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ORIGINAL ARTICLE

Prevalence of *Bacillus cereus* Virulence Genes in Some Dairy Desserts

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Abstract

BACKGROUND: Dairy products are ideal growth media for numerous bacteria, including psychrotrophic bacteria and spore-formers. Rice milk is made from various recipes and ingredients, such as milk a basic constituent, rice, corn starch, sugar, vanilla, nuts, raisins, and coconut to enhance its nutritive value, and dairy products are ideal growth media for numerous bacteria, including psychrotrophic bacteria and spore-formers. Milk pudding and creme caramel custard are popular dairy desserts consumed in Egypt that are processed and stored in conditions suitable for many microorganisms to flourish. One of these microorganisms is *Bacillus cereus*, a spore-forming pathogen that has been implicated in several foodborne outbreaks of two distinct types: diarrheal infections and emetic intoxication. Both types are caused by *B. cereus* harmful pathogenic toxins encoded by the virulence genes *Nhe, hbl, CytK* and *ces*.

OBJECTIVE: This study aimed to determine the prevalence of *B. cereus* toxin virulence genes in locally manufactured rice milk sold in Mansoura City.

PROCEDURES AND RESULTS: Some of the recovered isolates from rice milk were scanned for *B. cereus* virulence genes; it was found that *B. cereus* isolates obtained from the examined samples had *Nhe, hbl, CytK* and *ces* genes in different ratios.

CONCLUSION: Strict hygienic measures should be applied in food production, ensuring clean sources for constituents, sufficient heat treatments when cooking, and refrigeration of the products as soon as possible.

Keywords: **Bacillus cereus, Creme caramel custard, Food poisoning, Milk pudding, Virulence genes**

1. Introduction

Dairy products are ideal growth media for numerous bacteria, including psychrotrophic bacteria and spore-formers [1,2]. Rice milk is made from various recipes and ingredients, such as milk a basic constituent, rice, corn starch, sugar, vanilla, nuts, raisins, and coconut, to enhance its nutritive value and flavor [3,4]. These ingredients could be potential sources of microbiological contamination [5]. *Bacillus cereus* is a gram-positive psychrotrophic bacteria [6]. It is ubiquitous and abundant in nature, in plant-origin foods such as rice and vegetables, and is commonly present in dairy foods, vegetables, desserts and cakes [7–9]. Spore-creating skills enable *B. cereus* to survive most of the heating procedures performed in dairy production [10,11].

Bacillus cereus may be able to produce virulence toxins in food, making it a major foodborne pathogen incriminated in two different types of food poisoning [12,13] and emetic and diarrheal syndromes [14–17]. Usually, self-limiting, but severe and lethal consequences have been recorded in both types [18]. However, it is not a reportable illness [19]. *Bacillus cereus* food poisoning outbreaks have been related to all types of food products [20], specifically with the consumption of a variety of dairy products, starchy RTE food, and rice-based food [8,21,22].

So, this study aims to detect the prevalence of *B. cereus* virulence genes in some locally manufactured some dairy desserts that sold in Mansoura city in order to figure out the involvement of this product in food poisoning.

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2. Methods

2.1. Collection and preparation samples

One hundred samples of milk pudding, Creme Caramel Custard (50 samples each), were collected from different localities in Mansoura City, Egypt, during 2019 and 2020.

2.2. Isolation of Bacillus cereus

Every sample of rice milk was prepared According to Diane and Melody [23]. According to [24] aseptically inoculate 0.1 ml of each previously prepared dilution was inoculated directly onto the surface of *Bacillus cereus* selective agar base CM0617 plates. The plates were then incubated at 37 °C for 24 h. Typical colonies of *Bacillus cereus* were examined after 24 h if no colonies appeared, and a further 24 h incubation at 37 °C was performed. Typical *Bacillus cereus* colonies, which are approximately 5 mm in diameter, crenated, dull, and have the characteristic turquoise to peacock blue color, are surrounded by a good egg yolk precipitate of the same color because of the production of lecithinase [23].

2.3. Identification of Bacillus cereus

According to Diane and Melody [23], gram stain, anaerobic growth, hemolytic activity, and biochemical identification (acid production from sugar, nitrate reduction test) were performed on the isolates.

2.4. Molecular examination of Bacillus cereus isolates

According to Ehling-Schulz et al. [25], by scanning the isolates for some *Bacillus cereus* virulence toxin genes for the presence of the Nhe, Hbl, CytK, and ces genes after detecting the groEL gene in the scanned isolates.

2.4.1. Extraction of DNA

According to QIAamp DNA mini kit (Qiagen, USA).

2.4.2. Preparation of PCR master mix

Emerald Amp GT PCR Master Mix (Takara) kit and oligonucleotide primer sequences Metabion (Germany) (Table 1). The cycling conditions for the different primers used during PCR are presented in (Table 2).

2.4.3. Agarose gel electrophoreses [27,28] with modification

According to [28], Electrophoresis grade agarose (ABgene), DNA ladder Gel Pilot 100 bp plus ladder supplied by QIAGEN (USA), and gel documentation system (Alpha Innotech).

3. Results & discussion

Milk pudding and Creme Caramel Custard are popular desserts in Egypt that are palatable, rich in milk nutrients, healthy, served cool or hot, and cheap with all classes of consumers in different seasons [2]. Milk pudding, which is considered a ready-to-eat food (RTE), is made in a variety of recipes, constituents, cooking times, and temperatures, which are valuable media for the growth and flourishment of many microbes, especially spores that are able to withstand, survive, and grow to level hazards to public health [29]. Some studies have shown that RTE foods are contaminated with different pathogens through food preparation surfaces and packaging [30]. *Bacillus cereus* is commonly isolated from RTE food and milk products [31], and its toxins are common causes of symptoms associated with foodborne illnesses [32]. Therefore, the presence of *B. cereus* and its enterotoxigenic genes in milk products may pose a potential risk to public health [33].

B. cereus strains isolated from heated rice should be considered possible enterotoxin producers, in

Table 1. Oligonucleotide primers sequences source.

Target gene	Primer Sequences	Amplified product	Reference
Hbl	GTA AAT TAI GAT GAI CAA TTTC AGA ATA GGC ATT CAT AGA TT	1091 bp	[25]
Nhe	AAG CIG CTC TTC GIA TTC ITI GTT GAA ATA AGC TGT GG	766 bp	
cytK	ACA GAT ATC GGI CAA AAT GC CAA GTI ACT TGA CCI GTT GC	421 bp	
Ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271 bp	
groEL	TGCAACTGTATTAGCACAAGC T TACCACGAAGTTTGTTCACTACT	533 bp	[26]

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 s.	49 °C 1 min.	72 °C 1 min.	35	72 °C 10 min.
groEL gene	94 °C 5 min.	94 °C 30 s.	55 °C 40 s.	72 °C 45 s.	35	72 °C 10 min.

agreement with the results of PCR [34]. The consumption of rice-based and dairy products prepared for direct consumption has been reported to be strongly associated with *B. cereus* food poisoning outbreaks via toxin production [12,35−37]. The main factors leading to outbreaks are rice cooked and inefficiently refrigerated or stored at room temperature until consumption [38]. *B. cereus* is known to survive all unfavorable conditions via sporulation; therefore, routine heat treatment of products during processing is not completely efficient in eliminating *B. cereus* in foods, and some strains are psychrotolerant, thus limiting the quality of both pasteurized and refrigerated dairy products [39,40].

The number of toxin-related food poisoning outbreaks is largely underestimated because the disease is often mild and self-limiting and laboratory recognition (toxin testing) is not usually performed [41,42]. However, in Europe, *B. cereus* toxins are incriminated in approximately 600−700 definite foodborne outbreaks annually [43].

Because *B. cereus* contamination can occur from numerous sources throughout production, when food is not satisfactorily refrigerated and in the absence of competitive flora, *B. cereus* grows well after cooking [44]. In addition, handling and processing, and distribution mainly from improperly cleaned and sanitized equipment [35], this study aimed to investigate the potential hazards of food poisoning that may occur due to consumption of these products.

According in (Fig. 1) 20 *Bacillus cereus* isolates were chosen from those obtained from the examined samples and were molecularly scanned for groEL gene, which is the specific *Bacillus cereus* group gene, 18 (90%) of examined isolates found to harbor it.

B. cereus can produce at least four different toxins [45]. Diarrheal syndrome is linked to non-hemolytic enterotoxin (NHE), hemolysin BL (HBL), and cytotoxin K (CytK) [25,46], whereas emesis is produced by the action of cereulide toxin encoded by the ces gene. Detection of these toxins is principal to ensuring food safety; however, extraction of toxins from food is a difficult and time-consuming procedure, and some toxins are not directly detectable in food [32].

PCR has recently been useful for the quick recognition and differentiation of enterotoxin-encoded genes in *B. cereus* [47]. The recently identified cereulide synthetase genes permitted the development of a molecular assay that targets all toxins known to be involved in food poisoning in a single reaction using only four different groups of primers [25]. Different primer systems for PCR amplification are routinely used to detect the ces gene; however, cereulide and its non-antigenicity have resulted in difficulties in producing reliable detection methods [48].

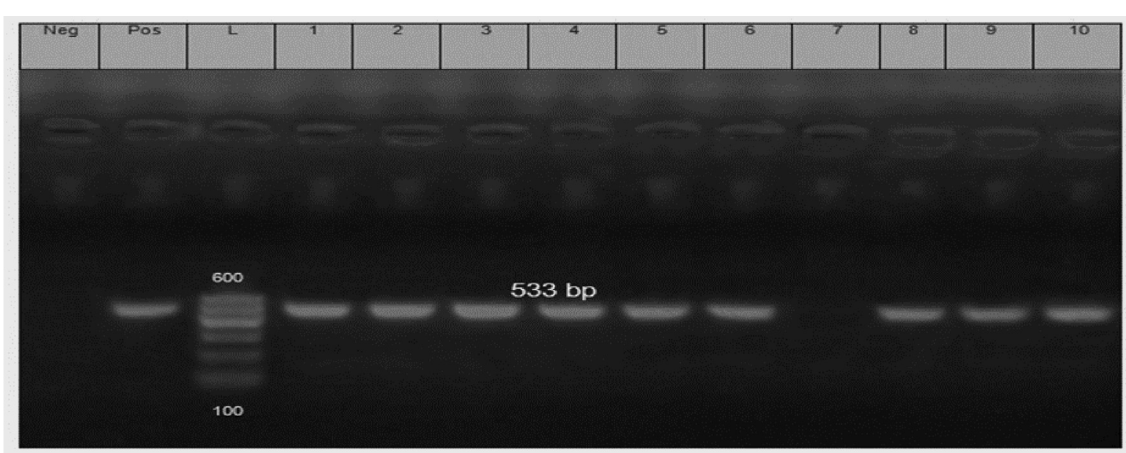

Fig. 1. Agarose gel electrophoresis of uniplex PCR of groEl gene 533 bp which is a diagnostic gene for B. cereus. Lanes 1,2,3,4,5,6,8,9,10: positive B. cereus isolates for groEl gene.

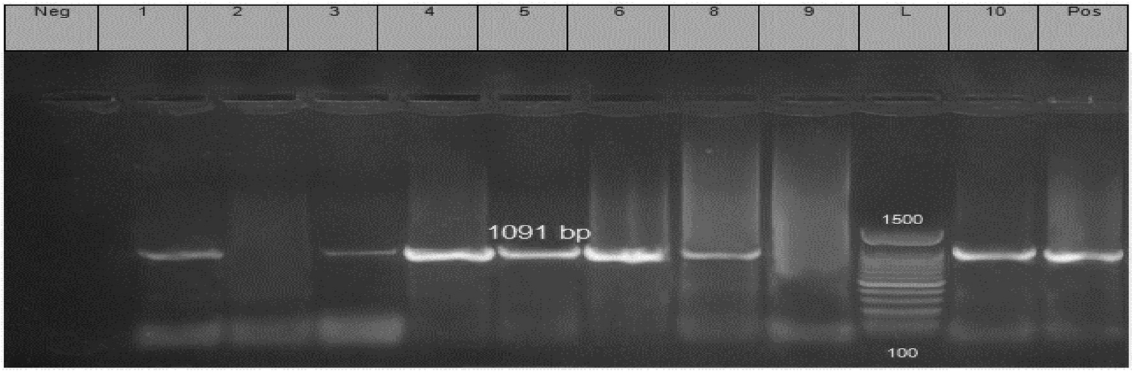

Fig. 2. Agarose gel electrophoresis of uniplex PCR ofhbl gene 1091 bp which is avirulence gene for b. cereus. lanes 1,3,4,5,6,8,10:positive b. cereus isolates for hbl gene.

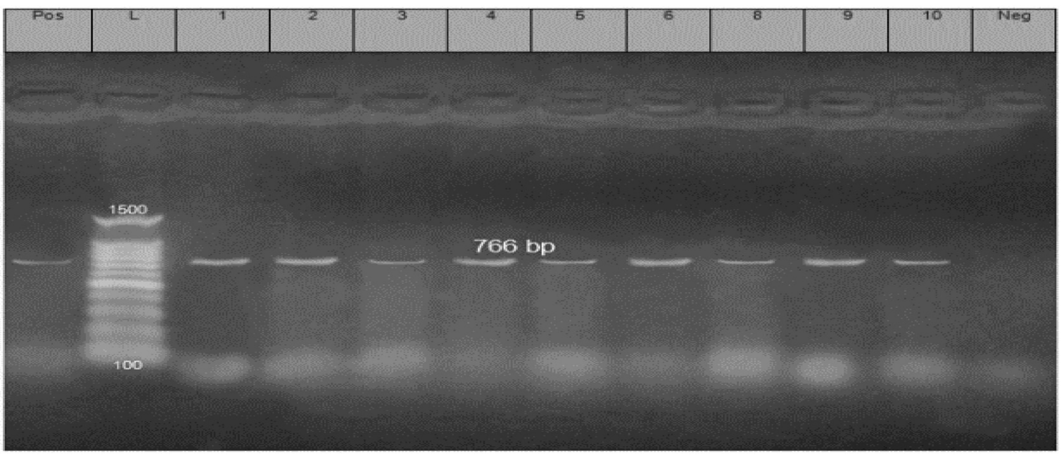

Fig. 3. Agarose gel electrophoresis of uniplex PCR of nhe gene 766 bp which is avirulence gene for b. cereus. Lanes 1,2,3,4,5,6,8,9,10: positive b. cereus isolates for nhe gene.

These 18 isolates, which were confirmed to be *Bacillus cereus*, were examined for the presence of potential toxin genes known to be responsible for the virulence of *B. cereus*. Emetic toxin ces gene could be detected in 25% 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 (Figs. 1–5).

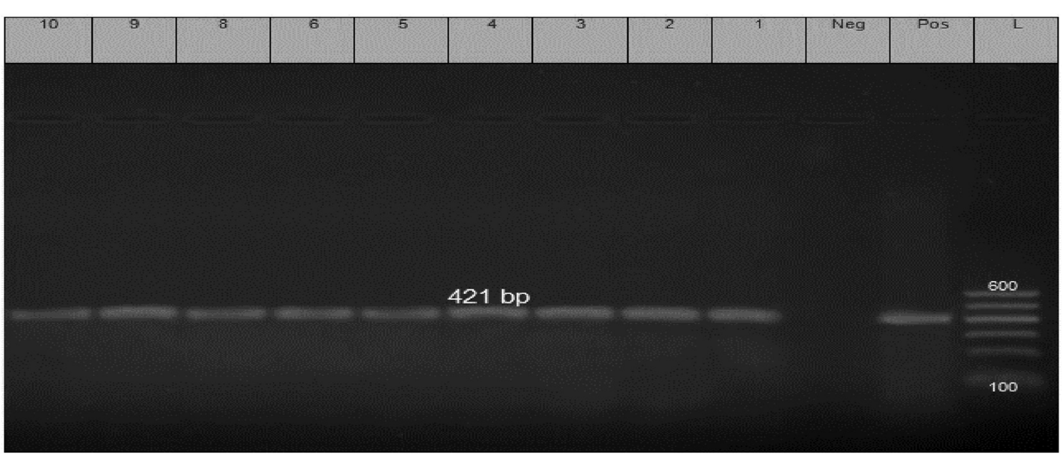

Fig. 4. Agarose gel electrophoresis of uniplex PCR of cytk gene 421 bp which is avirulence gene for b. cereus. Lanes 1,2,3,4,5,6,8,9,10: positive b. cereus isolates for cytk gene.

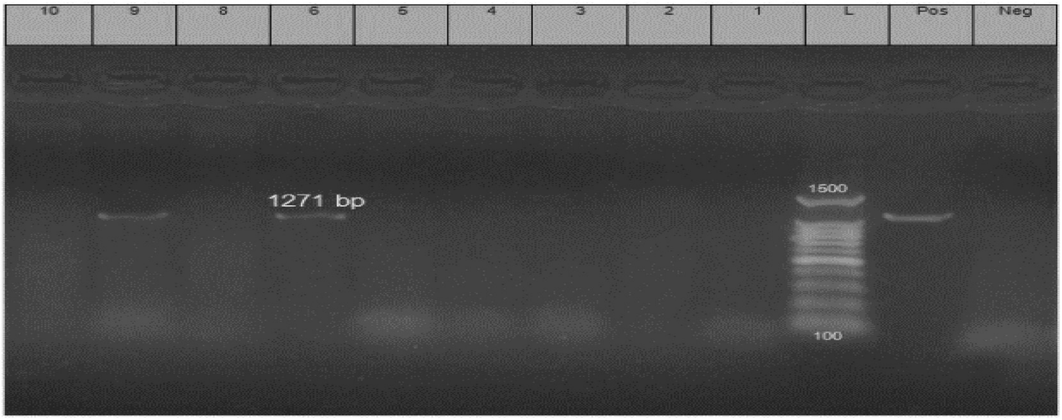

Fig. 5. Agarose gel electrophoresis of uniplex PCR of ces gene 1271 bp which is a virulence gene for b. cereus. Lanes 6, 9: positive b. cereus isolates for ces gene.

There is a connection between rice and the production of the toxin, known as the emetic toxin cereulide, by *B. cereus*. Cereulide toxins are believed to cause emetic food poisoning, which may lead to mortality. In most cases of emetic toxin, food poisoning is associated with precooked rice-based food held for too long at unsatisfactory storage temperatures (without refrigeration), which is ideal for inducing growth and cereulide synthesis by emetic *B. cereus* strains [21,49,50].

Strains having cereulide toxin genes were infrequent in dairy products [51,52]. [2,53], Kim et al. (2011) established that the ces gene is not present in isolates obtained from food samples.

In our study, based on molecular examination of *B. cereus* isolates, it was clear that emetic toxin ces gene was identified to be in 25% of the isolates (Fig. 5). Congruently with [54], who identified ces 21% of milk samples examined. But is a higher incidence than obtained by others; 1.0–3.8% [51], 1.1% [25], 1.1% [55], 4.7% [56], 5% [22], 4% [57], 0.9%, 6.8% in dairy and rice samples, respectively, [46], 3.1% of the isolates dairy products [58],7% [37], and 11.1% [59] in (Table 3).

Much higher incidence reported by [60], who identified ces in 66% of raw milk samples examined. The combination of dairy and cereal ingredients results in higher levels of cereulide production compared to rice and non-dairy ingredients [61,62]. Because cereulide is resistant to acidic conditions, proteolysis, and heat, it will not be destroyed by anything; therefore, emetic *B. cereus* is considered a potential hazard in dairy products [45]. The results in Fig. 5 are compatible with those of earlier studies, which reported that rice-containing desserts could accumulate high amounts of cereulide when stored at non-refrigerated temperature [63,64].

It is currently considered that the enterotoxins Hbl, Nhe, and CytK, which are produced by *B. cereus*, are the causative agents of *B. cereus* diarrheal foodborne disease and are genetically encoded by Hbl, Nhe, and CytK. They cause diarrhea by disrupting the integrity of the plasma membrane of the epithelial cells in the small intestine [65] (Table 3).

Diarrheal toxin genes Nhe, Hbl, and CytK could be detected in 100%, 77.8%, 100%, respectively of isolates obtained from rice milk samples in our study (Figs. 2–4). This indicated that diarrheal strains have a broader existence and a higher threat of *B. cereus* infection.

Nhe gene have been detected in 18 (100%) of isolates screened, which is in agreement with results obtained in other dairy products studies; Nhe identified in 84.3% of examined samples by [53], 96% [55],100% [66], 100% [67], 86.9% [2], 2017, 63% [54], 60% [68], 93% [22],100% [69], 98% [70], 71% [71], 82.7% [46], 100% [62], 83% [37], and 94.4% [59] in (Table 3).

Hbl was detected in 77.8% of rice milk isolates and this is a high prevalence indicating a serious problem for consumers. Almost agree with [46,70]78%

Table 3. Prevalence of B. cereus (groEL gene), B. cereus emetic strains (ces gene) and B. cereus diarrheal strains (Hbl, Nhe, CytK genes) from the examined samples based on molecular examination (cPCR).

	Number of examined isolates	Number of +ve isolates (%)
B. cereus (groEL gene)	20	18 (90%)
B. cereus Emetic strains (ces gene)	20	5 (25%)
B. cereus diarrheal strains (Hbl gene)	20	14 (77.8%)
B. cereus diarrheal strains (Nhe gene)	20	20 (100.0%)
B. cereus diarrheal strains (CytK gene)	20	20 (100.0%)

and 84.9%, respectively). However, higher than others: 13% [68], 11% in dairy products [71], 57.6% in rice [46], 9:30% in dairy food [62], 39% ready to eat food containing rice [37], and 11.1% [59] in (Table 3).

It is clear that the Nhe gene had a higher percentage than the Hbl gene in our isolates, and these results are compatible with [2,22,37]; all reported that the Hbl gene was less frequent than the Nhe gene in isolates from dairy foods. Other studies have found opposite results (Hbl gene had higher percentage than Nhe gene) 31.9%,15.9% hbl, nhe respectively, [56], 42%,34% hbl, nhe, respectively, [33], 57.6%,48% hbl, nhe, respectively, [46]. However, both are known to be major virulence toxins of *B. cereus*, causing food poisoning.

We identified cytK in 100% of our isolates. Several authors also detected cytK gene in dangerous percent 91.6% [67], 88% [54], 75% [68], 73% [22], 95.5%, and 100% of dairy products and rice, respectively, [71], 68.2% [58] and 75.9% [59]. However, our results contradict those found by [2] only 17.4% of dairy desserts have cytk, [56] cannot detect cytk (0%) in milk based desserts, and [69] identified cytk in 17% of Starchy/rice food examined. *B. cereus* isolates harboring the cytK gene should be considered extremely toxic because of the necrotizing effect of cytK toxin [72].

4. Conclusion

With regard to the results of molecular examination of our isolates, which showed toxigenic activity, strict hygienic measures should be applied in food production, ensuring clean sources for constituents, sufficient heat treatments during cooking, and refrigeration as soon as possible to the products. Rice should not be reserved for a long period of time. Confirms the requirement for Authorities and manufacturers must approve *B. cereus* as a reference for microbiological hazard control, principally for dairy food.

Ethical approval

All experimental procedures were approved by the Mansoura University Institutional Animal Care and Use Committee.

Author's contribution

All authors contributed equally to this work.

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Conflicts of interest

The authors declare no conflicts of interest, financial or otherwise.

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