

Characterization of *Bacillus Cereus* Isolated From Raw Milk and Milk Products

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Abstract

Bacillus cereus (*B. cereus*) is one of the common types of the group of bacteria responsible for foodborne illnesses, causing severe gastrointestinal disorders. In this study we quantify the rate of *B. cereus* contamination in food, particularly the raw milk and the milk products. To this end, a total of 100 samples of raw milk and milk products, were collected from Aswan Governorate in Egypt, and examined for the presence of *B. cereus*. 34 % of samples showed positive results on Mannitol egg yolk polymyxin (MEYP) agar and biochemical tests. The Polymerase chain reaction (PCR) tests, using specific primer for *B. cereus*(*cspf* gene), confirmed the results by biochemical identification. Different virulence genes were detected in *B. cereus* isolates; diarrheal type genes (*hbl* gene 47 %, *nhe* gene 53 % and *cytK* gene 33 %), while no isolates showed harbored emetic type genes (*ces* gene). These results highlighted the importance of including *B. cereus* in the disease control and the prevention programs, as well as in the schedule of clinical and food quality control of the Egyptian laboratories.

Keywords: *Bacillus cereus*; Milk; Milk Product; Emetic Gene; Enterotoxin Genes

Introduction

Milk is considered the most perfect single food for human from birth to senility providing major nutritional requirements to human at any age. However, milk is a nutritious medium that can support the growth of different types of microorganisms during a long chain of milk processing, personnel handling, distribution as well as storage [1]. Kareesh cheese, which is a soft cheese commonly made and consumed in Egypt, is also a rich source of many micronutrients, such as amino acids, protein, and vitamin. Similarly, the microbiological quality of this cheese is affected by all the production processes as well as packaging and handling [2]. These kind of Cheeses are produced in either a commercial facilities such as large planning that is well equipped or in a small scale facilities such as farmers homes, which are unlicensed facilities. The former represents a challenging in hygiene and coping with the safe production standards. Manufacturing cheese in such circumstances may lead to series hazards. For instance, microorganisms may contaminate the cheese during handling and distribution processes [2]. Ice cream is major produce of dairy industry and it has an increasing interest of a large segment of the population [3]. Unlike the other dairy products, the microbiological quality of ice cream during retail marketing mainly relies on the post production handling, especially the quality of the frozen storage. Due to the lack of the efficient frozen storage, there is a chance of temperature raise during transport and distribution of ice cream. Under warm tropical climatic conditions psychrotrophs can proliferate leading to occasional food poisoning events [4].

Among the contaminating microorganism, *B. cereus* is an opportunistic pathogen classified as the fifth main frequent bacterium causing foodborne infectious diseases and food poisoning in the USA [5]. It is a gram-positive, spore-forming, motile rod that is broadly dispersed in the environment and undoubtedly the most important of the aerobic spore-forming species found in milk [6]. *B. cereus* may produce emetic toxin and diarrheal enterotoxins, which may cause two types of gastrointestinal disease emesis and diarrhea. The emetic *B. cereus* is caused by cereulide, which comprised of the heat-stable cyclic dodecadepsipeptide encoded by the *ces* gene [7]. Three heat-labile enterotoxins that cause a diarrheal type are hemolysin BL, nonhemolytic enterotoxin, and cytotoxin K [7]. As a result of lack of effectual scrutiny, *B. cereus*-associated food poisoning may be generally unregistered and perhaps confused with *Staphylococcus aureus* and *Clostridium perfringens* food poisoning due to similar symptoms [8].

In the Middle East, there is an attainment in infant diarrhea cases which were attributed to unidentified etiology; especially when the analysis of fecal samples for the presence of *Salmonella*, *Shigella*, and *Entamoeba* produce negative results. *B. cereus* is not taken into consideration when diarrhea cases for infants or adults are analyzed [9]. Thus, the true occurrence of *B. cereus* in the Egyptian community is not plainly understood. The current analytical methods for *B. cereus* recommended in the Food and Drug Administration's Bacteriological Analytical Manual (BAM) and International Organization for Standardization is cultivation on Mannitol Egg Yolk Polymyxin (MYP) media but traditional method remain frail [10]. So, it is necessary to develop rapid methods to discriminate hazardous strains from non-toxic strains. The efficacy of polymerase chain reaction-based methods is noticeable by the 1999 guidelines issued by the National Committee for Clinical Laboratory Standards (NCCLS-1999) in the identification of *B. cereus* [11]. For the previous reasons, our study focused on determining the incidence of *B. cereus* in raw milk and milk products and the detection of the virulence genes encoded for the emetic and diarrheal syndrome.

Materials and Methods

Samples Collection

A total of 100 samples including raw milk (n = 30), ice cream (n = 30), and Kareesh cheese (n = 40) were collected from different shops in Aswan, Egypt during the summer season. All samples are collected in sterile bags and brought to the laboratory in an insulated icebox. They were kept in the refrigerator at 4 °C until tested.

Preparation of Samples

Ice Cream: Ten ml of ice cream (15±20 gm) were diluted with 90 ml of sterile saline than tenfold serial dilution was done. The preparation of the ice cream samples was performed according to Zhou, *et al.* [12].

Raw Milk: Raw milk samples were subjected to tenfold serial dilutions. The preparation of the raw milk samples is done according to Altekruse, *et al.* [13].

Kareesh Cheese: Ten gram of cheese was homogenized in 90 ml sterile saline solution (0.85 % NaCl) and then shaken vigorously for 5 min in 250 ml Erlenmeyer flasks. Tenfold serial dilutions up to 10⁶ were then prepared. The preparation of Kareesh cheese samples was done according to Ayoub, *et al.* [14].

Isolation and Identification of *B. cereus*

Isolation and identification of *B. cereus* were done according to Bottone, *et al.* [15]. Minutely, 0.1 ml aliquot of each dilution was spread on the surface of Mannitol yolk polymyxin (MYP) media (high media batch no 1427431172) and incubated at 37 °C for 24 hours. All growing *B. cereus*-like isolates (appear as a violet-red background and are surrounded by a zone of egg-yolk precipitate), were considered as presumptive *B. cereus* isolates and were streaked on sheep blood agar plates to observe haemolysis after incubation at 34 °C for 24h [16]. Colonies were identified by biochemical tests that include the production of catalase, arginine dihydrolase, reduction of nitrate, Voges Proskauer reaction, gelatin hydrolysis, acidification of glucose, hydrolysis of starch, and motility test.

DNA Extraction

The refreshment of suspected isolates was done in nutrient broth (oxid) at 30 °C (2 °C) for 16-24h. DNA was extracted from 5 ml of incubated broth for each isolate by QIAamp DNA Mini kit (Qiagen, Germany, GmbH) (Catalogue no.51304) according to the manufacture instructions.

Molecular Confirmation of *B. cereus* by Using PCR

Gene	Type	Sequence (53)	Size	References
cspF		F- CTT (C/T) TT GGCCTT CTT CTA A- R- GAG ATT TAA ATG AGC TGT AA	284bp	[12]
ces	Emetic type	F1 GGTGACACAT- TATCATATAAGGTG R2 GTAAGCGAACCT- TCTGTAACAACA	1271bp	[4]
hbl	Diarrheal type	F-GTA AAT TAI GAT GAI CAA TTTC R-AGA ATA GGC ATT CATAGA TT	1091bp	[10]
nhe	Diarrheal type	F-AAG CIG CTC TTC GIA TTC R- ITI GTT GAA ATA AGC TGTGG	766bp	[5]
cytk	Diarrheal type	F-ACA GAT ATC GGI CAA AAT GC R-CAA GTI ACT TGA CCIGTT G	421bp	[4]

Table 1: Oligonucleotide primer sequences

Amplification reaction was carried out according to Altayar and Sutherland [17] with slight modification, in a final volume of 25µl including 12.5 µl PCR Mastermix (Emerald Amp GT), 1 µl for each primer for *cspf* gene (Table 1), 6 µl of DNA Template and 4.5 µl of PCR grade water. Reactions were done in thermal cycler (MJ Research, Inc. Watertown, MA) with the following program: initial denaturation at 95 °C for 15 sec followed by 30 cycles of 50 °C for 30 sec, 72 °C for 30 sec and 72 °C for 1 min with a final extension at 72 °C for 2 min. PCR product was alienated by gel electrophoresis (1.5 % agarose gel (Bio shopR, Candainc) stained with ethidium bromide, then visualized in a UV transilluminator.

Detection of Virulence Genes in *B. cereus* Isolates by Using Multiplex PCR

Multiplex PCR was applied for detection of virulence genes encoded for, emetic type(*ces* genes) and diarrheal types (*hbl*, *nhe*, *cytk* genes) (Table 1), the PCR mixture was prepared as following: 25 µl PCR Taq green Mastermix (Thermo), 1 µl of F primer for each gene 1 µl of R primer for each gene and 8 µl DNA. The final volume was adjusted to 50 µl by adding PCR grade water (9 µl). Amplification profile was standardized in 95 °C for 7 min followed by 30 cycles at 94 °C or 30 sec then 60 °C for 30 sec and 72 °C or 45 sec with a final extension at 72 °C for 7 min. PCR amplicatons were alienated by gel electrophoresis (1.5 % agarose gel stained with ethidium bromide) then were visualized in a UV transilluminator.

Results

The bacteriological examination showed that 34 % of the total samples (n = 100) cleared a violet color with red background colonies. These colonies encircled by a zone of egg- yolk precipitate on MYP media. Also hemolysis was appeared with a ground glass-like appearance on the blood agar. This is a typical sign that those samples may be positive for *B. cereus*, i.e. they may contain this microorganism. We need then to confirm this result by biochemical tests.

The biochemical scheme used for identifying *B. cereus* revealed positive results in the following tests:

- Catalase,
- Arginine dihydrolase,
- Reduction of nitrate,
- Motility test,
- gelatin and starch hydrolysis,
- Acidification of glucose, and
- Voges Proskauer reaction

The highest incidence of contaminated samples were obtained from Kareesh cheese 16/40 (40 %) and raw milk 12/30 (40 %) followed by ice cream 6/30 (20 %), as shown in Table 2.

Type of samples	No. of samples	No. of positive samples for <i>B. cereus</i>
Raw milk	30	12 (40 %)
Kareesh cheese	40	16 (40 %)
Ice cream	30	6 (20 %)
Total samples	100	34

Table 2: Incidence of *B.cereus* isolated from examined samples

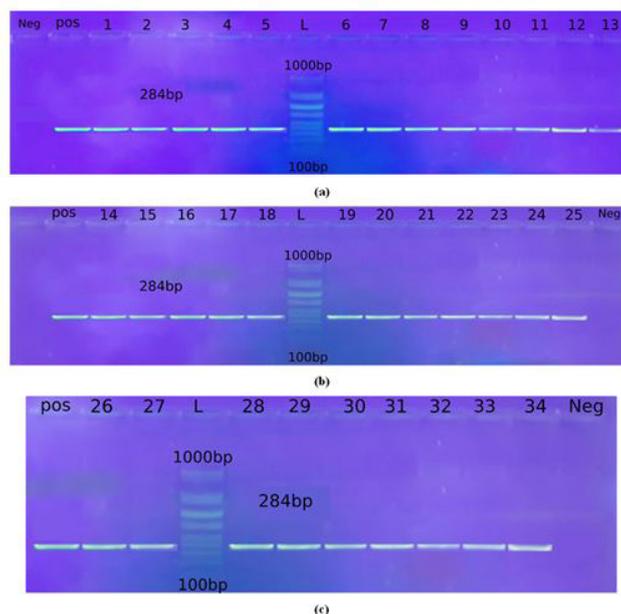


Figure 1: Amplification profile of *cspf* gene lane (L): molecular weight marker 100 bp DNA ladder Lane (pos): control positive Lane (neg): control negative Lanes (1-34) are positive isolates for *cspf* gene of *B. cereus* showing band at 284 bp. The lanes are shown in Figure 1-a are for raw milk (lanes 1-12) and kareish cheese (lane 13). For Figure 1-b, the lanes are for kareish cheese (lanes 14-25). The lanes shown in Figure 1-c are for Kareesh cheese (lanes 26-28) and ice cream (lanes 29-34)

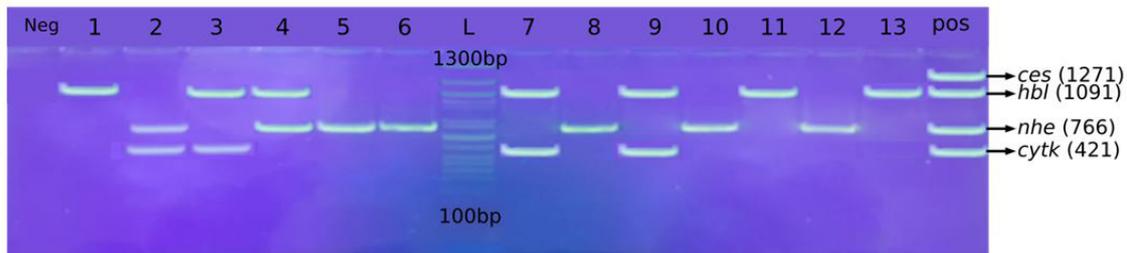


Figure 2: Lane (L): 100 bp DNA ladder; Lane (Pos): positive control; Lane (Neg): negative control. Agarose gel electrophoresis of PCR products of *Bacillus cereus* enterotoxin genes detected in raw milk and kareish cheese by multiplex PCR raw milk isolates from lane1: lane12. Lane 1: positive for *hbl* gene (1091bp) Lane2: positive for *nhe* (766 bp), *cytk* (421bp). Lane3: positive for *hbl*, *cytk*. Lane4: positive for *hbl*, *nhe*. Lane6: positive for *nhe*. Lane7: positive for *hbl*, *cytk*. Lane8: positive for *nhe*. Lane9: positive for *hbl*, *cytk*. Lane10: positive for *nhe*. Lane11: positive for *hbl* Lane12: positive for *nhe*. Kareish cheese isolates from lane 13: lane28 Lane 13: positive for *hbl*

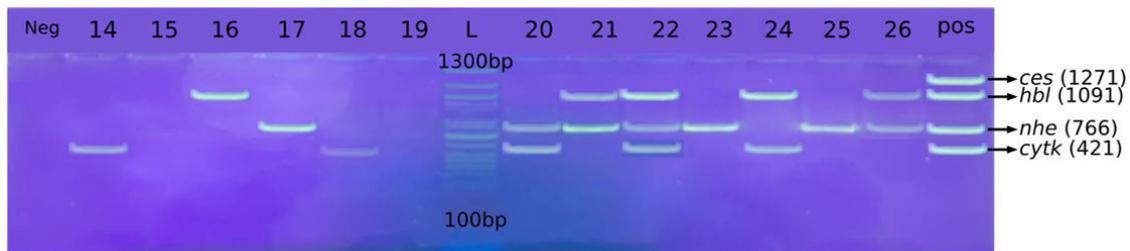


Figure 3: Lane (L): 100 bp DNA ladder; Lane (Pos): positive control; Lane (Neg): negative control. Agarose gel electrophoresis of PCR products of *B. cereus* enterotoxin genes Lane 14: positive for *cytk* Lane 15: negative for all genes Lane 16: positive for *hbl* Lane 17: positive for *nhe* Lane18: positive for *cytk* Lane19:negative for all genes. Lane20: positive for *nhe*, *cytk* lane21: positive for *hbl*, *nhe* Lane22: positive for *hbl*, *nhe*, *cytk* Lane23: positive for *nhe* Lane24: positive for two genes *hbl*, *cytk* lane25: positive for *nhe* Lane 26:positive for *hbl*, *nhe*

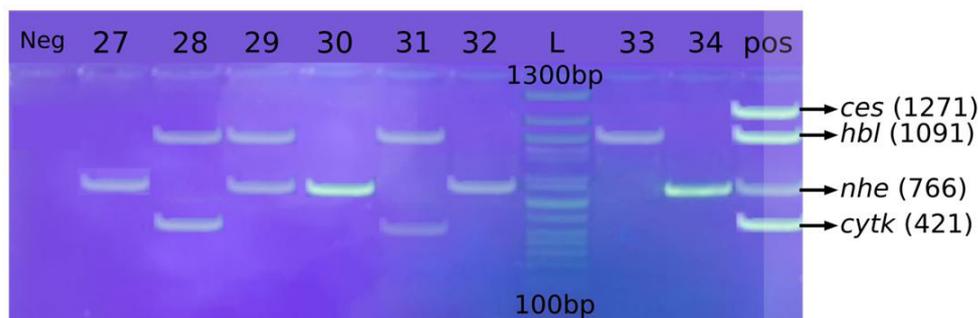


Figure 4: Lane (L): 100 bp DNA ladder; Lane (Pos): positive control; Lane (Neg): negative control. Agarose gel electrophoresis of PCR products of *B. cereus* enterotoxin genes Lane 27: positive for *nhe* Lane 28: positive for *hbl*, *nhe*. Ice cream isolates from lane 29 to lane 34, Lane 29: positive for *hbl*, *nhe*, Lane30: positive for *nhe*, Lane31: positive for *hbl*, *cytk* Lane32: positive for *nhe* detected Lane33: positive for *hbl* Lane34: positive for *nhe*

Toxigenic genes	Raw milk (n = 12)	Kareesh cheese (n = 16)	Ice cream (n = 6)	Total (n = 34)
Ces	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Hbl	6 (50 %)	7 (44 %)	3 (50 %)	16 (47 %)
Nhe	6 (50%)	8 (50 %)	4 (67 %)	18 (53 %)
Cytk	4 (34%)	6 (38 %)	1 (17 %)	11 (33 %)

Table 3: Distribution of virulence genes in *B. cereus* isolates

Further identification was performed by using the PCR technique. Specific primers (*cspf* gene) for *B. cereus* were used and inveterate the presence of the DNA of *B. cereus* in the biochemically positive isolates (n = 34 isolates), as shown in Figure 1. This confirms the previous step that these samples are biochemically positive for *B. cereus*. Two types of virulence genes were detected in this study, i.e metic gene, enterotoxin genes. The data reported in the Table 3 and the results shown in Figures 2,3 & 4 revealed that *hbl*, *nhe*, and *cytk* gene were detected in 47 %, 52 %, and 33 % of the tested isolates, respectively. While *ces* gene was absent in all the *B. cereus* isolates.

Discussion

The occurrence of *B. cereus* in food is a major cause for concern for human health. This pathogen is responsible for the spoilage of milk and other food products. It is a major foodborne pathogen [18]. So, the identification of this strain should be used as a part of a threat in the microbiological analysis. In this study, the prescriptive identification showed typical rough and bright pink colonies with a zone of egg yolk precipitation on mannitol egg yolk polymyxin agar in 34 % of the samples. The description of colonial morphology was similar to Yobouet, *et al.* [19]. Also the microscopic features showed gram-positive spore-forming rods of *B. cereus*. These findings were supported by Bottone, *et al.* [15]. In addition, the results of biochemical tests were similar to Shivalingsarj, *et al.* [20]. Milk produced from healthy cows would be deemed germs free, but dairy farms environments may be a source of contamination predominantly, during milking and cheese handling. In particular, *B. cereus* was prominent as responsible for raw milk spoilage [21].

In this study, the incidence of *B. cereus* contamination in raw milk was 40 % (Table 2). These results were supported by Shivalingsarj, *et al.* [20] who isolated the organism from milk and milk-based food with a percentage of 40 %. The lower results were gained by Giffel, *et al.* [22] and Hassan, *et al.* [23] who reported that 35 % and 30 % of the examined raw milk samples were contaminated with *B. cereus*, respectively. However, higher incidence rates of *B. cereus* in milk were reported by Rezende-Lago, *et al.* [24] and Owusu-Kwarteng, *et al.* [25], who reported values of 50 % and 45 %, respectively. *B. cereus* contamination of raw milk varied between studies, due to many factors, such as combined different farming practices, season, animal housing, ration, teat soiling, and bedding. Milk contamination with *B. cereus* spores was interrelated with the number of *B. cereus* spores in feces and silage [21].

The infection of milk and other dairy products with *B. cereus* is abandoned problem due to its effect on the quality of the products and the probable health hazards of the presence of toxigenic strains [19]. The results illustrated in Table 2 show that 20 % of the ice cream samples were contaminated by *B. cereus*. These results were similar to those reported by Ayoub, *et al.* [14] and Atasever, *et al.* [26]. They found that 20 % and 19 % of tested raw milk samples and ice cream were contaminated with *B. cereus*, respectively. However, higher incidence rates of *B. cereus* in ice cream were recorded by Ahmed, *et al.* [27], Wong, *et al.* [28], and Zhou, *et al.* [12] as 48 %, 52 %, 26.6 %, and 79.4 %, respectively. *B. cereus* may be present in high rate at the raw feed or may have conditions that are generally less than standard [29]. Noticeably, a raising growth rate through the manufacturing. Additionally, the view cabinet, machines, and processing station may be contaminated by *B. cereus*.

Forty percentage of Kareesh cheese samples were contaminated by *B. cereus*, as shown in Table 2. This increase in contamination rate due to the traditional soft curd cheeses cannot be subjected to temperatures more than 40 °C. The hazard of causing food poisoning increases if cheeses are made from raw milk, and the safety of processing tools alone as the main pathogen control step in cheese making at this time under interest [28]. Our results were nearly parallel to Owusu-Kwarteng, *et al.* [25] who illustrated that 38.7 % of Kareesh cheese samples were tainted with *B. cereus*, while lower results were noted by Enan, *et al.* [30] 25 %. However, higher incidence rates were reported by Daur, *et al.* [31] (45 %). In general *B. cereus* appear to be survived in the cheese-making process. In most cases, concentrations decreased during storage, probably due to the pH reductions and the growth of competitive microflora [13].

B. cereus is currently the focus of rising awareness due to its ability to produce a range of enterotoxins [32]. The PCR test is designed as a rapid and reliable method for the detection of the presence of specific organisms even when the organism is present in a low number and the sample is contaminated with some other organisms [33]. In the current study, the PCR confirmed the presence of *B. cereus* DNA in 34 isolates out of 34 isolates identified serologically, by using specific primers for *B. cereus* at 284 bp (Figure 1). These findings were bolstered by Organji, *et al.* [34]. *B. cereus* harbored different virulence targeted genes encoding enterotoxins and emesis. These genes included haemolytic (*hbl*) and non-haemolytic (*nhe*) enterotoxins, cytotoxin K (*cytk*), and cereulide (*ces*) genes, as shown in Figures 2,3 & 4. The distribution of these virulence genes amid 34 *B. cereus* isolates are demonstrated in Table 2. Several studies have been established the presence of enterotoxigenic *B. cereus* group in dairy products [35-37].

The diarrheal syndrome is caused by three enterotoxins; *hbl*, *nhe* and/or *cytk* formed in the intestine by a high number of cells or spores ingested. The enterotoxins are heat labile and sensitive to acid conditions. They are destroyed during cooking or gastro-intestinal digestion. Food containing at least 10³ cfu/l is considered to be harm to destroy by digestion, but instead colonize the gut of the host and produce adequate enterotoxin to cause diseases [38]. *hbl* is the main virulence factor. The vast majority of research on the pathogenic nature of *B. cereus* has been deliberate for identifying and characterizing this *hbl* diarrhea enterotoxin [39]. In this study, *hbl* gene was detected in 47 % of *B. cereus* isolates related results were supported by Wiwat, *et al.* [40]. The lower percentage was recorded by Zhou, *et al.* [12] who found that 45.8 % of *B. cereus* isolates have the *hbl* gene. Higher results were reported by Wijnands, *et al.* [41], Ngamwongsatit, *et al.* [42], and Tewari, *et al.* [43] who showed that 66 %, 65.9 % and 55 % of tested isolates harbored *hbl* gene.

The incidence rate of *nhe* gene was 53 % in tested isolates, as shown in Table 2. Similar results were reported by Owusu-Kwarteng, *et al.* [25] who showed that 60 % of tested *B. cereus* isolates contained a *nhe* gene. The higher results of *nhe* were reported by Zhou, *et al.* [12], Wiwat, *et al.* [40] and Ngamwongsatit, *et al.* [42] who showed that 97 %, 100 %, 87.5 % of tested *B. cereus* isolates contained *nhe* gene, respectively. In the previous studies enforced our result in the following: it was demonstrated that the gene of the *hbl* was generally less common than this of the *nhe* [36,31]. Our result clarified that *cytk* gene was detected in 11 isolates of *B. cereus* (33 %). Nearly similar results were noted by Vyletelova, *et al.* [44] who showed that 29 % of tested *B. cereus* isolates harbored *cytk* gene. The higher percentage was recorded by Owusu-Kwarteng, *et al.* [25], Wijnands, *et al.* [41], Ngamwongsatit, *et al.* [42] and Tewari, *et al.* [43] who found different percentage 50 %, 88.8 %, 41.4 % and 100 %, respectively. The lower percentage was recorded by Zhou, *et al.* [12] who detected *cytk* in 8.3 % of tested isolates.

The emetics syndrome is caused by the emetic toxin or cereulid, which is highly resistant to temperature and heat. It is performed in the food and therefore will not be ruined either by cooking or by digestion. It is considered as a real danger [45]. In the present work, *ces* gene was absent in all samples, this result was supported by Arslan, *et al.* [37]. Also, Kim, *et al.* [46] and Ankolekar, *et al.* [47] showed that the *ces* gene encoding the emetic toxin was not detected in all isolates from clinical and food samples. On the other hand, *ces* gene was detected by Owusu-Kwarteng, *et al.* [25] in raw milk (21 %) and traditional cheese (13 %). This is in disparity with prior findings of the high incidence of emetic strain of *B. cereus* in rice fields and the processing environment, due to rice is a selective area for growth of the emetic strain of *B. cereus* [34]. Because *B. cereus* is mostly normal flora of soil and often related to farm environments and the fecal shedding of cattle, there is a higher threat of contamination of milk, and consequent entry into the dairy food chain where they can encourage spoilage and diseases.

Conclusion

The present study concluded that *B. cereus* is widespread in the raw milk and the traditional dairy products in Egypt. Different enterotoxin genes related to the virulence of *B. cereus* are common among isolates. However, emetic toxin was rare among the isolate. It is clear from this study that the threat of food poisoning caused by *B. cereus* in dairy products should not be ignored. There is therefore the need to observe good hygienic and manufacturing practice by milk producers and traditional dairy processors to prevent contamination and subsequent potential disease outbreak by *B. cereus*.

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