HYGIENIC STATUS OF INFANT MILK SOLD AT THE MARKETS

Aman, I.M.1; Esraa M. Abbas2; Walaa M. El Kassas2

2 Food hygiene dep. Animal Health Research Institute, Kafrelsheikh branch, Egypt.

Abstract

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 ± 5.2x10⁵ 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 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 and 7 isolates had both genes. Of the eleven B. cereus spore strains, 4 isolates had hblC gene, 2 isolates had cytK gene 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 hblC gene, 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 ranged from 5x10 to 1.6x10² cfu/g reconstituted milk. The inoculated milk samples were incubated at 25 ºC and examined for B. cereus count each 2 hours until 6 hours storage. There was a remarkable increase of B. cereus organisms 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 \textit{Enterobactersakazakii (FAO/WHO, 2006)}.

Powdered milk formula is important source of \textit{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 \textit{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 \textit{B. cereus} (vegetative and spore former), \textit{E. sakazakii} and Salmonellae and detection of enterotoxin production genes of \textit{B. cereus} (\textit{hblc} and \textit{cytk}) in infant milk powder and to study the effect of storage time on the growth of \textit{B. cereus} in reconstituted infant milk powder stored at room temperature.

\textbf{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.

\textbf{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 °c 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:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hblC</td>
<td>FHbIC (F)</td>
<td>5’ CCTATCAATACCTCTCGCA’3</td>
<td>565</td>
<td><em>Nagamwongsatit et al. (2008)</em></td>
</tr>
<tr>
<td></td>
<td>FHbIC (R)</td>
<td>5’ TTTTCTTGTATACGCTG’3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytK</td>
<td>FCytK (F)</td>
<td>5’ CGACGTCACAAGTTGTAACA’3</td>
<td>695</td>
<td><em>Nagamwongsatit et al. (2008)</em></td>
</tr>
<tr>
<td></td>
<td>FR2ytK (R)</td>
<td>5’ CGTGTGATAATACCCCAAGTT’3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 broth 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* – *vehblC&cytK*, *B. cereus* +*vehblC*, *B. cereus* +*vecytK* and *B. cereus* +*vehblC&cytK*, each strain in each flask. The flasks were efficiently corked, incubated at 25ºC and examined each 2 hours until 6 hours of storage for *B. cereus* count.

**RESULTS**

Table (1): Statistical analytical results of *Bacillus cereus* count (vegetative form) in the examined infant milk powder samples on PEMBA agar media.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of examined samples</th>
<th>Positive Samples</th>
<th>result / g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>Minimum</td>
</tr>
<tr>
<td>Infant Milk Powder</td>
<td>100</td>
<td>19</td>
<td>1 × 10^2</td>
</tr>
</tbody>
</table>
Table (2): Statistical analytical results of *Bacillus cereus* (spore former) count by M.P.N/g in the examined infant milk powder samples.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of examined samples</th>
<th>Positive Samples</th>
<th>M.P.N/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Infant Milk Powder</td>
<td>100</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of positive samples</th>
<th>No. of isolates</th>
<th>Positive hblC gene Only</th>
<th>Positive cytK gene Only</th>
<th>Positive hblC &amp; cytK genes</th>
<th>Negative hblC &amp; cytK genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of isolates</td>
<td>%</td>
<td>No. of isolates</td>
<td>%</td>
</tr>
<tr>
<td>Infant Milk Powder</td>
<td>19</td>
<td>19</td>
<td>8</td>
<td>42</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of positive samples</th>
<th>No. of isolates</th>
<th>Positive <em>hblC</em> gene Only</th>
<th>Positive <em>cytK</em> gene Only</th>
<th>Positive <em>hblC&amp;cytK</em> genes</th>
<th>Negative <em>hblC&amp;cytK</em> genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant Milk Powder</td>
<td>11</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36.4</td>
<td>18.2</td>
<td>18.2</td>
<td>27.3</td>
</tr>
</tbody>
</table>

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.


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.

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

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>No. of examined samples</th>
<th>Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant milk powder</td>
<td>100</td>
<td>3</td>
</tr>
</tbody>
</table>

Aman, I.M. et., al.

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

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of isolates</th>
<th>Biochemical tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant milk powder</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table (7):** Comparison of the isolated pathogens from infant milk powder samples with *FDA (1996)* and *CAC (2008)* standards (n=100).

<table>
<thead>
<tr>
<th>Pathogenes</th>
<th>Infant milk powder samples</th>
<th>Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compatible samples</td>
<td>Incompatible samples</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><em>B. cereus</em> (vegetative)</td>
<td>15</td>
<td>79</td>
</tr>
<tr>
<td><em>B. cereus</em> (sporulated)</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td><em>E. sakazakii</em></td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td><em>Salmonellae</em></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Storage Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero time</td>
</tr>
<tr>
<td></td>
<td>cfu/ml</td>
</tr>
<tr>
<td>Control –ve</td>
<td>-ve</td>
</tr>
<tr>
<td>-vehblC&amp;cytK</td>
<td>1.6 x 10^2</td>
</tr>
<tr>
<td>+vehblC</td>
<td>5.0 x 10</td>
</tr>
<tr>
<td>+vecytK</td>
<td>1.1 x 10^2</td>
</tr>
<tr>
<td>+vehblC&amp;cytK</td>
<td>8.0 x 10</td>
</tr>
</tbody>
</table>

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 ([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 $1 \times 10^1$ to $9 \times 10^2$ and a mean value of $1.2 \times 10^3 \pm 5.2 \times 10^2$. Higher results were reported by [Dovilèt al. (2012)](#) and [Angelaet al. (2013)](#), while the lower results were obtained by [Becker et al. (1994)](#) and [Azza et 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 $2.3 \times 10^2$ to $1.1 \times 10^4$ 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 [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 \textit{B. cereus} spores (Becker et al., 1994). The infectious dose for \textit{B. cereus} may vary from about $1 \times 10^5$ to $1 \times 10^8$ viable cells or spores/g. Generally, presence of \textit{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).

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

The primers designed by \textit{Nagmongsatit et al. (2008)} were used under specific multiplex PCR conditions for detection of enterotoxin genes (\textit{hblC} and \textit{cytK}) in selected strains. DNA band visualized by ethidium bromide in agarose gel at the expected molecular size for \textit{hplC} and \textit{cytK} genes at 565bp and 695 bp respectively were detected.

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

Moreover, 11 \textit{B. cereus} spore strains isolated were analyzed for the presence of \textit{hblC} and \textit{cytK} genes using the PCR primers listed in fig. (2), \textit{hblC} gene was detected in 4 isolates (36.4\%), \textit{cytK} gene only was in 2 isolates (18.2\%), \textit{hblC&cytK} genes was in 2 isolates (18.2\%) and no \textit{hblC} and \textit{cytK} genes was detected in 3 isolates (27.3\%) (table 4). \textit{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 25°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.

**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 Kafr elsheikh 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.

**REFERENCES**

Hygienic Status Of Infant Milk Sold At The Markets.


Hygienic Status Of Infant Milk Sold At The Markets.


- **FDA (1996): Food and Drug Administration.** Microbiological standards for infant formula proposed in 1996 ANPR.


Hygienic Status Of Infant Milk Sold At The Markets.

- **ICMSF (1998):** Microorganisms in Foods. 6 Microbial Ecology of Food Commodities, Blackie Academic and Professional, London.


- **PN-EN ISO 21871 (2007):** Microbiology of food and feedstuffs. Horizontal method of determination of low numbers of presumptive *Bacillus cereus*. Detection and determination of most probable number (in Polish).


The hygiene status of infant milk sold at the markets.

Ibrahim Mohamed Amer, Eman Mohamed Yasseen, and Waleed Mohamed el-Qassas

1 Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt
2 Department of Food Safety, Animal Health Research Institute, Kafr El-Sheikh, Egypt

The study investigated the hygiene status of infant milk sold at the markets. The samples were analyzed for different indicators of hygiene.

100 samples of infant milk were analyzed for different indicators of hygiene. The results showed that 19% of the samples contained bacterial spores at a concentration of 2 x 10^2 to 2 x 10^5 cfu/mL. PCR analysis also revealed the presence of cytK and hblC genes in 4% of the samples. These findings highlight the need for improved hygiene practices in infant milk production.

KAFR EL-SHEIKH VET. MED. J. VOL. 14 NO. 1 (2016) 239