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Original Research Article

Prevalence, antibiotic-resistance properties and enterotoxin gene profile of Bacillus cereus strains isolated from milk-based baby foods

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Abstract

Purpose: *To investigate the prevalence, distribution of enterotoxins and antibiotic resistance of B. cereus in milk-based infant foods.*
Methods: *Three-hundred milk-based infant foods were collected and immediately transferred to the laboratory. Samples were cultured and B. cereus isolates were also confirmed using polymerase chain reaction (PCR)-based detection of gyrB gene. B. cereus strains were subjected to disk diffusion and PCR-based detection of enterotoxigenic genes.*
Results: *Prevalence of B. cereus in infant foods was 3 %. Contamination was in the range of 12.5 – 41.5 CFU/g. Brand D had the highest prevalence of B. cereus (6.2 %). NheA (88.8 %), nheC (55.5 %) and entFM (55.5 %) were the most commonly detected enterotoxigenic genes. Bacteria showed the highest prevalence of resistance against penicillin (100 %), tetracycline (77.7 %) and oxacillin (66.6 %). Prevalence of resistance against two antibiotics were 100 %.*
Conclusion: *Considerable prevalence of resistant and toxigenic B. cereus and high consumption of milk-based infant foods in Iran, represent an important public health issue which should be considered for further preventive approaches.*

Keywords: *Prevalence, Bacillus cereus, Antibiotic resistance, Enterotoxigenic genes, Milk-based infant food*

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INTRODUCTION

Infants and young children are mainly susceptible to food-borne diseases because they have weak immune system. They are at a great risk of gastrointestinal (GI) disorders mostly caused by pathogenic agents. It has been documented that about 5 billion episodes of GI diseases and disorders occur worldwide each year in children under 4 years old [1]. About 2 million infants are at risk of death due to food-borne diseases [1].

Bacillus cereus (*B. cereus*) is a Gram-positive, facultative anaerobic, rod-shape and spore forming bacterium that is widely survived in hard conditions like pasteurization and sterilization [2,3]. It is also responsible for foodborne diseases, diarrhea, emesis, abdominal pain, and meningitis [2,3]. Soup, porridge, meat, rice, spaghetti, noodle, milk powder and infant formula are the main sources of *B. cereus* [2,3]. Different extracellular factors like protein complexes (hemolysin bl (*hbl*)), non-hemolytic enterotoxin (*nhe*), hemolytic enterotoxin *hbl*, and cytotoxin K are the main pathogenic genes responsible for

adhesion, colonization and invasion of *B. cereus* to gastric epithelial cells [4,5]. Occurrence of antibiotic resistance is a considerable risk factor in treatment of diseases caused by *B. cereus*. Documented data revealed that *B. ceuers* strains harbored high levels of resistance against several types of antibiotics and especially tetracycline, β-lactamase, and quinolones [6-8]. To study the epidemiological and microbiological aspects of the *B. cereus* in milk-based infant foods, evaluation of the profile of enterotoxigenic genes and antibiotic resistance pattern are required. Therefore, the present investigation was done to study the prevalence of *B. cereus* in milk-based infant foods as well as study the distribution of enterotoxigenic genes and antibiotic resistance pattern of bacterial isolates.

EXPERIMENTAL

Ethical issue

This study was approved by Ethical Agency of Research of the Baqiyatallah University of Medical Sciences, Tehran, Iran (consent ref no. 110523745). Sampling procedure was approved by Professors Reza Ranjbar and Ebrahim Rahimi (approval ref no. Med 3802017). Identifying information of each brand of milk-based infant foods were kept secret.

Sample collection and *B. cereus* identification

From May to September 2015, a total of 300 milk-based infant food samples were collected from the shopping centers of Tehran, Iran. Samples were collected from four different brands of milk-based infant foods. Each pasteurized can or package of milk-based infant food was determined as a single sample. All samples were directly transferred to the laboratory at 4 ^{o}C. Ten grams of samples were added into 90 ml 0.1 % (wv^{-1}) peptone water (Merck, Germany). Samples were mixed and homogenized at room temperature for 3 min. Ten-fold dilution was prepared in 20 % (vv^{-1}) glycerol–peptone water. A 50 µl aliquot from the dilution was inoculated into the 5 ml Nutrient Broth (NB, Merck, Germany) and incubated at 37 ^{o}C for 24 h with shaking at 150 rpm. To eliminate growth of non-sporulating bacteria, tubes were pasteurized at 80 ^{0}C for 10 min. The suspension was streaked onto chromogenic *B. cereus* agar (BCA, Merck, Germany) supplemented with chromogenic *B. cereus* selective supplement (Oxoid, UK). The plates were incubated at 37 ^{o}C overnight and blue/green colonies were subcultured on chromogenic BCA until obtaining a pure culture. Bacterial colonies were also verified using the biochemical tests like Gram staining and catalase test.

DNA extraction and PCR confirmation of *B. cereus*

A single colony was inoculated on 5 ml of brain heart infusion broth (BHI, Merck, Germany) and incubated over night at 37 ºC. Genomic DNA was extracted from the bacterial colonies using the genomic DNA extraction and purification kit (Fermentas, Germany). DNA extraction procedure was done according to the manufacture's instruction. DNA concentration was determined by measuring absorbance of the sample at 260 nm using spectrophotometer [9]. *B. cereus gyrB* gene was detected using the PCR technique [10]. PCR procedure was done according to the method described by Park *et al* [10]. A pair of primers ((forward: 5'-TCATGAAGAGCCTGTGTACG-3' and reverse: 5'-CGACGTGTCAATTCACGCGC-3') (475 bp)) was used in this study. A programmable thermal cycler device (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) was applied in all PCR reactions.

Study of the distribution of enterotoxigenic genes

Table 1 represents the sequence of primers and PCR conditions used for detection of eneterotoxigenic genes of *B. cereus* [11-15]. PCR products were stained with SYBR DNA gel stain, separated electrophoretically in 1.5 % agarose gels, and imaged using an UV transilluminator and digital capture system. To confirm the presence of previously identified targets. *B. cereus* ATCC 10987 and sterile distilled water were used as positive and negative controls, respectively.

Disk diffusion analysis

The simple disk diffusion technique was done to study the antibiotic resistance pattern of *B. cereus* strains. The Mueller–Hinton agar (Merck, Germany) was used for this goal. Antibiotic resistance pattern of the *B. cereus* strains was studied against 25 frequently used antibiotics including ampicillin (10 µg/disk), amoxicillin (25 µg/disk), streptomycin (10 µg/disk), chloramphenicol (30 µg/disk), enrofloxacin (5 µg/disk), tetracycline (30 µg/disk), gentamicin (10 µg/disk), meropenem (10 µg/disk), imipenem (10 µg/disk), vancomycine (5 µg/disk), ciprofloxacin (5 µg/disk), ceftriaxone (30 µg/disk), linezolid (30 µg/disk), tigecycline (15 µg/disk), rifampicin (5 µg/disk), clindamycin (2 µg/disk), *trimethoprim-sulfamethoxazole* (25 *µg/disk*),

Table 1: Sequence of primers and PCR conditions used for detection of enterotoxigenic genes of the *B. cereus* strains isolated from milk-based infant food [11-15]

Target gene	Primer sequence (5'-3')*	PCR product (bp)	PCR program	PCR volume (50µL)
nheA	F-TACGCTAAGGAGGGGCA R-GTTTTTATTGCTTCATCGGCT	499	1 cycle: 95 ^{0C} ---------- 5 min. 30 cycle: 95 ^{0C} ---------- 1 min 58 ^{0C} ---------- 1 min 72 ^{0C} ---------- 1 min 1 cycle: 72 ^{0C} ---------- 10 min	5 µL PCR buffer 10X 2 mM $Mgcl_2$ 200 µM dNTP (Fermentas) 1 µM of each primers F & R U Taq DNA polymerase (Fermentas) 2.5 µL DNA template
nheB	F-CTATCAGCACTTATGGCAG R-ACTCCTAGCGGTGTTCC	769		
nheC	F-CGGTAGTGATTGCTGGG R-CAGCATTCGTACTTGCCAA	581		
hblA	F-AAGCAATGGAATACAATGGG R-AGAATCTAAATCATGCCACTGC	1154		
hblB	F-AAGCAATGGAATACAATGGG R-AATATGTCCCAGTACACCCG	2684		
hblC	F-GATACTAATGTGGCAACTGC R-TTGAGACTGCTGTCTAGTTG	740		

nalidixic acid (30 µg/disk), penicillin G (10 u/disk), oxacillin (1 µg/disk), erythromycin (15 µg/disk), bacitracin (10 ug/disk), levofloxacin (5 µg/disk), moxifloxacin (5 µg/disk), and azithromycin (15 µg/disk) antibiotic agents (Oxoid, UK). Instruction of the Clinical and Laboratory Standards Institute [16] was used to study the antibiotic resistance properties of *B. cereus* strains. All media were incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured and interpreted based on the instruction of the CLSI [16]. *B. cereus* ATCC 10987 and *Escherichia coli* ATCC 8739 were used as quality control organisms.

Statistical analysis

Statistical analysis was performed using the SPSS 21.0 software. The chi-square and Fisher's exact tests were performed on obtained data to identify any significant differences for the prevalence of *B. cereus*, enterotoxigenic genes and antibiotic resistance pattern. Statistical significance was set at $p < 0.05$.

RESULTS

Prevalence and enumeration of *B. cereus*

Table 2 represents the total prevalence of *B.* cereus in milk-based infant foods. Nine out of 300 samples studied (3 %) were positive for *B. cereus*. Contamination had a range of 12.5 – 41.5 CFU/g. *B. cereus* isolates were also confirmed using the PCR-based detection of *gyrB* gene. Figure 1 shows the gel electrophoresis of the *gyrB* gene (475 bp) of the *B. cereus*. We found that the Brand D had the highest prevalence of *B. cereus* (6.2 %), while Brand B and C had the lowest (1.4 %). Statistically significant difference was seen between brand of samples and prevalence of *B. cereus* ($p < 0.05$).

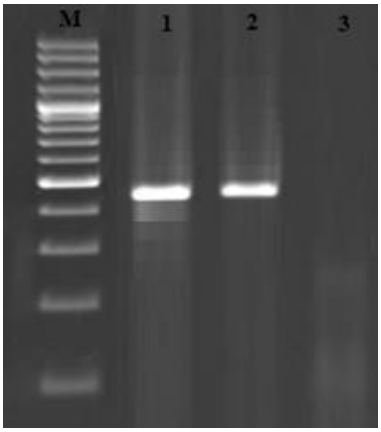

Figure 1: Gel electrophoresis of the PCR products of *gyrB* gene of *B. cereus* isolated from milk-based infant foods. M: 100 bp ladder, 1: Positive sample (475 bp), 2: Positive control and 3: Negative control

Table 2: Prevalence of *B.* cereus in milk-based infant food samples in Iran

Sample brand	No. of samples collected	No. of positive samples (%)	PCR-confirmation of *B. cereus* (%)	Numbers of *B. cereus* (CFU/g)	
				Mean	Range
Brand A	80	2 (2.5)	2 (2.5)	***22.5***	***14.0-31.2***
Brand B	70	1 (1.4)	1 (1.4)	***12.5***	***12.5***
Brand C	70	1 (1.4)	1 (1.4)	***16.4***	***16.4***
Brand D	80	5 (6.2)	5 (6.2)	***37.4***	***17.4-41.5***
Total	300	9 (3.0)	9 (3.0)	***22.2***	***12.5-41.5***

Frequency of enterotoxigenic genes

Table 3 represents the total distribution of enterotoxigenic genes in the *B. cereus* strains of milk-based infant foods of four different brands in Iran. The most commonly detected enterotoxigenic genes in the *B. cereus* strains of milk-based infant foods were *nheA* (88.8 %), *nheC* (55.5 %) and *entFM* (55.5 %). There were no positive results for the *hblA*, *hblB* and *bceT* genes.

Antibiotic resistance pattern

Table 4 represents the antibiotic resistance pattern of *B. cereus* strains isolated from milk-based infant foods. *B. cereus* isolates had the highest levels of resistance against penicillin (100 %), tetracycline (77.7 %), oxacillin (66.6 %), amoxicillin (55.5 %), ceftriaxone (55.5 %), azithromycin (44.4 %), *trimethoprim-sulfamethoxazole* (44.4 %), ampicillin (44.4 %) and enrofloxacin (44.4 %). Statistically significant difference was seen between the brand of samples and prevalence of antibiotic resistance (p <0.05). Figure 2 represents the pattern of multi-drug resistance in the *B. cereus* strains isolated from milk-based infant foods. We found that all of the *B. cereus* strains of infant foods harbored at least resistance against 2 antibiotics (100 %), while prevalence of resistance against more than 8 antibiotics was 11.1 %.

Table 3: Total distribution of enterotoxigenic genes in *B. cereus* strains isolated from milk-based infant foods in Iran

Sample (no. positive)	Distribution of enterotoxigenic genes (%)							
	hblA	***hblB***	***hblC***	***nheA***	***nheB***	***nheC***	***entFM***	***bceT***
Brand A (2)	-	-	-	2 (100)	-	1 (50)	1 (50)	-
Brand B (1)	-	-	-	1 (100)	-	1 (100)	1 (100)	-
Brand C (1)	-	-	-	1 (100)	-	1 (100)	1 (100)	-
Brand D (5)	-	-	1 (20)	4 (80)	2 (40)	2 (40)	2 (40)	-
Total (9)	-	-	1 (11.1)	8 (88.8)	2 (22.2)	5 (55.5)	5 (55.5)	-

Table 4: Antimicrobial resistance pattern of the *B. cereus* strains isolated from milk-based infant foods of four producing factories in Iran

Antibiotic agent	Antibiotic resistance pattern of *B. cereus* strains (%)				
	Brand A (2*)	***Brand B (1)***	***Brand C (1)***	***Brand D (5)***	***Total (9)***
Ampicillin	1 (50)	-	-	3 (60)	4 (44.44)
Amoxicillin	1 (50)	-	1 (100)	3 (60)	5 (55.55)
Streptomycin	1 (50)	-	-	2 (40)	3 (33.33)
Chloramphenicol	-	-	-	1 (20)	1 (11.11)
Enrofloxacin	1 (50)	1 (100)	-	2 (40)	4 (44.44)
Tetracycline	1 (50)	1 (100)	1 (100)	4 (80)	7 (77.77)
Gentamicin	1 (50)	-	-	1 (20)	2 (22.22)
Meropenem	-	-	-	-	-
Imipenem	-	-	-	1 (20)	1 (11.11)
Vancomycine	1 (50)	-	-	1 (20)	2 (22.22)
Ciprofloxacin	1 (50)	-	-	2 (40)	3 (33.33)
Ceftriaxone	1 (50)	1 (100)	-	2 (40)	4 (44.44)
Linezolid	-	-	1 (100)	2 (40)	3 (33.33)
Tigecycline	-	-	-	2 (40)	2 (22.22)
Rifampicin	1 (50)	-	-	1 (20)	2 (22.22)
Clindamycin	1 (50)	-	-	2 (40)	3 (33.33)
Trimethoprim-sulfamethoxazole	1 (50)	-	1 (100)	2 (40)	4 (44.44)
Nalidixic acid	-	1 (100)	-	1 (20)	2 (22.22)
Penicillin G	2 (100)	1 (100)	1 (100)	5 (100)	9 (100)
Oxacillin	1 (50)	1 (100)	1 (100)	3 (60)	6 (66.66)
Erythromycin	1 (50)	-	-	2 (40)	3 (33.33)
Bacitracin	1 (50)	-	-	1 (20)	2 (22.22)
Levofloxacin	-	-	-	2 (40)	2 (22.22)
Moxifloxacin	-	-	-	1 (20)	1 (11.11)
Azithromycin	1 (50)	1 (100)	-	2 (40)	4 (44.44)

*Number of positive samples

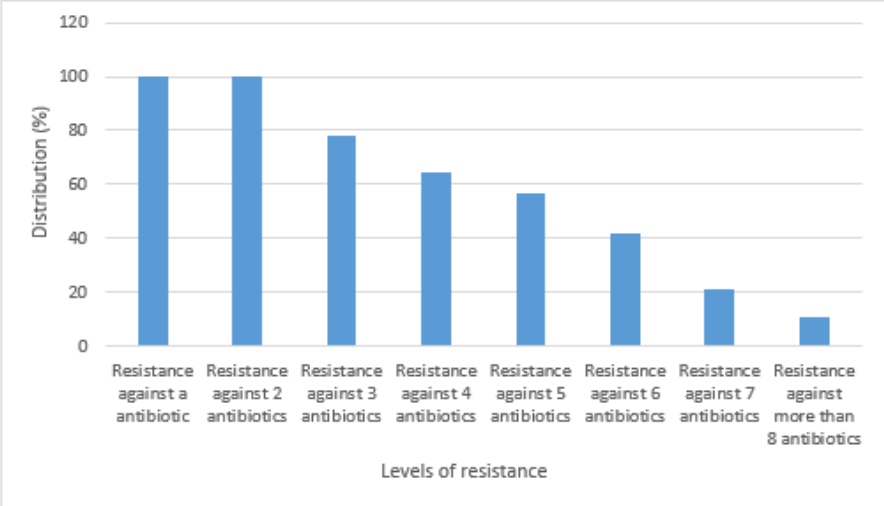

Figure 2: Pattern of multi-drug resistance in the *B. cereus* strains of milk-based infant foods

DISCUSSION

Results of the present investigation showed that Iranian milk-based infant food samples had an acceptable quality based on the low prevalence of *B. cereus*. This finding is in conflict with the results of other researchers which showed higher prevalence of *B. cereus* [17,18]. Rahimi *et al* [17] reported that eighty-four of two hundred infant food samples (42 %) were contaminated with *B. cereus* with a range of 30 – 93 CFU/g which was higher than our findings. They showed that rice and milk-based baby foods had the highest prevalence of *B. cereus* (62 %), while wheat, banana and milk based-baby foods had the lowest (20 %). Becker *et al* [18] reported higher prevalence of *B. cereus* in infant foods than those of our study and also research of the Rahimi *et al* [17]. They showed that 54 % of infant food samples were contaminated with *B. cereus* with a range of 30 – 93 CFU/g. The prevalence of Bacillus species in baby food samples of Egyptian study [19] was 31.8 %. Organji *et al* [19] reported that the prevalence of *B. cereus* in baby food samples were 54.2 %. Reyes *et al* [20] showed that 35 out of 56 baby food with rice and milk based (62.5 %) were contaminated with *B. cereus*. They showed that contamination had a range of 3 to 1000 spore per gram which was higher than our results.

We found that *B. cereus* strains of our investigation harbored high numbers of enterotoxigenic genes. *EntFM*, *nheC*, *nheA*, *hblC* and *nheB* were the most commonly detected enterotoxigenic genes among the *B. cereus* strains of infant food samples. Simultaneous presence of some of these genes together in some strains of *B. cereus* indicated an important public health problem facing Iranian infant food industry. Simultaneous presence of *entFM*, *nheC*, *nheA*, *hblC* and *nheB* genes in infant food samples was also reported previously [17,19,]. Rahimi *et al* [17] reported that the prevalence of *entFM*, *nheC*, *bheA*, *hblC* and *nheB* enterotoxigenic genes in the *B. cereus* strains of infant food samples were 61.9 %, 51.1 %, 44.0 %, 34.5 % and 33.3 %, respectively. Total prevalence of *hblD* and *hblA* enterotoxigenic genes among the *B. cereus* strains recovered from the baby food samples of Mantynen and Lindstrom [21] and Hansen and Hendriksen [22] investigations were 64 % and 52 %, respectively. Samapundo *et al* [23] showed that 52.5 % of all *B. cereus* strains isolated from the baby food samples in Belgium harbored all *hblA*, *hblB*, *hblC*, *nheA*, *nhrB* and *nheC* enterotoxigenic genes. High prevalence of *nhe* gene in our investigation and also results of other researchers is due to the variability in the *nhe* operons which facilitate its detection.

We found that all of our tested *B. cereus* strains harbored resistance against several types of antibiotics. A probable reason for the high prevalence of resistance against ampicillin, amoxicillin, ceftriaxone, oxacillin, and penicillin is maybe the synthesis of β-lactamase. We found that 11.1 % of *B. cereus* strains harbored resistance against chloramphenicol. High levels of resistance of *B. cereus* strains against chloramphenicol showed its unequal and illegal use in veterinary. High prescription of this antibiotic and its yield into the milk is the main factor for presence of resistance against this drug. Tewari *et al* [24] reported that *B. cereus* strains harbored a high prevalence of resistance against carbenicillin, kanamycin and ampicillin which was similar with our findings. They showed that all isolates were resistant to bacitracin and

penicillin G. Similar findings have been reported by Park *et al* [25]. Enayat *et al* [26] reported that *B. cereus* strains of foods with animal origins harbored the highest levels of resistance against ampicillin, tetracycline and *trimethoprim-sulfamethoxazole which was similar to our findings. Prevalence of resistance against chloramphenicol was 19 % which was higher than our findings.* Tansuphasiri *et al* [27] reported that high numbers of the *B. cereus* isolates of food samples were resistant to tetracycline (55.4 %) and ciprofloxacin (50.9 %). Whong and Kwaga [28] reported that the *B. cereus* strains of foods in Nigeria had the high prevalence of resistance against penicillin G (82 %), cefotaxime (56.7 %), ceftriaxone (53.3 %) and ampicillin (44 %). Prevalence of resistance against tetracycline (6.7 %), nalidixic acid (3 %) and gentamicin (1 %) was low.

CONCLUSION

High prevalence of *B. cereus*, a moderately high distribution of enterotoxigenic genes and antibiotic resistance show a latent public health hazard of Iranian milk-based infant foods. Each of the *B. cereus* isolate, irrespective of their source, had at least one of the major enterotoxin genes indicating their pathogenic nature. Using hygienic and high quality raw milk can reduce the risk of the presence of *B. cereus* in milk-based infant foods. Regular and appropriate prescription of meropenem, imipenem and moxifloxacin based on the findings of antibiogram test would be an effective approach to eliminate the risk of food-poisoning in infants caused by *B. cereus*. Based on the high prevalence of resistant and toxigenic strains of B. cereus and high consumption of milk-based infant foods in Iran, it is essential to continuously control the level of B. cereus contamination in these Iranian food products.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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