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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 poisoning 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

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(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].

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 °C 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 °C 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 °C for 24–48 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 °C 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 °C 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 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 2 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 °C 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 °C 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.*