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Safety of Infant Milk Powder Sold at Kafrelsheikh Governorate Markets-Egypt

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Research Article

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ABSTRACT

A total of one hundred infant milk powder samples were collected from different pharmacies in Kafrelsheikh Governorate for bacteriological examination. The obtained results revealed that *B. cereus* was detected in 19% and 11% (sporulated form) of the examined samples with average counts of $1.2 \times 10^2 \pm 5.2 \times 10^2$ and $4.0 \times 10^2 \pm 1.4 \times 10^2$ cfu/g, respectively. Subsequently, PCR assay to identify 2 virulent genes in 30 of the isolated strains was applied. The PCR targets selected were the *hblc* gene using FHBLC (F) and FHBLC (R) primers, and *cytK* gene using FCytK (F) and FR2ytK (R) primers. Eight (42%) of vegetative *B. cereus* isolates had *hblC* gene, and 3 isolates had *cytK* gene, while 7 isolates had both genes. Of the eleven *B. cereus* spore strains, 4 isolates had *hblC* gene, 2 had *cytK* gene and 2 had both genes. Additionally, *E. sakazakii* could be isolated from 3% of the examined samples, while salmonellae failed to be detected in any of the examined samples. Furthermore, 4 strains, one carrying *hblC* gene, one carrying *cytK* gene, one carrying both genes and one do not carry any of the genes were experimentally inoculated into reconstituted milk powder at concentration raged from 5×10 to 1.6×10^2 cfu/ml. The inoculated milk samples were incubated at 25ºC and examined for *B. cereus* count each 2 h up to 6 h storage. There was a remarkable increase of *B. cereus* organism's count without significance difference between the *B. cereus* inoculated genes.

The results concluded that infant milk powder in spite of its low moisture content may at times suitable for supporting the growth of these organisms and subsequently be responsible for food poisoning to infants. The public health importance of the isolated microorganisms was discussed.

INTRODUCTION

Powdered Infant formula (PIF) has been used to feed millions of infants for years, and it constitutes the majority of infant formula worldwide. This product is formulated to mimic the nutritional profile of human breast milk. As PIF is not a sterile product, it is an excellent medium to support bacterial growth may be contaminated with pathogenic microbes that can cause serious illness in infants [1].

It has not possible by current technology to produce PIF that were devoid of low levels of microorganisms. Post processing contamination is a major factor impacting on contamination of milk powders, as the raw material is often subjected to lethal temperatures, which eliminate vegetative cells of pathogens. Milk powder outbreaks demonstrate that failure in preventive systems such as presence of water which allow microbial multiplication, or presence of zones difficult to maintain and to clean is the origin of contamination [2].

B. cereus was among the primary microorganisms associated with PIF contamination as reported by FAO/WHO Expert Consultations [3] and Low numbers of *B. cereus* present in infant formula are due to contamination of raw milk from the environment [4].

B. cereus has been reported to produce 5 enterotoxins and 1 emetic toxin, of them, heamolysine BL (HBL) and non heamolytic enterotoxin (Nhe) which consists of 3 different exo-proteins while the other toxins, Ent FM, cyt K and Bce T which consist of a single protein [5].

In 2004, an expert meeting convened by the Food and Agriculture Organization of the United Nations and the World Health Organization concluded that the microorganisms of greatest concern in PIF are *Salmonella enterica* and *Enterobacter sakazakii* [6].

Powdered milk formula is important source of *E. sakazakii* infection. This bacterium is resistance to drying and acid pH, heat, biofilm formation and persistence on food preparation surfaces and new-born infections of *E. sakazakii* were associated with infant formula and milk powder [7]. Low–level contamination of powdered infant milk formula with salmonellae has been associated with infection in infant [8].

To the best of our knowledge, there is a little data pertaining to the ecology and virulence in a variety of *B. cereus* detected in infant milk powder in Kafrelsheikh governorate, Egypt. Therefore, the objective of this study is to determine the prevalence of *B. cereus* (vegetative and spore former), *E. sakazakii* and Salmonellae and detection of enterotoxin production genes of *B. cereus* (*hblc* & *cytk*) in infant milk powder and to study the effect of storage time on the growth of *B. cereus* in reconstituted infant milk powder stored at room temperature to guarantee safe consumption of infant milk powder.

MATERIALS AND METHODS

This study was carried out with one hundred random samples of infant milk powder collected from local different pharmacies in Kafrelshiekh Governorate, Egypt during the period from January to July 2015. Samples were transferred to the laboratory in their packages to be examined bacteriologically.

Preparation of serial dilution [9]

Each infant milk powder packages was mixed well before being aseptically opened. 11 g of well mixed milk powder were transferred to 89 ml of sterile 0.1% peptone water (40ºC-45ºC) using a dry and sterile metal spatula to give a dilution of 1:10 and then ten-fold serial dilutions were prepared.

Bacteriological examination

Enumeration, isolation and identification of vegetative form of B. cereus: This was done according to Holbrook and Anderson [10] using polymyxine puruvate - egg yolk - mannitol bromothymol - blue agar (PEMPA) and bacterial isolates were identified as *B.cereus*

Enumeration (MPN/g), isolation and identification of spore former B. cereus: This was performed according to Polish standard PN - EN ISO 21871 [11]. Growth - positive tubes (turbid) were sub-cultured on PEMPA medium (Oxoid), the plates were incubated at 30ºC for 48 h. The total count of *B. cereus* group spores in 1 g of infant milk powder was determined by the MPN (Most Probable Number) method. Biochemical identification of the isolated organisms was done according to Koneman et al. [12].

Detection of hblC & cytK genes of the isolated strains of vegetative and spore former B. cereus by using PCR technique: Application of PCR for identification of heamolysin BL (*hblC*) and cytotoxic K (*cytK*) genes of *B. cereus* was performed essentially by using Primers (Pharmacia Biotech) as shown in **Table 1.**

Table 1. Identification of hblC & cytK genes of *B.cereus* was performed using primers.

Target gene	Primers	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
hblC	FHblC (F)	5′ CCTATCAATACTCTCGCAA ′3	695	[13]
	FHblC (R)	5′ TTTCCTTTGTTATACGCTGC ′3		
cytK	FCytK (F)	5′ CGACGTCACAAGTTGTAACA ′3	565	[13]
	FR2ytK (R)	5′ CGTGTGTAAATACCCCAGTT ′3		

Isolation and identification of E. sakazakii: according to FDA [14].

Isolation and identification of Salmonellae: according to FDA [15].

Growth characters of B. cereus in reconstituted milk powder

Bacterial stock culture: *B. cereus* strain was cultured in 10 ml of sterile Tryptic Soy Broth (TSB). The broth is incubated at 37 °C for 24 h and then centrifuged at 3000 rpm. The supernatant is removed and the remaining cells are re-suspended in sterile dist. water. Serial dilutions were prepared from each stock tube and 100 ul from each tube were spread on previously prepared PEMPA plates. The plates were incubated at 35ºC for 24 h. and the colonies forming unit/ml were calculated.

Experimental inoculation: 1000 ml of reconstituted milk powder were added into five sterile flasks (200 ml each). The

flasks were inoculated with *B. cereus* –ve *hblC* & *cytK*, *B. cereus* +ve *hblC*, *B. cereus* +ve *cytK* and *B. cereus* +ve *hblC* & *cytK*, each strain in each flask. The flasks were efficiently corked, incubated at 25°C and examined each 2 h until 6 h of storage for *B. cereus* count.

RESULTS

Table 2. Statistical analytical results of *Bacillus cereus* count (vegetative form) in the examined infant milk powder samples on PEMBA agar media.

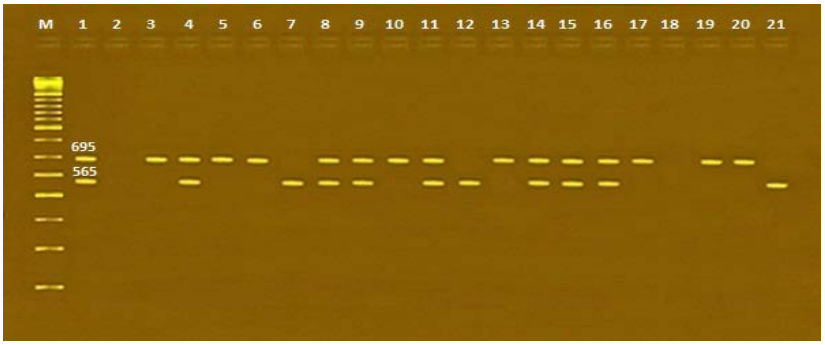
Type of sample	No. of examined samples	Positive Samples		result/g			
		No.	%	Minimum	Maximum	Mean	SEM ±
Infant Milk Powder	100	19	19	1 × 10	9×10^2	1.2×10^2	5.2 × 10

Table 3. Statistical analytical results of *Bacillus cereus* (spore former) count by MPN/g in the examined infant milk powder samples.

Type of sample	No. of examined samples	Positive Samples		MPN/g			
		No.	%	Minimum	Maximum	Mean	SEM ±
Infant Milk Powder	100	11	11	2.3 × 10	1.1×10^3	4.0×10^2	1.4×10^2

Table 4. Detection of enterotoxin genes (*hblC* and *cytK*) in *Bacillus cereus* (vegetative form) isolates from examined infant milk powder samples.

Type of sample	No. of isolates	Positive *hblC* gene Only		Positive *cytK* gene Only		Positive *hblC* & *cytK* genes		Negative *hblC* & *cytK* genes	
		No of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Infant Milk Powder	19	8	42	3	15	7	36	1	5.4



Lane M: 100 bp ladder as molecular size DNA marker

Lane 1: Control positive *B. cereus* for *hblC* and *cytK* genes

Lane 2: Control negative

Lanes 3, 5, 6, 10, 13. 17, 19 & 20: Positive *B. cereus* strains for *hblC* gene

Lanes 7, 12 & 21: Positive *B. cereus* strains for *cytK* gene

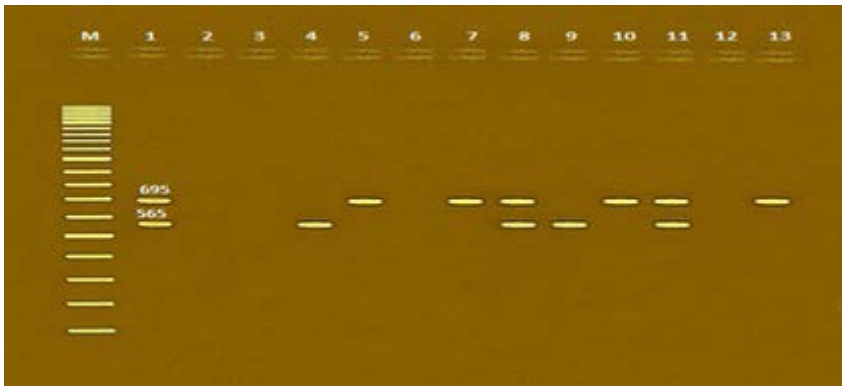
Lanes 4, 8, 9, 11, 14, 15 & 16: Positive *B. cereus* strains for *hblC* and *cytK* genes

Lane 18: Negative *B. cereus* strains for *hblC* and *cytK* genes

Figure 1. Agarose gel electrophoresis of multiplex PCR of *hblC* (695bp) and *cytK* (565 bp) virulent genes for characterization of vegetative *B. cereus*.

Table 5. Detection of enterotoxin genes (*hblC* and *cytK*) in *B. cereus* (spore former) isolates from examined infant milk powder samples.

Type of sample	No. of isolates	Positive *hblC* gene Only		Positive *cytK* gene Only		Positive *hblC* & *cytK* genes		Negative *hblC* & *cytK* genes	
		No of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Infant Milk Powder	11	4	36.4	2	18.2	2	18.2	3	27.3



Lane M: 100 bp ladder as molecular size DNA marker

Lane 1: Control positive *B. cereus* for *hblC* and *cytK* genes

Lane 2: Control negative

Lanes 5, 7, 10 & 13: Positive *B. cereus* strains for *hblC* gene

Lanes 4 & 9: Positive *B. cereus* strains for *cytK* gene

Lanes 8 & 11: Positive *B. cereus* strains for *hblC* and *cytK* genes

Lanes 3, 6 & 12: Negative *B. cereus* strains for *hblC* and *cytK* genes

Figure 2. Agarose gel electrophoresis of multiplex PCR of *hblC* (695bp) and *cytK* (565 bp) virulent genes for characterization of sporulated *B. cereus*.

Table 6. Incidence of *E. sakazakii* in the examined infant milk powder samples on VRBG media.

Type of samples	No. of examined samples	Positive samples	
		No.	%
Infant milk powder samples	100	3	3

Table 7. Comparison of the isolated pathogens from infant milk powder samples with FDA [16] and CAC [17] standards (n=100).

Pathogenes	Infant milk powder samples				
	Compatible samples		Incompatible samples		Standards
	No.	%	No.	%	
B. cereus (vegetative)	15	79	4	21	≤ 100/g. FDA [16]
B. cereus (sporulated)	4	36	7	64	≤ 100/g. FDA [16]
E. sakazakii	97	97	3	3	Absent in 10 g sample. CAC [17]
Salmonellae	100	100	0	0	Absent. FDA [16]

Table 8. Incidence of Salmonella in examined infant milk powder samples (n=100).

Type of samples	No. of isolates	Biochemical tests	
		No.	%
Infant milk powder	5	0	0

Table 9. Effect of storage at room temperature (25°C-30°C) on the growth of *B. cereus* having certain virulent genes in reconstituted milk.

Strains	Storage Time						
	Zero time	2 h		4 h		6 h	
	Cfu/ml	cfu/ml	% of increase	cfu/ml	% of increase	cfu/ml	% of increase
Control -ve	-ve	-ve	0	-ve	0	-ve	0
-ve hblC & cytK	1.6×10^2	6.9×10^2	431.2	5.1×10^3	3187.5	2.3×10^3	1437.5
+ve *hblC*	5.0×10	2.1×10^2	420	1.4×10^3	2800	6.9×10^3	13800
+ve *cytK*	1.1×10^2	4.7×10^2	427.3	3.2×10^3	2909	1.5×10^4	13636
+ve *hblC & cytK*	8.0×10	3.3×10^2	412.5	2.1×10^3	2625	1.0×10^4	12500

N.B. % of increase was calculated from zero time counts.

DISCUSSION

B. cereus is classified as category C or low risk, its prevalence in infant formula is sufficiently high to cause food borne

infection outbreaks [18].The enterotoxin (diarrhoeal syndrome) of *B. cereus* poisoning is caused by ingestion of large number of cells and the subsequent production of the toxin in the small intestine. However the emetic syndrome of *B. cereus* food poisoning occurs after the ingestion of food in which the organism has grown and formed its toxins [19].

Results presented in the **Table 2** shows that 19% of examined infant milk powder samples were positive for *B. cereus* with counts ranged from 1 × 10 to 9 × 10^2 and a mean value of 1.2 × 10^2 ± 5.2 × 10 cfu/ml reconstituted milk. These results agree nearly to results obtained by Azza et al. [20], Wong et al. [21] while higher results were reported by Sameer et al. [22] and Angela et al. [23].

Results in **Table 3** declare that the *B. cereus* spores were detected in 11% of examined infant milk powder samples with counts ranged from 2.3 × 10 to 1.1 × 10^3 and a mean value of 4.0 × 10^2 ± 1.4 × 10^2 spores/ml. These results agree with results obtained by Aman et al. [24], Reyes et al. [25] and Juan et al. [26].

According to FDA (1996) standard which stipulate that *B. cereus* must be less than and or equal 100/g , so it is clear that 21% and 64% of infant milk powder samples failed to comply the standard limit regarding counts of vegetative and spore formers respectively.

Dried milk products are known to be frequently contaminated with *B. cereus* spores [26]. The infectious dose for *B. cereus* may vary from about 1 × 10^5 to 1 × 10^8 viable cells or spores/g. Generally presence of *B. cereus* greater than 10^6 organisms/g in the food is indicative of growth and proliferation of the organisms and considers a potential hazard to health [27].

Fernandes et al. [28] found that about 40% of *B. cereus* strains harbor the *hblc* genes responsible for the HBL codification while Lund et al. [29] recorded an outbreak of a strain expressing the *cytk* toxin produced severe symptoms with bloody diarrhea.

The primers designed by Ngamwongsatit et al. [13] were used under specific multiplex PCR conditions for detection of enterotoxin genes (*hblc* and *cytk*) in selected strains. DNA band visualized by ethidium bromide in agarose gel at the expected molecular size for *hblc* and *cytk* genes at 695 bp and 565 bp respectively were detected.

Nineteen *B. cereus* vegetative strains isolated from infant milk powder samples were analyzed for the pressence of *hblc* and *cytk* genes as in **Table 4** using the PCR primers listed in **Figure 1**, *hblc* genes was detected in only in 8 isolates (42.1%), *cytk* genes only was in 3 isolates (15.8%), *hblc* and *cytk* genes was in 7 isolates (36.8%) and *hblc* and *cytk* genes was not detected in one (5.2%) isolate.

Moreover, 11 sporulated *B. cereus* spore strains isolated were analyzed for the presence of hblc and cytk genes using the PCR primers listed in **Figure 2**, hblc gene was detected in 4 isolates (36.4%), cytk gene was detected in 2 isolates (18.2%), *hblc* & *cytk* genes was detected in 2 isolates (18.2%) and both *hblc* and *cytk* genes failed to be detected in 3 isolates (27.3%) **(Table 5).** Angela et al. [23]; Ji-Yeon and Jong-Hyun [30]; Arsalan et al. [31]; Hussein [32] and Chon et al. [33] could detect both *hblc* and *cytk* genes, at varying percentages ranged from 20 to 77% of screened isolates.

The results summarized in **Table 6** show that 3% of examined infant milk powder samples were contaminated with Gram - negative *E. sakazakii*. Our findings are consistent with Heuvelink et al. [34] while higher findings were obtained by Aigbekaen et al. [35]. On the other hand El-Sharoud et al. [36] failed to detect *E. sakazakii* in any samples examined. According to CAC [17] standard which sets a limit of absence of *E. sakazakii* in 10 g of infant milk powder, so it is clear that 3% of infant milk powder samples failed to comply the standard limit **(Table 7).** Infant formula and milk powder have been the most common vehicles implicated in neonatal *E. sakazakii* infections [37]. Historically, Enterobacter have been implicated in newborn and infant infections, causing meningitis, necrotizing enterocolitis (NEC) and bacteremia or sepsis [38].

Salmonella organisms failed to be detected in all of examined infant milk powder samples **(Table 8).** These findings were nearly similar to results obtained by Matug et al. [39], and agree with the European and Egyptian, FDA [16] standards which stipulated a limit of zero salmonella in 25 g of dry milk products [40]. On the other hands Zagare et al. [41] could detect salmonellae in infant milk powder.

Results in **Table 9** reveal that the survival characteristics of *B. cereus* carrying *hblc* gene, *cytk* gene and both *hblc* & *cytk* genes in reconstituted milk powder stored at 25°C for 6 h. All batches were examined each 2 h. A steady increase in cfu/ml reconstituted milk was clearly observed but without significant difference between the growth characters of different *B. cereus* carrying genes inoculated and reached the infectious dose in less than 6 hours. The results which slightly agree with the Food Standard Australian New Zealand [4] who stated that formula prepared with initial levels of 100 cfu *B. cereus*/g may reach infectious dose when stored at room temperature for greater than 4 h. Therefore, FDA, FAO/WHO and CDC forcefully advocate the mother- feed over bottle feed to avoid the possible life threatening illness to neonates and infants caused by microbial contamination and reduce the delay between preparation and consumption of infant milk powder.

CONCLUSION

This study indicated high incidence of toxigenic *B. cereus* strains and *E. sakazakii* in infant milk powder sold in Kafrelsheikh governorate and a possible high risk of food borne infections especially for infants. Therefore, special attention should be given to

the importance of including *B. cereus* and *E. sakazakii* in disease control and prevention programs in Egypt that may constitute a public health hazard. Multiplex PCR is considered as an alternative method for rapid identification of *B. cereus* in milk products.

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