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Occurrence and characterization of toxigenic *Bacillus cereus* in food and infant feces

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ABSTRACT

Objective: To investigate the true incidence of *Bacillus cereus* (*B. cereus*) in food and children diarrhea cases.
Methods: A total of 110 samples of various dairy products such as raw milk, long life pasteurized milk, yoghurt and infant powdered milk formulas, raw rice, and feces were examined for the presence of *B. cereus* by selective plating on mannitol-egg-yolk-polymyxin agar. Confirmation of *B. cereus* was carried out by biochemical tests and PCR. Identification of non-*B. cereus* isolates was carried out by 16S rDNA sequencing. Antimicrobial susceptibility was done by disk diffusion method.
Results: Overall 35 samples (31.8%, n = 110) yielded *Bacillus*-like growth. Of which 19 samples (54.28%) were positive for *B. cereus*. All isolates were positive for enterotoxin production. No psychrotolerant *B. cereus* strains were detected in all samples. All *B. cereus* isolates were resistant to penicillin G, but susceptible to vancomycin, erythromycin and clindamycin.
Conclusions: The results of this study confirm the importance of including *B. cereus* in disease control and prevention programs, as well as in routine clinical and food quality control laboratories in both Saudi Arabia and Egypt.

1. Introduction

Bacillus cereus (*B. cereus*) is a facultative anaerobic, Gram-positive, spore forming bacterium; that is widely distributed in the environment due to its ability to resist hostile conditions[1,2]. *B. cereus* is a common food contaminant, it can be found in different types of raw food such as rice, meat, vegetables, raw milk, dairy products as well as cooked dishes[3-8]. The presence of *B. cereus* in food is usually associated with food spoilage[9] as well as food poisoning that usually occur in two types of illness: the emetic and diarrheal syndromes. The emetic syndrome is due to a small molecular weight toxin, the cereulid, whereas the diarrheal syndrome results from the production of enterotoxins[10].

B. cereus has been implicated in various foodborne outbreaks worldwide[10-12]. Due to lack of effective surveillance, *B. cereus*-associated food poising may be largely under-reported, and probably confused with *Staphylococcus aureus* and *Clostridium perfringens* food poisoning due to similar symptoms[10]. In the Middle East, particularly Saudi Arabia, there is an increase in infant diarrhea cases[13], which were usually attributed to unknown etiology; particularly when the analysis of fecal samples for the presence of *Salmonella*, *Shigella* and *Entamoeba* yields negative results. *B. cereus* is not taken into consideration when diarrhea cases (infant or adults) are diagnosed in Saudi Arabia[13]. Thus, the true occurrence of *B. cereus* in the Saudi and Egyptian communities is not clearly understood.

The routine detection and identification of *B. cereus* in food and feces involves the use of selective solid media such as mannitol-egg-yolk-polymyxin agar (MYP) and polymxin pyruvate-egg yolk-mannitol-bromothymol blue-agar that usually facilitates the detection of *B. cereus* lecithinase production (precipitate zones with egg yolk) and lack of mannitol fermentation. Other routine identification for *B. cereus* isolates include detection of motility, observation of haemolyisis on blood agar, acidification of glucose[10]. Detection of *B. cereus* enterotoxin is made commercially available by using the BCET-RPLA kit (Oxoid), a semi-quantitative reversed antibody agglutination assay that detects the L2 component of the Hbl cytotoxin[10]. Various PCR protocols have been developed to identify *B. cereus* isolates obtained from food, fecal and environmental samples. A combination of PCR primers BcAPR1 and BcFF2 can detect the *cspF* gene in both mesophilic and psychrotrophic *B. cereus* strains[5].

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The aim of the present study is to determine the true incidence of *B. cereus* in food, particularly infant milk powder formulas, and infant fecal samples in both Saudi Arabia and Egypt. All *B. cereus* isolates were characterized by phenotypic traits, PCR, production of enterotoxin and antibiotic susceptibilities. All non-*B. cereus* isolates were identified by 16S rDNA PCR and sequencing.

2. Materials and methods

2.1. Sampling

In the present study we examined a total of 110 different samples for the presence of *B. cereus*; these samples were collected from the city of Makkah (Saudi Arabia) and the city of Fayoum (Egypt), samples were collected from October 2012 to March 2013. The 110 samples were comprised of raw rice (14 samples), raw milk (7 samples), long life pasteurized milk (2 samples), pasteurized milk (6 samples), yoghurt (5 samples), various formulas of powdered infant milk (20 samples) and infant and children feces (ages between eight days and two years old) (56 samples).

Raw rice, dairy products and infant milk powder samples were purchased from local supermarkets and groceries. Raw milk samples were obtained from local farms. Infant and children diarrhea fecal samples were obtained from Maternity and Children Hospital. Raw milk and fecal samples were transported to the laboratory in sterile conditions and on ice. All samples were processed within six hours of collection at the same day of sampling.

2.2. Isolation of B. cereus

For the isolation of *B. cereus* all samples were devitalized at 80 ℃ for 10 min in a water bath to kill vegetative cells and recover bacterial spores[14]. Preparation of samples for devitalization was as follow: a volume of 100 mL of pasteurized milk and yoghurt was used directly; for raw rice and infant milk powder, a 10 g of each sample was added to 90 mL of sterile pure water, and 2.0 g of each fecal sample was homogenized in 18 mL of sterile pure water prior to heat treatment. An aliquot of 1.0 mL of each devitalized sample was added to a universal bottle containing 9.0 mL of nutrient broth (Oxoid, Basingstoke, UK) for primary enrichment. Enrichment cultures were incubated at 34 ℃ for 24 h. Observation of turbidity in enrichment cultures was considered as a presumptive positive result. All presumptive positive enrichment cultures were streaked on MYP agar plates (Oxoid), incubation of the plates was at 34 ℃ for 24 to 48 h[15].

2.3. Identification of B. cereus isolates

All *B. cereus*-like isolates growing on MYP agar plates (appear as blue colonies surrounded by egg yolk precipitate), were considered as presumptive *B. cereus* isolates and were streaked on sheep blood agar plates (Oxoid) to observe haemolysis after incubation at 34 ℃ for 24 h[16].

Colonies growing on blood agar plates (large, grayish to greenish, circular colonies with a β-haemolytic and ground glass appearance) were identified by biochemical tests that include production of catalase, arginine dihydrolase, reduction of nitrate, Voges Proskauer reaction, gelatin hydrolysis, acidification of glucose, hydrolysis of starch and motility[11,16]. Incubation at 7 ℃ for 7-10 days was done to recover psychrotolerant strains[17]. *B. cereus* ATCC11778 (Oxoid) was used as control strain throughout the study.

2.4. Identification of B. cereus by PCR

Colonies growing on blood agar were also subjected to identification by PCR, in parallel with biochemical tests. The PCR method was described by Altayar and Sutherland[5], using primers BcAPR1 [CTT (C/T) TT GGC CTT CTT CTA A] and BcFF2 (GAG ATT TAA ATG AGC TGT AA) (Bioneer Corp., Korea), amplification of DNA using these primers was shown to give a single PCR band of 284 bp PCR reaction mixture (50 μL in volume) contained: 5.0 μL of 10 × PCR buffer, 2.0 mmol/L $MgCl_2$, 5.0 pmol of each primer, 0.2 mmol/L of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 1 IU of *Taq* DNA polymerase (Qiagen, UK) and a pin-head-sized aliquot of bacterial target DNA. PCR amplification was then done with 30 cycles at 95 ℃ for 15 seconds, 50 ℃ for 30 seconds and 72 ℃ for 30 seconds, followed by a final extension step at 72 ℃ for 7 min.

2.5. Detection of B. cereus enterotoxin and antibiotic susceptibility

The diarrheal toxin was detected from colonies growing on blood agar plates by immunolatex assay using the BCET-RPLA kit (Oxoid) according to the manufacturer instructions[18].

The antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method. All isolates were grown in brain heart infusion broth (Oxoid) for 18 h at 34 ℃ followed by spreading on Mueller-Hinton agar (Oxoid)[19]. Six commercially antibiotic disks were used (Oxoid): vancomycin (30 μg/mL), clindamycin (2.0 μg/mL), erythromycin (15 μg/mL), gentamicin (10 μg/mL), oxacillin (1.0 μg/mL) and penicillin (10 units). All Mueller-Hinton plates were incubated at 34 ℃ for 18-24 h[19].

2.6. Identification of non-B. cereus isolates by 16S rDNA PCR and sequencing

All *Bacillus*-like isolates that did not yield PCR products with primers BcAPR1 and BcFF2 were subjected to identification by means of 16S rDNA sequencing. PCR amplification of 16S rDNA was performed using the following primers[20]: F785 (5'-GGATTAGATACCCTGGTAGTC-3') and R1510 (5'-GGCTACCTTGTTACGA-3'), (Bioneer Corp., Korea). The PCR protocol was carried out as described by Assaeedi *et al.*[20]. Sequencing of PCR products was performed by the team of Al-Jawhara Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.

3. Results

The aim of the current study was to investigate the true incidence of *B. cereus* in food, children diarrhea cases and infant milk formulae products in both Saudi Arabia and Egypt. A total of 110 samples were screened for the presence of *B. cereus*, overall 35 samples (31.8%) yielded *Bacillus*-like growth. Of which 19 samples (54.28%) were positive for *B. cereus* (Table 1). *B. cereus*

were recovered from raw rice (75%), raw milk (66.6%), infant milk powder (33.3%), and infant and children (less than two years old) diarrhea cases (62.6%) (Table 1).

Table 1

Occurrence of *B. cereus* in food and infant fecal samples in Saudi Arabia and Egypt.

Sample type	No. of samples tested	No. of *Bacillus*-like colonies [*n* (%)]	No. of confirmed *B. cereus*	% of samples with *B. cereus*
Raw rice	14	4 (28.5)	3	75.0
Raw milk	7	3 (42.8)	2	66.6
Infant milk formula	20	12 (60.0)	4	33.3
Infant feces	56	16 (28.5)	10	62.6
Yoghurt	5	0	0	0.0
Long life pasteurized milk	2	0	0	0.0
Pasteurized milk	6	0	0	0.0
Total	110	35 (31.8)	19	54.2

As shown in Table 2, all *B. cereus* isolates were confirmed by their biochemical prosperities, and gave a 284 bp PCR product with primers BcAPR1 and BcFF2 (Figure 1). It was noted that all these confirmed isolates were also positive for BCET-RPLA test which confirms the presence of *B. cereus* enterotoxin. None of all confirmed *B. cereus* isolates were able to grow at 7 ˚C (Table 2) which means none of these isolates is psychrotolerant.

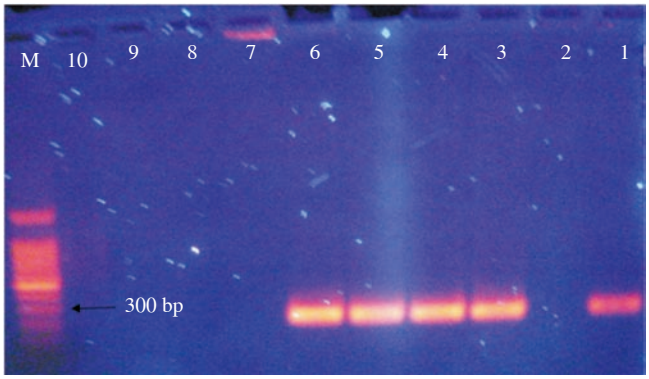

Figure 1. PCR amplification of 284 bp fragments using BcAPR1 and BCFF2 primers.

Lanes 1, 3, 4, 5 and 6 are positive while lanes 2, 7, 8, 9 and 10 are negative. M: DNA ladder 100 bp from Promega (Madison, WI, USA). Lane 1: R1; Lane 2: R8; Lane 3: M4; Lane 5: IM5; Lane 6: F25; Lane 7: M3; Lane 8: IM1; Lane 9: F10.

Table 2

Characteristics of *B. cereus* strains isolated from food and infant feces from Saudi Arabia and Egypt.

Isolate	Source	Haemolysis	Motility	Starch	Nit	Gel	VP	Glu	ADH	Cat	Growth at 7 ˚C	Toxin	PCR
R1	Raw rice	+	+	+	+	+	+	+	+	+	-	+	+
R3	Raw rice	+	+	+	+	+	+	+	+	+	-	+	+
R6	Raw rice	+	+	+	+	+	+	+	+	+	-	+	+
M4	Raw milk	+	+	+	+	+	+	+	+	+	-	+	+
M5	Raw milk	+	+	+	+	+	+	+	+	+	-	+	+
IM4	Infant milk formula	+	+	+	+	+	+	+	+	+	-	+	+
IM5	Infant milk formula	+	+	+	+	+	+	+	+	+	-	+	+
IM7	Infant milk formula	+	+	+	+	+	+	+	+	+	-	+	+
F4	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F14	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F25	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F33	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F35	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F40	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F42	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F51	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F52	Feces	+	+	+	+	+	+	+	+	+	-	+	+
F55	Feces	+	+	+	+	+	+	+	+	+	-	+	+
26 man	Infant milk formula	+	+	+	+	+	+	+	+	+	-	+	+

Nit: Nitrate reduction; Gel: Gelatin hydrolysis; VP: Voges Proskauer reaction; Glu: Acidification of Glucose; ADH: Arginine dihydrolase; Cat: Catalase.

Table 3

Identification of other non-*B. cereus* isolates by 16S rDNA.

Isolate	Source	Haemolysis	Motility	Growth at 7 ˚C	PCR	Toxin	16S rDNA identification	Similarities shared with GenBank isolates
R8	Raw rice	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
M3	Raw milk	-	+	-	-	-	*B. subtilis*	(100%) *B. subtilis* FJ984438.1
IM1	Infant milk	+	+	-	-	-	*B. licheniformis*	(97%) *B. lichenformis* (w611) KC441866.1
IM2	Infant milk	+	+	-	-	-	*B. licheniformis*	(97%) *B. lichenformis*(w611) KC441866.1
IM6	Infant milk	+	+	-	-	-	*B. licheniformis*	(97%) *B. lichenformis* (w611) KC441866.1
F10	Feces	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
F17	Feces	+	+	-	-	-	*B. lichenformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
F29	Feces	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
F31	Feces	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
F39	Feces	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
F50	Feces	+	+	-	-	-	*B. licheniformis*	(97%) *B. licheniformis* (xfbak) KC429647.1
1 man	Infant milk	+	+	-	-	-	*B. licheniformis*	(97%) *B. lichenformis* (w611) KC441866.1
5 man	Infant milk	+	+	-	-	-	*B. licheniformis*	(97%) *B. lichenformis* (w611) KC441866.1
24 man	Infant milk	-	+	-	-	-	*B. sphaericus*	(88%) *B. sphaericus* I14011.1
25 man	Infant milk	-	+	-	-	-	*B. subtilis*	(100%) *B. subtilis* FJ984438.1
7 man	Infant milk	-	+	-	-	-	*B. polymyxa*	(93%) *B. polymyxa* JN409465.1

All other *Bacillus*-like isolates (16 in total) that were not confirmed by PCR using primers BcAPR1 and BcFF2 as *B. cereus* were identified by means of 16S rDNA PCR and sequencing. These isolates were identified as *Bacillus licheniformis* (*B. licheniformis*), *Bacillus polymyxa* (*B. polymyxa*), *Bacillus sphaericus* (*B. sphaericus*) and *Bacillus subtilis* (*B. subtilis*) (Table 3). Among these isolates, it can be noted that *B. licheniformis* was more abundant (12 out of 16 isolates), particularly in infant fecal samples and infant milk formulas.

Screening *B. cereus* isolates for their antimicrobial susceptibilities showed that all isolates were resistant to penicillin G, but susceptible to all other antibiotics tested (Table 4).

Table 4

Antibiotic susceptibilities of *B. cereus* isolates.

Antibiotics	Number of isolates [*n* (%)]		
	Resistant	Intermediate resistance	Sensitive
Oxacillin	0 (0.0)	0 (0.0)	19 (100.0)
Vancomycin	0 (0.0)	0 (0.0)	19 (100.0)
Clindamycin	0 (0.0)	0 (0.0)	19 (100.0)
Penicillin G	19 (100.0)	0 (0.0)	0 (0.0)
Gnetamicin	0 (0.0)	0 (0.0)	19 (100.0)
Erythromycin	0 (0.0)	0 (0.0)	19 (100.0)

4. Discussion

B. cereus is an important underestimated foodborne pathogen that is ubiquitous in the environment[2]. Although, substantial amount of data are available about the incidence of *B. cereus* in various types of food and food poisoning cases worldwide[10], data concerning the occurrence of this pathogen in food and its involvements in diarrhea cases in Saudi Arabia and Egypt are sparse. Therefore, the results presented in this study report for the first time the incidence of toxigenic *B. cereus* in food and fecal samples from infants and children diarrhea cases in Saudi Arabia and Egypt.

The contamination of milk and other dairy products with *B. cereus* is a common problem due to its effect on the quality of the products and the potential health hazards of the presence of toxigenic strains[21]. In the current study, two out of seven raw milk samples were found to be contaminated with toxigenic *B. cereus*. Other studies reported relatively high percentage of *B. cereus* contamination of raw milk[15,21,22]. The source of contamination of raw milk samples is probably the soil in the milking area, feed and bedding material could be as well potential sources of contamination[21,22]. In this study, raw milk samples were obtained from different farms, four out of seven samples were negative for *Bacillus*-like growth. This may indicate that with good code of practice and hygiene, contamination of raw milk with *Bacillus* spp. in general could be avoided. Drinking unpasteurized raw milk purchased from local farms could be considered as a customary habit for young and adults in Saudi Arabia, with the incidence of toxigenic *B. cereus* in raw milk, food poisoning is likely to occur.

In Saudi Arabia, infant and children diarrhea cases after the consumption of powdered milk formulas is a common under reported problem. Usually, the parents are advised to change the product if they encountered this problem without further diagnosis. In most of the cases the diarrhea stops when the milk formula is changed and therefore the entire case is neither diagnosed nor being documented. In the current study we report for the first time the occurrence of toxigenic *B. cereus* strains in infant powdered milk formulas available in the market of both Saudi Arabia and Egypt. It is possible that the occurrence of diarrhea cases after the consumption of these products is due to their contamination with toxigenic *B. cereus*. The contamination of infant milk formulas with *B. cereus* is well documented[23-26]. Contamination of infant milk formulas with *B. cereus* is unavoidable considering the current manufacturing technologies associated with inappropriate handling practices, however it can be reduced if a hazard analysis and critical control point system is implemented during manufacturing[25,27].

It is established that cooked rice is associated with *B. cereus* food poisoning[28], a number of studies reported the incidence of toxigenic *B. cereus* in raw and cooked rice dishes[4,6,7]. In the current study we managed to isolate toxigenic *B. cereus* from imported white raw rice in Saudi Arabia. *B. cereus* spores may survive cooking temperature, and the spores may heat-shocked to germinate, if these dishes left to cool at ambient temperature, germination and vegetative growth may begin, thus, if these vegetative cells are toxigenic, infection is most likely to occur[7]. Rice is the main and daily dish in Saudi cuisine, with various cooking and dietary habits together with improper handling and kitchen hygiene, it is possible to expect potential health hazard due to *B. cereus* food poisoning.

The diagnosis of diarrhea stool samples in clinical laboratories in Saudi Arabia is solely based on the detection of *Salmonella*, *Shigella*, *Vibrio* and *Entamoeba*[13]. Thus, diarrhea caused by other pathogens such as *Campylobacter*, *Escherichia coli* O157 and *B. cereus* may not be reported and usually if stool cultures were negative for the sought after pathogens, then the diarrhea case could be reported as "unknown etiology" and/or "viral infection". In this study we managed to isolate *B. cereus* for the first time from infant stool samples. We assumed that these infant diarrhea cases were caused by the consumption of *B. cereus*-contaminated infant milk formula, especially that we found toxigenic *B. cereus* strains in these products available in the market. The incidence of enterotoxin and emetic toxin-producing *B. cereus* strains from stool samples of diarrhea patients is widely reported[18,28,29].

In the current study, psychrotolerant *B. cereus* isolates were not recovered from all types of samples. This might suggest the absence of emetic toxin-producing *B. cereus* and agree with the findings of Samapundo *et al*.[8] that the incidence of psychrotolerant *B. cereus* (growing at 7 °C) is generally low.

We examined all *B. cereus* isolates recovered in this study for their antimicrobial susceptibility. All isolates were resistant to penicillin G, while sensitive to vancomycin, clindamycin, erythromycin, gentamicin and oxacillin. Our results support the broad picture of antibiotic susceptibility patterns of *B. cereus* and confirm resistance to penicillin G and susceptibility to clindamycin, vancomycin and erythromycin[30].

A total of 16 *Bacillus*-like isolates were recovered from various samples, but were not confirmed as *B. cereus* by biochemical tests and PCR. We identified these isolates by 16S rDNA sequencing and revealed that *B. licheniformis* were more prevalent than other *Bacillus* species, and were found in raw rice, raw milk, infant fecal samples and infant milk formula. *B. licheniformis* have been recovered from raw and infant milk powder, and implicated in food poisoning cases[31,32]. *B. licheniformis* have shown to exhibit pathogenic properties and they may pose a health hazard[33,34].

In conclusion, the occurrence of toxigenic *B. cereus* strains in rice, raw milk and infant milk formulas in Saudi Arabia and Egypt indicates possible high risk of foodborne infections that could occur as a result of the consumption of these products. This was confirmed by the recovery of *B. cereus* strains from the fecal samples of infants and children under the age of three years old, where these cases had been diagnosed as "unknown etiology". The results of this study confirm the importance of including *B. cereus* in disease control and prevention programs, as well as in routine clinical and food quality control laboratories in both Saudi Arabia and Egypt. The result of this research is very relevant to other countries in the Middle East and other developing countries where data on the occurrence of toxigenic *B. cereus* in food and diarrhea cases are sparse and difficult to obtain, where *B. cereus* food poising is underestimated. To the authors' best knowledge, this is the

first report on the incidence of toxigenic *B. cereus* in food and feces in both Saudi Arabia and Egypt.

Conflict of interest statement

We declare that we have no conflict of interest.

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