HYGIENIC STATUS OF INFANT MILK SOLD AT THE MARKETS

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ABESTRACT

One hundred of infant milk powder samples were collected from different pharmacies in Kafrelsheikh Governorate for bacteriological examination. The obtained results revealed that 19% of examined samples contained B. cereus in average count of 1.2x102± 5.2x10 cfu/g while 11% of the samples had B. cereus (sporulated form)with an average count of 4.0x102± 1.4x102 cfu/g . 30 strains isolated were screened by PCR technique for hblCgene 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 cytKgene and 7 isolates had both genes. Of the eleven B. cereus spore strains, 4 isolates had hblC gene, 2 isolates had cytKgene and 2 isolates had both genes. E. sakazakii could be isolated from 3% of the examined samples while salmonellae failed to be detected in any of the examined samples. 4 strains , one carrying hblCgene, one carrying cytK gene, one carrying the both genes and one do not carry any of the genes were inoculated into reconstituted milk powder at concentration raged from 5x10 to 1.6x102 cfu/g reconstituted milk. The inoculated milk samples were incubated at 25℃ and examined for B. cereus count each 2 hours until 6 hours storage.There was a remarkable increase of B.cereusorganisms count without significance difference between the B.cereus inoculated genes.

The results allow concluding that infant milk powder in spite of its low moisture content may at times be responsible for food poisoning to infants. The public health importance of the isolated microorganisms was discussed.

Keywords: Infant milk powder, *B.cereus*, enterotoxins, *E.sakazakii*, Salmonellae.

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 ***(Breeuwer et al., 2003).***

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 are the origin of contamination ***(ICMSF, 1998).***

B.cereus was among the primary microorganisms associated with PIF contamination as reported by FAO/ WHO Expert Consultations ***(Wang et al., 2009)*** and Low numbers of *B.cereus* present in infant formula are due to contamination of raw milk from the environment ***(Food standards Australia New Zealand,2004)***.

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 exoproteins while the other toxins, Ent FM, cyt K and Bce T which consist of a single protein ***(Hansen et al., 2003).***

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 *Enterobactersakazakii* ***(FAO/ WHO, 2006).***

Powdered milk formula is important source of *E. sakazakii* infection (Drudy et al., 2006). This bacterium is resistance to drying and acid PH, heat , biofilm formation and persistance on food preparation surfaces and new-born infections of *E.sakazakii*were associated with infant formula and milk powder ***(Iversen et al.,2003)***. Low – level contamination of powdered infant milk formula with salmonellae has been associated with infection in infant ***(Bornemann et al., 2002).***

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* and *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 .

MATERIALS AND METHODS

One hundred random samples of infant milk powder collected from local different pharmacies in Kafrelshiekh Governorate and transferred to the laboratory in their packages to be examined bacteriologically.

1- Preparation of serial dilution *(APHA.,1992)*:

Each infant milk powder packages was mixed well before being aseptically opened . 11 g of well mixed milk powder were transferred to 99 ml of sterile 0.1% peptone water (40-45ºc) using a dry and sterile metal spatula to give a dilution of 1:10 and then ten fold serial dilutions were prepared .

2-Bacteriological Examination:

2.1- Enumeratio, isolation and identification of vegetative form of *B.cereus* was done according to ***Holbrook and Anderson (1980)*** using polymyxinepuruvate- egg yolk- mannitolbromothymol- blue agar (PEMPA).

2.2- Enumeration (MPN/g), isolation and identification of spore former *B.cereus* was performed according to Polish standard ***PN-EN ISO 21871(2007).*** Growth- positive tubes (turbid) were subcultured on PEMPA medium (Oxoid), the plates were incubated at 30 ºc for 48h**.** The total count of *B.cereus* group spores in 1g 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. (1992).***

2.3- Detection of *hblC* and *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 the following table:

Target gene	Primers	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
hblC	FHblC (F)	**5′** CCTATCAATACTCTCGCAA **′3**	565	***Nagamwongsatitet al. (2008)***
	FHblC (R)	**5′** TTTCCTTTGTTATACGCTGC **′3**		
cytK	FCytK (F)	**5′** CGACGTCACAAGTTGTAACA **′3**	695	***Nagamwongsatitet al. (2008)***
	FR2ytK (R)	**5′** CGTGTGTAAATACCCCAGTT **′3**		

2.4- Isolation and identification of *E.sakazakii* :according to ***FDA (2002).***

2.5-Isolation and identification of Salmonellae:according to ***FDA (2006).***

3- Growth characters of *B.cereus* in reconstituted milk powder:

3.1- Bacterial stock culture:

B.cereus strain was cultured in 10 ml of sterile Tryptic soy broth (TSB). The borth is incubated at 37ºc for 24 hours and then centrifuged at 300 rpm. The supernatant is removed and the remaining cells are resuspended in sterile distilled water. Serial dilutions were prepared from each stock tube and 100µl from each tube were spread on previously prepared PEMPA plates. The plates were incubated at 35 ºc for 24 h and the colonies forming unit / ml was calculated.

3.2- 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* +vehblC&cytK , each strain in each flask. The flasks were efficiently corked , incubated at 25℃ and examined each 2 hours until 6 hours of storage for *B. cereus* count.

RESULTS

Table (1): Statistical analytical results of *Bacillus cerus* 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	1×10^2	9×10^3	1.2×10^3	5.2×10^2

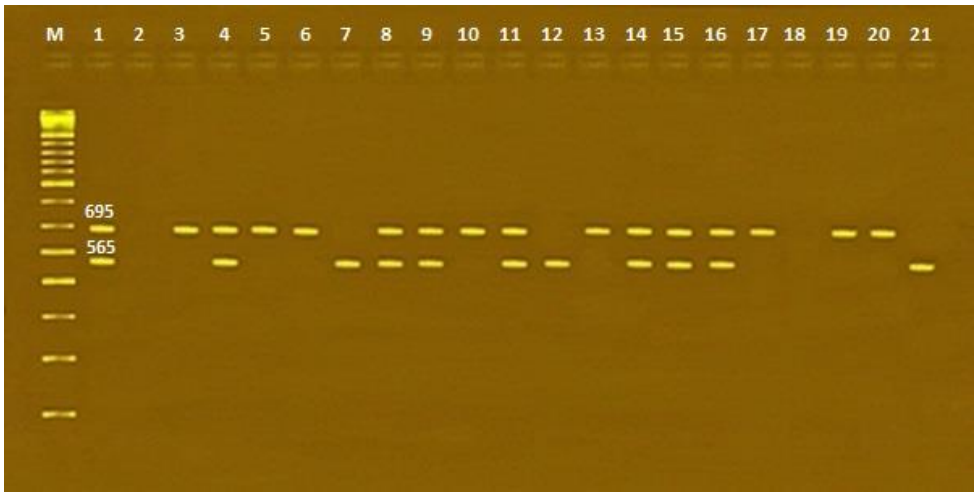
Table (2): Statistical analytical results of *Bacillus cerus* (spore former) count by M.P.N/g in the examined infant milk powder samples.

Type of sample	No. of examined samples	Positive Samples		M.P.N / g			
		No.	%	Minimum	Maximum	Mean	SEM ±
Infant Milk Powder	100	11	11	2.3×10^2	1.1×10^4	0.4×10^4	1.4×10^3

Table (3): Detection of enterotoxin genes (*hblC*and *cytK*) in *B.Cereus* (vegetative form) isolates from examined infant milk powder samples.

Type of sample	No. of positive samples	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	19	8	42	3	15	7	36	1	5.4

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



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.

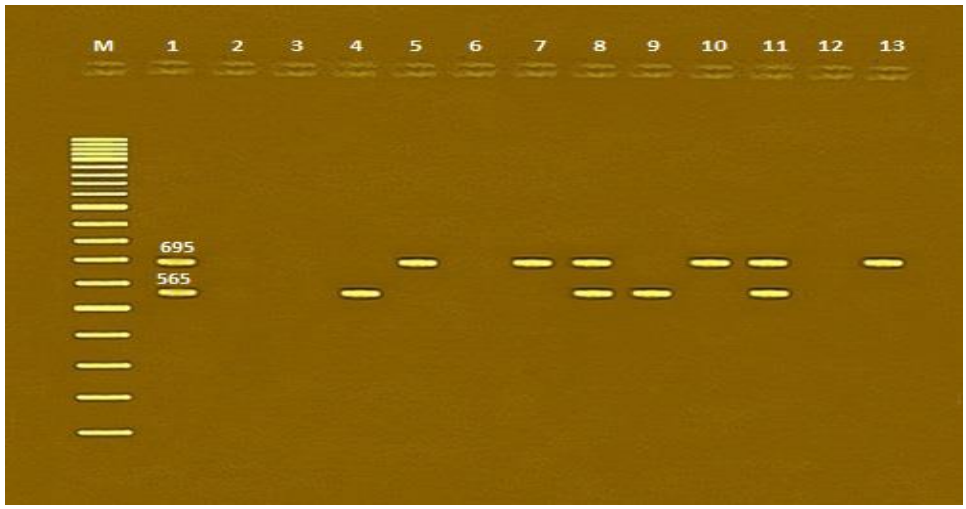
Lanes 3, 5, 6, 10, 13. 17, 19 & 20: Positive *B. cereus* strainsfor *hblC*gene.**Lanes 7, 12 & 21:** Positive *B. cereus* strainsfor *cytK* gene.

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.

Table (4): Detection of enterotoxin genes (*hblC* and *cytK*) in *B.cereus* (spore former) isolates from examined infant milk powder samples.

Type of sample	No. of positive samples	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	11	4	36.4	2	18.2	2	18.2	3	27.3

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



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

Table (5): Incidence of *E.Sakazakii* in the examined infant milk powder samples on V.R.B.G media

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

Table (6): Incidence of Salmonella in examined infant milk powder samples (n = 100).

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

Table(7): Comparison of the isolated pathogens from infant milk powder samples with ***FDA (1996)*** and ***CAC (2008)*** 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 (1996)
B.cereus (sporulated)	4	36	7	64	≤ 100/g. FDA (1996)
E.sakazakii	97	97	3	3	Absent in 10 g sample. CAC (2008)
Salmonellae	100	100	_	0	Absent FDA (1996)

Table (8): Effect of storage at room temperature (25°C) on the growth of *B. cereus* having certain virulent genes in reconstituted milk

Strains	Storage Time						
	Zero time	2 hours		4 hours		6 hours	
	cfulml	cfu/ml	% of cfu/ml	cfu/ml	% of increase	cfu/ml	I% of increase
Control –ve	-ve	-ve	-	-ve	-	-ve	-
-vehblC&cytK	$1.6x\ 10^2$	$6.9x10^2$	331	5.1×10^3	3088	2.3×10^3	14275
+vehblC	5.0x 10	$2.1x\ 10^2$	320	1.4×10^3	2700	6.9×10^3	13500
+vecytK	$1.1x\ 10^2$	$4.7x\ 10^2$	327	3.2×10^3	2809	1.5×10^4	13536
+vehblC&cytK	8.0x 10	$3.3x\ 10^2$	312	2.1×10^3	2525	1.0×10^4	12400

DISCUSSION

B.cereus is classified as category C or low risk, its prevalance in infant formula is sufficiently high to cause food borne infection outbreaks ***(Animal and plant quarantine Agency, 2013).***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 ***(ICMSF, 1996).***

Results presented in the table(1) show that 19 % of examined infant milk powder samples were positive for *B.cereus* with counts ranged from 1x10 to 9x102 and a mean value of $1.2 \times 10^3 \pm 5.2 \times 10^2$. higher results were reportedby ***Dovilėet al. (2012)*** and ***Angelaet al. (2013),*** while the lower results were obtained by ***Becker et al.(1994)*** and ***Azzaet al. (2010).***

Results in table (2) declare that the *B.cereus* spores were detected in 11% of examined infant milk powder samples with counts ranged from 2.3×10^2 to 1.1×10^4 and a mean value of$0.4 \times 10^4 \pm 1.4 \times 10^3$ spores/g. These results agree with results obtained by by ***Reyes et al. (2007) and Juan et al. (2007)*** while lower results obtained by ***Aman et al. (1998).***

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 (table 7).

Dried milk products are known to be frequently contaminated with *B.cereus* spores ***(Becker et al ., 1994).*** The infectious dose for *B.cereus* may vary from about 1x 10^5 to 1x10^8 viable cells or spores/g. Generally presence of B.cereus greater than 10^6 organisms/ g in a food is indicative of growth and proliferation of the organisms and consider a potential hazard to health ***(Nortermans and Batt ,1998).***

Fernandes et al ., 2014 found that about 40% of *B.cereus* strains harbour the *hblC* genes responsible for the HBL codification while ***Lund et al ., (2000)*** recorded an outbreak of a strain expressing the cytk toxin produced severe symptoms with bloody diarrhea.

The primers designed by ***Nagmwongsatit et al. (2008)*** 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 *hplc*and *cytk* genes at 565bp and 695 bp respectively were detected.

19 *B.cereus* vegetative strains isolated from infant milk powder samples were analyzed for the pressence of hblc and cytk genes as in table (3) using the PCR primers listed in fig. (2), *hblC* genes was dtected in only in 8 isolates (42%), cytk genes only was in 3 isolates (15%), *hblC* and *cytK* genes was in 7 isoaltes (36%) and *hblC* and *cytK* genes was not detected in one (5.2%) isolate.

Moreover, 11 *B.cereus* spore strains isolated were analyzed for the presence of *hblC* and *cytK* genes using the PCR primers listed in fig. (2), hblc gene was detected in 4 isolates (36.4%), *cytk* gene only was in 2 isolates (18.2%), *hblC*&*cytk* genes was in 2 isolates (18.2%) and no *hblC* and *cytk* genes was detected in 3 isolates (27.3%) (table 4). ***Angela et al.***

(2013); Ji-Yeon and Jong-Hyun. (2014);, Arsalan et al. (2014) and Hussein (2015) and Chon et al. (2012) could detect both *hblC* and *cytK* genes, at varying percentages ranged from 20 to 77 % of screened isolates.

The results summarized in table (5) show that 3% of examined infant milk powder samples were contaminated with Gram-negative *E.sakazakii*. Our findings are consistent with ***Heuvelink et al. (2001)*** while higher findings were obtained by ***Aigbekaen et al. (2010)***. On the other hand, ***El-Sharoud et al. (2009)*** failed to detect *E.sakaazaki* in any samples examined. According to ***CAC (2008)*** standard which sets a limit of absence of *E.sakazakii* in 10g 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 ***(Gökmen et al., 2010).*** Historically, Enterobacter have been implicated in newborn and infant infections, causing meningitis, necrotizing enterocolitis (NEC) and bacteremia or sepsis ***(Healy et al., 2010).***

Salmonella organisms failed to be detected in all of examined infant milk powder samples (table6). These findings were nearly similar to results obtained by ***Matuget al. (2015),*** and agree with the European and Egyptian, ***FDA (1996)*** standards which stipulated a limit of zero salmonella in 25 g of dry milk products ***(Food Standard Australia New Zealand, 2006).*** On the other hands ***Zagare et al. (2012)*** could detect salmonellae in infant milk powder.

Results in table (8) reveal that the survival characteristics of *B.cereus* carrying *hblC* gene, *cytK*gene and both hblc&cytk genes in reconstituted milk powder stored at 25C for 6 hours and examined each 2 hours, increase in counts and no significant difference between the growth characters of different B. cereus carrying genes and reached the infectious dose in less than 6 hours. ***Food Standard Australian New Zealand (2004)*** stated that Formula prepared with intial levels of 100 cfu/g, B.cereus may reach infectious dose when stored at room temperature for greater than 4 hours.

CONCLUSION

From the results of this study, I can conclude that the occurrence of toxigenic *B.cereus* strains and *E.sakazakii* in infant milk powder in Kafrelsheikh governorate indicates possible high risk of food borne infections especially for infants and the importance of including *B.cereus* and *E.sakazakii* in disease control and prevention programs in Egypt is required. Moreover, 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 .

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الحالة الصحية لألبان الأطفال المباعة فى الأسواق

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تستخدم ألبان الأطفال الجافة لكثير من الأطفال الرضع لأنها الغذاء البديل للبن الأم والتى قد تحتوى على ميكروب الباسيلس سيرس والإنتيروباكترساكازاكى والسالمونيلا وكذلك السموم البكتيرية التى تهدد حياة الأطفال الرضع لذلك أجريت هذه الدراسة للكشف عن هذه الميكروبات فى ألبان الأطفال الجافة.

لذا اشتملت هذه الدراسة على فحص 100عينه من ألبان الأطفال الجافة التى تم تجميعها عشوائيا من مختلف الصيدليات بمحافظة كفر الشيخ والتى كانت ضمن فتره الصلاحية وتم نقلها إلى المختبر لفحصها بكتريولوجيا. وقد أظهرت النتائج وجود ميكروب الباسيلس سيرس (النامية) بنسبة 19% من العينات المفحوصة بمتوسط عددى $2و1x10^2 \pm 2و5x10$ بينما الباسيلس سيرس (spore) وجدت بنسبة 11% بمتوسط عددى $4و1x10^2 \pm 4x10$. وتم عمل PCR للمعزولات للكشف عن جينات hblc و cytK المسئولة عن إفراز السموم وأظهرت النتائج أن معظم المعزولات قد احتوت على الأقل واحد من هذه الجينات. وقد تواجد ميكروب الإنتيروباكترساكازاكى بنسبة 3 % من العينات المفحوصة بينما لم يتم عزل ميكروب السالمونيلا فى أى من العينات التى تم فحصها.

وقد تم حقن ميكروب الباسيلس سيرس 4 معزولات بها الجينات المسئولة عن إفراز السموم (hblC&cytK) ($5x10$ to $1.6x10^2$خلية/جم) فى عينة لبن أطفال جاف تم تحضينها عند درجة حرارة الغرفة (25 درجة مئوية) حيث وجد أن لا يوجد اختلاف فى نمو الميكروب الذى يحمل الجينات والذى لا يحمل تلك الجينات المسئولة عن إفراز السموم لميكروب الباسيلس سيرس .

وتم مناقشة الأهمية الصحية للميكروبات المعزولة حيث أنها تمثل خطورة على صحة الأطفال وقد أظهرت النتائج الحاجة الى السيطرة على هذه الميكروبات والحد من تواجدها فى ألبان الأطفال الجافة للحفاظ على صحة الأطفال من حدوث تسمم غذائي.