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

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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 1.2x102± 5.2x10 and 4.0x102± 1.4x102 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 5x10 to 1.6x102 cfu/ml. The inoculated milk samples were incubated at 25oC and examined for B. cereus count each 2 hours up to 6 hours 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.

Key words: Infant milk powder, B.cereus, enterotoxins, E.sakazakii, Salmonellae.

I. 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 consists 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 Enterobacter sakazakii (FAO/ WHO, 2006).

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

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 was planned to determine the prevalence of B. cereus (vegetative and spore former), E.sakazakii and Salmonellae and also detection of enterotoxin production genes of B .cereus (hblc and cytk) in infant milk powder; and to study the effect

of storage on the growth of B. cereus in reconstituted infant milk powder stored at room temperature to guarantee safe consumption of infant milk powder.

II. 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.

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

Each infant milk powder package 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 (40-45ºc) using a dry and sterile metal spatula to prepare a dilution of 1:10 and then ten-fold serial dilutions were prepared .

B. Bacteriological Examination:

- Enumeratio, isolation and identification of vegetative form of *B.cereus*: Samples were cultured on polymyxine puruvate- egg yolk- mannitol bromothymol- blue agar (PEMPA), and bacterial isolates were identified as *B.cereus* according to Holbrook and Anderson(1980).
- Enumeration (MPN/g), isolation and identification of spore former of *B.cereus* were performed according to Polish standard *PN-EN ISO 21871(2007).* Growth- positive tubes (turbid) were sub-cultured 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)*.
- 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 :

Table (1): primer and target gene and size

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

- Isolation and identification of *E.sakazakii* was done according to FDA(2002).
- Isolation and identification of Salmonellae was done according to FDA (2006).

C. - 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) and incubated at 37oC for 24 h. and then centrifuged at 300 rpm. The supernatant was removed and the remaining cells were re-suspended in sterile distilled 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 35 ºc 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 25oC, and examined each 2 h. until 6 hrs. of storage for B. cereus count.

III. RESULTS

Table (2): 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	9×10^2	1.2×10^2	5.2×10

Table (3): 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 x 10	1.1×10^3	4.0×10^2	1.4×10^2

Table (4): Detection of enterotoxin genes (*hblC* and *cytK*) in *B.Cereus* (vegetative form) isolates from the examined infant milk powder samples.

Type of sample	Total 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

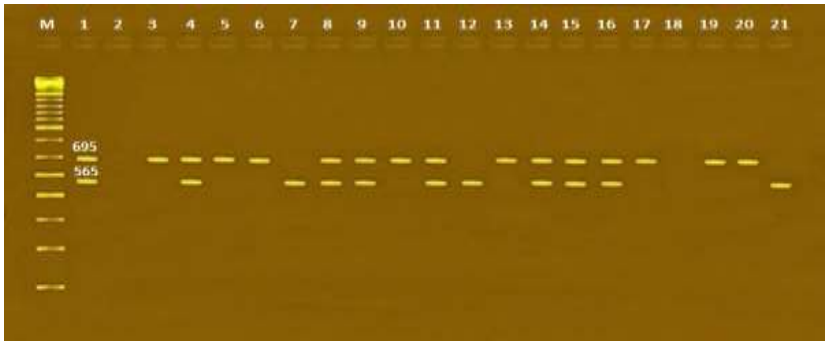

Figure (1): Agarose gel electrophoresis of multiplex PCR of *hblC* (695bp) and *cytK* (565 bp) virulent genes for characterization of vegetative *B. cereus*.

Lane M: 100 bp DNA ladder
.**Lane 1:** Control positive for hblC and cytK genes.
Lane 2: Control negative.
Lanes 3, 5, 6, 10, 13. 17, 19 & 20: Positive *hblC* gene.
Lanes 7, 12 & 21: Positive *cytK* gene.
Lanes 4, 8, 9, 11, 14, 15 & 16: Positive *hblC* and *cytK* genes
.**Lane 18:** Negative *hblC* and *cytK* genes.

Table (5): Detection of enterotoxin genes (*hblC* and *cytK*) in *B.cereus* (spore former) isolates from the 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

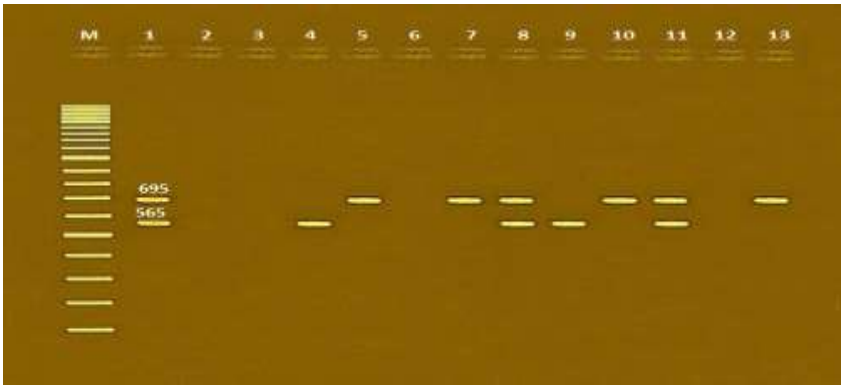

Figure (2): Agarose gel electrophoresis of multiplex PCR of *hblC* (695bp) and *cytK* (565 bp) virulent genes for characterization of sporulated *B. cereus*.

Lane M: 100 bp DNA ladder
.**Lane 1:** Control positive for hblC and cytK genes.
Lane 2: Control negative.
Lanes 5, 7, 10 & 13: Positive hblC gene.
Lanes 4 & 9: Positive cytK gene.
Lanes 8 & 11: Positive hblC and cytK genes
.Lanes 3, 6 & 12: Negative hblC and cytK genes.

Table (6): Incidence of *E.Sakazakii* in the examined infant milk powder samples on V.R.B.G medium

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

Table (7): Incidence of Salmonella in the examined infant milk powder samples (n = 100).

Type of sample	No. of isolates	Positive samples	
		No.	%
Infant milk powder	5	0	0

Table (8): 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 (25-30°C) on the growth of *B. cereus* having certain virulent genes in the 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	-
-ve hblC &cytK	$1.6x\ 10^2$	$6.9x10^2$	5.3×10^2	5.1×10^3	49.4 x 102	2.3×10^3	14275
+ve hblC	5.0x 10	$2.1x\ 10^2$	1.6×10^2	1.4×10^3	13.5 x 102	6.9×10^3	13500
+ve cytK	$1.1x\ 10^2$	$4.7x\ 10^2$	327	3.2×10^3	2809	1.5×10^4	13536
+ve hblC & cytK	8.0x 10	$3.3x\ 10^2$	312	2.1×10^3	2525	1.0×10^4	12400

IV. DISCUSSION

B.cereus is classified as category C or of 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 subsequent the production of the toxin in food. 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 (2) showed 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 x 102 ± 5.2 x 10 cfu/mlk. These results agreed nearly to results obtained by Azza et al. (2010), Wong et al. (2015); while higher results were reported by Angela et al. (2013) and Sameer et al. (2015).

Results in table (3) declared that *B. cereus* spores were detected in 11% of examined infant milk powder samples with counts ranged from 2.3x10 to 1.1x103 and a mean value of 4.0 x 102 ± 1.4x 102 spores/ ml. These results agreed with those obtained by Aman et al. (1998), Juan et al. (2007) and Reyes et al. (2007).

According to FDA (1996) standard which stipulated 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 8).

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 harbor 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.

Ninteen *B. cereus* vegetative strains isolated from infant milk powder samples were analyzed for the presence of hblc and cytk genes as in table (4) using the PCR primers (fig. 3), hblc gene was detected in only in 8 isolates (42%), cytk gene only in 3 isolates (15%), hblc and cytk genes in 7 isolates (36%) and hblc and cytk genes was not (?) detected in one (5.2%) isolate.

Moreover, 11 sporulated *B. cereus* strains were analyzed for the presence of hblc and cytk genes using the PCR primers listed in fig. (3). hblc gene was detected in 4 isolates (36.4%), cytk gene only in 2 isolates (18.2%), hblc & cytk genes in 2 isolates (18.2%) and none of the hblc and cytk genes were detected in 3 isolates (27.3%) (Table 4). Chon et al. (2012); Angela et al. (2013); Arsalan et al. (2014); Ji-Yeon and Jong-Hyun (2014) and Hussein (2015) could detect both hblc and cytk genes at varying percentages ranged from 20 to 77 % of screened isolates.

The results summarized in table (6) showed that 3% of the 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. sakazakii* in any samples examined. CAC (2008) standard sets the 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 8). Infant formula and milk powder have been the most common vehicles implicated in neonatal *E. sakazakii* infections (Gökmen et al., 2010). Historically, *E. sakazakii* 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 (Table 7). These findings were nearly similar to those obtained by Matug *et al*. (2015), and agreed 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 (9) revealed that the survival characteristics of *B. cereus* carrying hblc gene, cytk genes and both hblc&cytk genes in reconstituted milk powder stored at 25^{o}C 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 initial levels of 100 cfu/g, *B. cereus* may reach infectious dose when stored at room temperature for greater than 4 hours. 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.

V. 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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