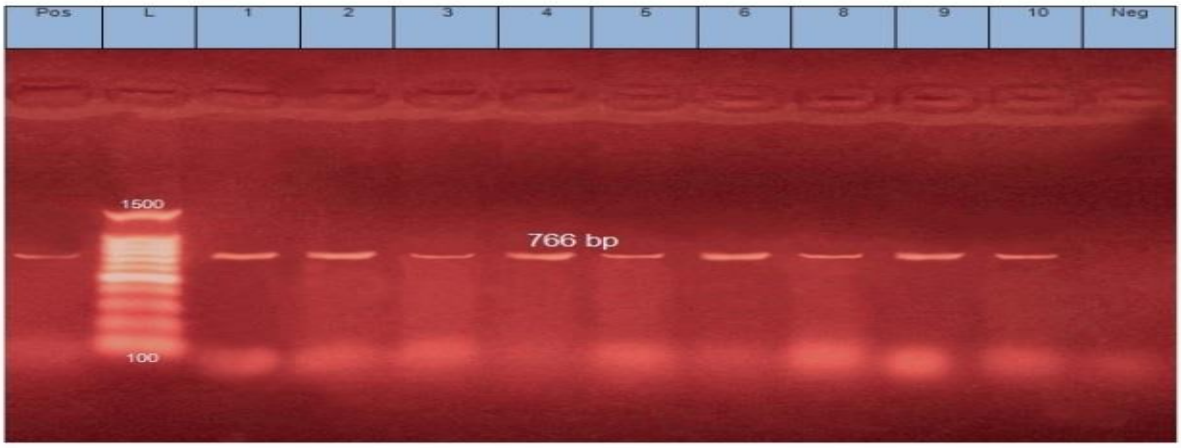


Photo 3. Agarose gel electrophoresis of uniplex PCR ofnhe gene766 bp which is avirulence gene for b.cereus : L: Ladder, +ve: positive control for nhe gene, -ve: negative control for nhe gene. Lane 1-10: B. cereus isolates obtained from examined dairy desserts samples .lanes 1,2,3,4,5,6,8,9,10 :positive b. cereus isolates for nhe gene.

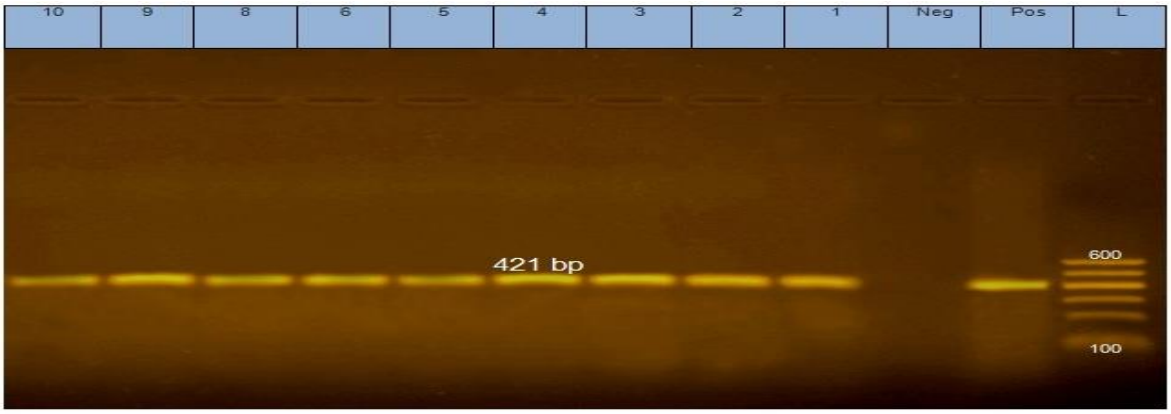


Photo 4. Agarose gel electrophoresis of uniplex PCR of cytk gene 421bp which is avirulence gene for b.cereus : L: Ladder, +ve: positive control for cytk gene, -ve: negative control for cytk gene. Lane 1-10: B. cereus isolates obtained from examined dairy desserts samples .lanes 1,2,3,4,5,6,8,9,10 :positive b. cereus isolates for cytk gen

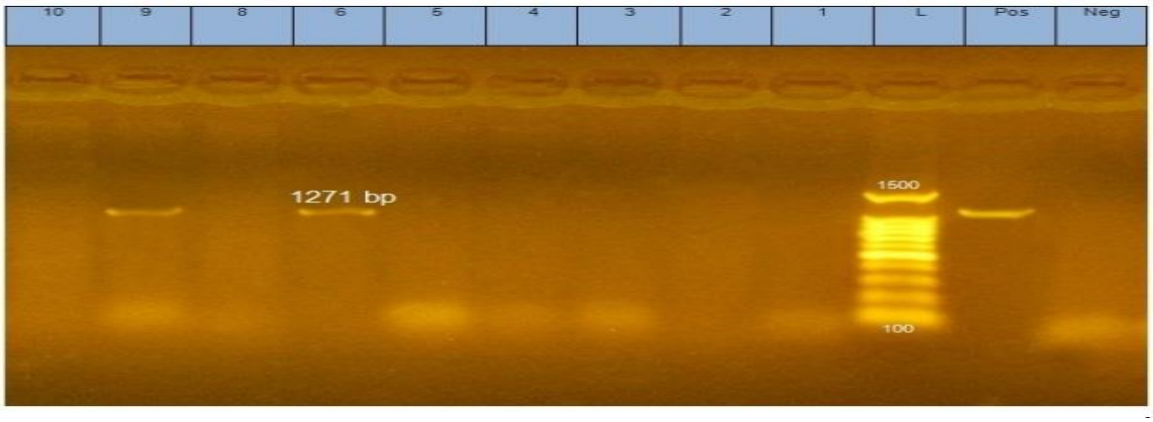


Photo 5. Agarose gel electrophoresis of uniplex PCR of ces gene1271 bp which is avirulence gene for b.cereusL: Ladder, +ve: positive control for ces gene, -ve: negative controlfor ces gene. Lane 1-10: B. cereus isolates obtained from examined dairy desserts samples .lanes 6,9 :positive b. cereus isolates for ces gene .

Conclusion

It can be concluded that the prevalence of *Bacillus cereus* in the examined dairy desserts indicates bad hygienic measures during their manufacture, inefficient heat treatment, improper storage conditons and the products are hold without refrigeration after processing that lead to product unfit for human consumption, public health hazard. So it is recommended to establish HACCP system or any equivalent system in the dairy chain and strict hygienic and manufacturing practice.

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تواجد الباسيلس سيريوس في بعض الحلويات اللبنية

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الملخص العربي

نظرا للشعبيه التي تحظي بها الحلويات اللبنية في مصر لذا قد أجريت هذه الدراسة لتقدير خطورة تواجد ميكروب الباسيلس سيريوس كمسبب للتسمم الغذائي في بعض الحلويات اللبنية التي تباع بمدينة المنصورة. لقد قمنا بتجميع عدد 100 عينه من الأرز بلبن والمهلبيه (50 عينه لكل منهما) بغرض اختبار مدي تواجد ميكروب الباسيلس سيريوس بها وقد وجد بها النتائج الآتية: تواجدت الباسيلس سيريوس في عينات المهلبية بنسبة 58% بمستوي عدّ من 10×2.18^{3} إلي 10×2.9^{6} ومتوسط عدّ $10\times8.38^{5}\pm10\times1.8^{5}$. أما في عينات الأرز بلبن فقد وجد بها أن ميكروب الباسيلس سيروس متواجد بنسبة74% وعدّ يتراوح من 10×1.01^{4} إلي 10×3.9^{7} ومتوسط عدّ $10\times4.4^{6}\pm10\times1.4^{6}$. وقد وجدت هذه الجينات ces , hbl , nhe , cytk بالعزلات عند فحصها جزيئيا بحثا عن وجود جينات الضرواة بميكروب الباسيلس سيروس وكلها فعالة وتتسبب في انتاج السموم التي بدورها تسبب حالات التسمم الغذائي .وقد أوضحت الدراسة أهمية الحفاظ علي صرامة الممارسات الصحية اثناء الإنتاج والتصنيع لهذه المنتجات لتفادي حدوث التسمم الغذائي .